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The relationship between cyto-genotoxic damage and oxidative stress produced by emerging pollutants on a bioindicator organism (*Allium cepa*): The carbamazepine case

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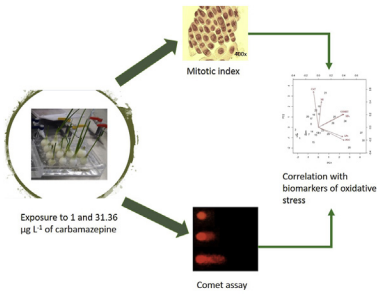
HIGHLIGHTS

- Relationship between oxidative stress with cyto and genotoxic effects of carbamazepina were studied.
- Environmentally relevant concentrations of carbamazepine (1 and 31.36 μg L^{-1}) decreased mitotic index.
- Concentrations equivalent to 31.36 μg L^{-1} of carbamazepine increased DNA damage in *A. cepa.*
- The genetic damage produced by carbamazepine is related to lipid and protein oxidation.

GRAPHICAL ABSTRACT



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ABSTRACT

The carbamazepine (CBZ) is one of the most frequently detected anticonvulsant drugs in water bodies. Although there are reports of its ecotoxicological effects in the scientific literature, toxicity studies have not focused on establishing the mechanism by which CBZ produces its effect at environmentally relevant concentrations. The objective of this work was to evaluate cyto-genotoxicity and its relationship with oxidative stress produced by carbamazepine in the *Allium cepa* model. The cytotoxicity and genotoxicity, as well as the biomarkers of oxidative stress were analyzed in the roots of *A. cepa*, exposed to 1 and 31.36 μg L^{-1} after 2, 6, 12, 24, 48 and 72 h. The results show that genotoxic capacity of this drug in the roots of *A. cepa* is related to the generation of oxidative stress, in particular with production of hydroperoxides and oxidized proteins. Also, the cytotoxic effect has a high correlation with DNA damage. The

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Emerging contaminants
Mitotic index
Plant bioassays

results of the present study clearly indicate that bioassays with sensitive plants such as *A. cepa* are useful and complementary tools to evaluate the environmental impact of emerging contaminants.

1. Introduction

An increase in anthropogenic activities has contributed to a rapid and global increase in biosphere pollutants, raising doubts about the possible consequences for biota, including humans. Recognizing that all forms of life are interconnected and that human health is strongly linked to ecosystem health, a clear understanding of all the dangers posed by environmental pollutants to both human populations and ecological systems is necessary (Geras'kin et al., 2011).

Emerging contaminants – i.e. contaminants of recent interest – comprise a wide range of chemical compounds, such as pharmaceuticals, personal care products, surfactants, plasticizers, and industrial additives that are not included in environmental monitoring programs (Petrovic et al., 2008). These types of compounds are continuously incorporated into air, water, or soil due to domestic and industrial wastewater. Thus, they have become cause of great concern amongst the scientific community and regulatory agencies (Herrero et al., 2012). However, despite researchers' efforts to understand possible effects on ecosystems, information is still limited. Pharmaceuticals are one of the emerging groups of environmental pollutants most frequently detected in surface water, groundwater and soil worldwide (Liu et al., 2012). Some drugs – such as non-steroidal anti-inflammatory drugs (NSAIDs), antibiotics, anticonvulsants and cholesterol-lowering agents – have been found in effluents from Europe and North America. Many of these have low elimination rates during water treatment processes. Incomplete elimination of pharmaceuticals causes concentrations in the range of 0.0001–5.97 μg/mL in surface water and 0.00199–42.7 μg/mL in treated drinking water (Glassmeyer et al., 2017; Petrie et al., 2015).

Among the most commonly detected pharmaceuticals in water bodies is carbamazepine (CBZ), a drug used to treat epilepsy and bipolar disorder. After administration, approximately one third of dosed CBZ is excreted unaltered (Fenet et al., 2012). This explains its presence in domestic and pharmaceutical effluents and later in receiving water bodies. In previous studies, this drug was characterized as a marker of aquatic contamination, since it is very resistant to photo and biodegradation. It has been found that less than 10% is removed by sewage sludge in water treatment plants (Ternes, 1998; Zhang et al., 2008). CBZ is frequently found in surface water, groundwater, treated water and drinking water in a concentration range of 0.03–11.6 μg L^{-1} (Bahlmann et al., 2012; Calisto et al., 2011; Li, 2014). Accumulation of CBZ has been reported in algae, zooplankton, invertebrates, fish and plants, both in field monitoring and in laboratory experiments (Freitas et al., 2016, 2015a; Tanoue et al., 2015; Valdés et al., 2016; Xie et al., 2015). However, only a few studies have addressed the integration of early damage biomarkers that delve into the mechanisms of action of CBZ, particularly those related to oxidative stress and cyto/genotoxicity.

Oxidative stress (OS) is the alteration resulting from the imbalance between the generation of reactive oxygen species (ROS) and free radicals (RL), and the antioxidant defense mechanisms in a biological system. This causes damage to cellular components and tissues. It is known that increased formation of ROS and RL can result in DNA damage, reduced DNA repair capabilities and increased susceptibility to apoptosis, factors that can lead to cytotoxic, mutagenic or carcinogenic events (García-Medina et al., 2013; Valavanidis et al., 2006). The results obtained with respect to oxidative stress and genotoxicity are considered useful indicators of the impact of pollutants on ecosystems. This is due to the fact that these types of effects may be associated with organic alterations that may affect fertility, health and the biological cycle of the organism involved (Theodorakis et al., 2000).

Knowledge of the existence of an environmental stress situation is a prerequisite for its solution or improvement. With this in mind, considerable efforts have been made to develop effective methods for environmental health assessment. The use of plants as test organisms has been indicated and validated by several environmental agencies, such as the United Nations Environment Programme, the World Health Organization and the United States Environmental Protection Agency. In particular, plant testing has been widely used to detect the genotoxicity of chemical compounds and for *in situ* monitoring of genotoxic environmental contaminants (Athanásio et al., 2014; Geras'kin et al., 2011; Grant, 1982; Lanier et al., 2015).

Onion (*Allium cepa*) is one of most used plant species in toxicity and genotoxicity tests, as it allows the evaluation of different endpoints, such as chromosomal aberrations and micronuclei. This test system provides important information to evaluate the mechanisms of action of a toxic on genetic material (clastogenic and/or aneugenic effects). It has also been used as an important tool for environmental monitoring studies, where satisfactory results have been reported (Fiskesjö, 1985; Gupta and Ahmad, 2012; Leme and Marin-Morales, 2009).

The objective of this work was to evaluate cytogenotoxicity and its relationship with oxidative stress produced by carbamazepine in the *Allium cepa* model.

2. Materials and methods

2.1. Test organisms

Allium cepa bulbs of the same size were used; 25–30 mm in diameter (2n = 16). The loose outer scales were removed and the dry bases were scraped to expose the primordial roots. These were germinated in distilled water at room temperature (28 ± 1 °C) for 12 h light/dark, for a period of 24 h.

2.2. Acute toxicity assay

To determine the appropriate concentrations for the cytogenotoxicity and oxidative stress tests, a root growth inhibition test was performed as described by Fiskesjö (1985), but with modifications. The tests were performed on 500 mL polyethylene containers, with the capacity to expose 12 bulbs, under a 12 h light/dark hours photoperiod, at room temperature. The poisoning systems were static without media renewal. Sprouted bulbs were exposed to 0 (negative control), 20, 30, 50, 60 and 75 mg L^{-1} of CBZ for 72 h. The CBZ (number CAS 298-46-4,>99% purity) was proportioned by Armstrong Laboratories of Mexico (Lote 11,010,076). At the end of the treatment, all germinated roots were measured in each exposed bulb and subsequently, the average length of each was obtained. Of the twelve replicates used, only ten

average measurements were used, as two more extreme values were discarded (Fiskesjö, 1985). With these data, the percentage of growth inhibition was calculated for each one of the concentrations. It was considered that the average length of the roots of the negative control represents 100% growth.

As a quality control test for acute toxicity, cytotoxicity and genotoxicity tests, a positive control consisting of organisms exposed to a concentration of 3.9 mg L^{-1} aluminium was performed simultaneously.

2.3. Sublethal toxicity assay

2.3.1. Mitotic index (MI)

Three groups of five previously germinated bulbs in water were formed, where control group remained in distilled water and the bulbs from the other two groups were treated with 2, 6, 12, 24, 48 and 72 h with one environmentally relevant concentration (1 μg L^{-1}) and 1/1000 IC_{50}, obtained from the acute bioassay (31.36 μg L^{-1}). Afterwards, ten roots of the exposed bulbs were cut and fixed in 3:1 (ethylic ethanol: acetic acid), and conserved in ethanol 70% at 4 °C. For slide preparation, the rootlets were hydrolyzed in 1 N HCl for 15 min, washed in distilled water, then submitted to the squashing technique, and finally colored with acetic orcein 2%. Slides of the onion root-tips were made and for each group of bulbs, 5000–5500 cells were analyzed, calculating the MI (Fiskesjö, 1988).

2.3.2. Comet assay

The root meristem cells of *A. cepa* were exposed to the same concentrations as the ones used for MI analysis. After treatment, 25 meristems were cut into thin layers and were placed in a 60 mm Petri dish kept on ice with 500 μL of Tris–$MgCl_2$ buffer (0.2 M Tris, 4 mM $MgCl_2$–$6H_2O$, pH 7.5 and 0.5% w/v Triton X-100). The roots were immediately chopped with a fresh razor blade and the isolated root nuclei (20 μL) was collected and mixed with 100 μL of 0.8% of low melting point agarose (LMPA) at 37 °C was placed 20 μL of the nuclear suspension over the microscope slides. Slides were immersed for 1 h at 4 °C in a lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% w/v Triton X-100 and 10% w/v DMSO, pH 10). Slides were placed in a solution consisting of 30 mM NaOH and 200 mM EDTA, pH > 13 for 20 min. Subsequent to DNA unwinding, electrophoresis was then conducted for 20 min at 300 mA and 25 V in a horizontal gel electrophoresis chamber cooled on ice. The process was finished by rinsing the slides in trizma base 0.4 M at pH 7.4, dried for 1 h at room temperature and stained with 50 μL ethidium bromide for 5 min and covered with a cover slip. For each slide, 100 randomly chosen nuclei were analyzed using a fluorescence microscope (Motic BA410) equipped with digital camera (Moticam Pro CCD). The length to width index in 100 nucleoids per concentration/time was determined by measuring the image length (T) and dividing the result by the nucleus diameter to obtain the T/N index (Cariño-Cortés et al., 2010; García-Medina et al., 2011; Liman, 2013).

2.3.3. Determination of OS

In order to determine oxidative stress, the bulbs of *A. cepa* were exposed pre-germinated for 24 h. Exposure to the drug was carried out under the same concentrations (1 and 31.36 μg L^{-1}) and times used in the comet assay and MI analysis. We worked with the roots of seven bulbs of *A. cepa*. After exposure time elapsed, we cut the roots and weighed 713.92 ± 8.17 mg. The roots were homogenized in 2.5 mL of PBS and centrifuged at 12,500 rpm at 4 °C for 15 min. The supernatant was used to evaluate the content of carbonated proteins (PCC) and the activity of the catalase enzyme (CAT). The rest of the homogenized was used to determine the levels of lipoperoxidation (LPx) and hydroperoxides (HPx). The total protein content was determined by Bradford (1976) method and used to express the results of the evaluated oxidative stress biomarkers.

2.3.3.1. Determination of LPx. LPx was determined using the thiobarbituric acid-reactive substances method Buege and Aust (1978). Tris-HCl buffer solution with pH 7.4 was to 300 μL of homogenized added until a 1 mL volume was attained. Samples were incubated at 37 °C for 30min; 2 mL TBA-TCA reagent (0.375% thiobarbituric acid in 15% trichloroacetic acid was added and samples were shaken in a vortex. They were then heated to boiling point for 15 min, allowed to cool, and the precipitate removed by centrifugation at 1000 rpm for 10 min. Absorbance was read at 535 nm against a reaction blank. Malondialdehyde (MDA) content was calculated using the molar extinction coefficient (MEC) of MDA (1.56×10^5 M/cm). Results were expressed as μmol MDA/mg protein (Buege and Aust, 1978).

2.3.3.2. Determination of HPx. HPx was determined by the Jiang et al. (1992) method. To 400 μL of supernatant (previously deproteinized with 15% trichloroacetic acid), 600 μL of the reaction mixture were added (0.25 mM $FeSO_4$, 25 mM H_2SO_4, 0.1 mM xylenol orange and 4 mM butyl hydroxytoluene in 90% (v/v) of methanol). The mixture was incubated during 60 min at room temperature and absorbance was read at 560 nm against a blank containing only reaction mixture. Results were interpolated on a type curve and expressed as nM CHP (cumene hydroperoxide)/mg protein (Jiang et al., 1992).

2.3.3.3. Determination of PCC. PCC was determined using the method of Levine et al. (1994) as modified, 200 μL of 10 mM DNPH in 2 M HCl were added to 150 μL of supernatant, and the resulting solution was incubated at room temperature for 1 h in darkness. Then, 300 μL of 20% trichloroacetic acid were added and the solution was allowed to rest for 15 min at 4 °C. The precipitate was centrifuged at 11,000 rpm for 5 min. The bud was washed several times with 1:1 ethanol: ethyl acetate, then dissolved in 1 mL of 6 M guanidine solution (pH 2.3) and incubated at 37 °C for 30 min. Absorbance was read at 366 nm. Results were expressed as nM reactive carbonyls formed (C=O)/mg protein, using the MEC of 21,000 M/cm (Burcham, 2007; Levine et al., 1994; Parvez and Raisuddin, 2005).

2.3.3.4. Determination of CAT activity. CAT activity was determined by the Radi et al. (1991) method. 1 mL isolation buffer solution (0.3 M saccharose, 1 mM EDTA, 5 mM HEPES and 5 mM KH_2PO_4), plus 0.2 mL of a hydrogen peroxide solution (20 mM) were added to 100 μL of supernatant. Absorbance was read at 240 nm after 0 and 60 s. Results were derived by substituting the absorbance value obtained for each of these times in the formula: CAT concentration = (A_0 - A_{60})/MEC) where the MEC of H_2O_2 is 0.043 mM cm^{-1}, and were expressed as μM H_2O_2/mg protein (Radi et al., 1991).

2.3.3.5. Determination of total protein content. 75 μl deionized water and 2.5 mL Bradford's reagent (0.05 g Coommassie Blue dye, 25 mL of 96% ethanol, and 50 mL H_3PO_4, in 500 mL deionized water) were added to 25 μl of supernatant. The test tubes were shaken and allowed to rest for 5 min prior to evaluation of absorptivity at 595 nm and interpolation on a bovine albumin curve (Bradford, 1976).

2.4. Determination of CBZ

For CBZ determination in the root of *A. cepa*, the same exposure

systems used in the determination of oxidative stress biomarkers were used. After 72 h of exposure, 1 g of the root was weighed and the CBZ was quantified according to what was indicated by Gasca-Pérez et al. (2019). CBZ levels were also quantified at time 0. The study was conducted in triplicate (Gasca-Pérez et al., 2019).

2.5. Statistical analysis

The IC_{50} was calculated using the program CompuSyn version 1.0. All variables were expressed as the mean ± standard error. Data normality and homoscedasticity were verified by Shapiro-Wilk and Bartlett tests, respectively. Results were statistically evaluated by one-way analysis of variance (ANOVA) and Tukey post hoc test (parametric data). For the non-parametric data, the Kruskal-Wallis ANOVA on Ranks were used. The Dunn post hoc test was used to evaluate significant differences, with p set at ≤ 0.05. The SigmaPlot 12.3 software was used. Finally, a correlation analysis was performed between the MI and the comet assay with the different biomarkers of oxidative stress for each concentration tested and a principal component analysis (PCA). A significant correlation between the variables was found, using the software R studio, version 1.1.456.

3. Results

The effect of CBZ on the growth of *Allium cepa* roots is summarized in Table 1. A significant and concentration-dependent reduction in the length of the roots of the bulbs exposed to all the tested concentrations of the drug was observed, obtaining an inhibitory concentration (IC_{50}) of 31.36 mg L^{-1}. During exposure and under the experimental conditions tested, there were no evident macroscopic changes in root growth.

In order to evaluate cytotoxicity, the MI of the different experimental groups was determined throughout the whole 72 h (Fig. 1) of exposure. The mean MI of the control group (negative control) was 6.681 ± 0.961 and the positive control was 0.638 ± 0.093 at all times evaluated. For the concentration of 1 μg L^{-1} of CBZ in most times, no significant difference with respect to the negative control was observed, with the exception of 12 h where a significant decrease of 29% was presented. In the case of the lot of bulbs treated with 31.36 μg L^{-1} of CBZ, a significant decrease of 51% was found at 12 h, as well as a significant increase of 47% at 48 h of exposure.

Regarding genotoxicity detected using the comet assay (Fig. 2), we observed a low and homogeneous T/N index in the meristematic cells of the control group over the evaluated time (1.051 ± 0.0434) and a significant increase in positive control (2.454 ± 0.151). On the other hand, at 48 h a significant increase of 13% over the negative control was observed in the meristematic cells that were exposed to 1 μg L^{-1} of CBZ. For the highest concentration (31.36 μg L^{-1}) a statistical significance was shown in comparison with the value of the control group at 12, 24, 48 and 72 h. At these times an increase of 19, 27, 65 and 60% was noticed, respectively. An example of the comets observed are shown in Fig. 3A to C.

Fig. 4 presents the results of the different oxidative stress biomarkers evaluated. Regarding LPx levels (Fig. 4A), an increase is observed at all times and tested concentrations. It presents a significant maximum value ($p < 0.05$) of almost nine times more than the control group at 48 h in organisms exposed to the lowest concentration. For the concentration of 31.36 μg L^{-1} of CBZ, the increase was time-dependent, being significant from 24 to 72 h. Regarding levels of HPx (Fig. 4B) for the group exposed to 1 μg L^{-1} CBZ, a tendency to increase up to eight times more with respect to the control at 48 h was found, presenting a maximum in this last time compared to the control group. For the highest concentration of CBZ, a time dependent increase from 12 to 72 h was exhibited. This increase became significant at 48 and 72 h ($p < 0.05$) with respect to the control negative roots. In the case of oxidation to

Table 1
Determination of the percentage of root growth inhibition of *Allium cepa* at different concentrations of carbamazepine.

Carbamazepine concentration (mg L^{-1})	Length of the roots mean ± SD (mm)	Percentage of inhibition
20	15.23 ± 4.25*	34.20
30	10.3 ± 2.29*	55.55
50	9.5 ± 1.53*	58.96
60	7.41 ± 1.68*	67.96
75	6.05 ± 1.15*	73.87
Negative control	23.15 ± 9.89	–
Positive control	4.40 ± 2.52*	80.99

IC_{50} = 31.3629 mg L^{-1}, r^2 = 0.96178.
ANOVA and Tukey post hoc (* indicates difference from the negative control, $p < 0.05$). SD = standard deviation, IC_{50} = inhibitory concentration 50, r^2 = coefficient of determination. Positive control, 3.9 mg L^{-1} of aluminum. Negative control, water.

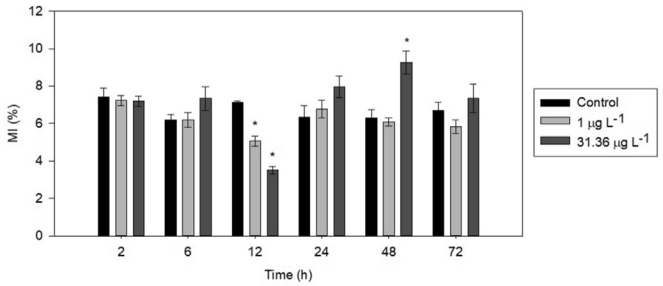

Fig. 1. Mitotic index of meristems of *Allium cepa* at different exposure times to carbamazepine. Each time represents the average of five bulbs with 10 meristems, * indicates the significant difference from its control using the ANOVA and Tukey test ($p < 0.05$).

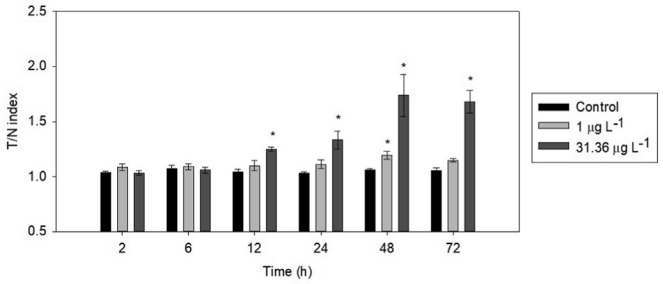

Fig. 2. DNA damage in nuclei of root meristems of A. cepa exposed to 1 and 31.36 μg L^{-1} of carbamazepine at different times. N = 5, * indicates the significant difference from its controls by means of the non-parametric ANOVA test, post hoc Dunn test ($p < 0.05$).

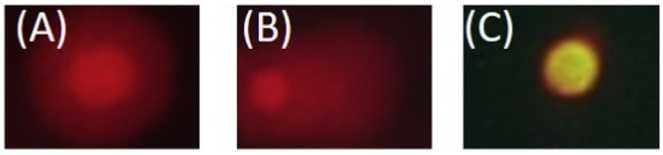

Fig. 3. Some comets obtained after exposure to carbamazepine (A) and positive control (B); and DNA in cell of negative control (C) in meristematic cells of *A. cepa*.

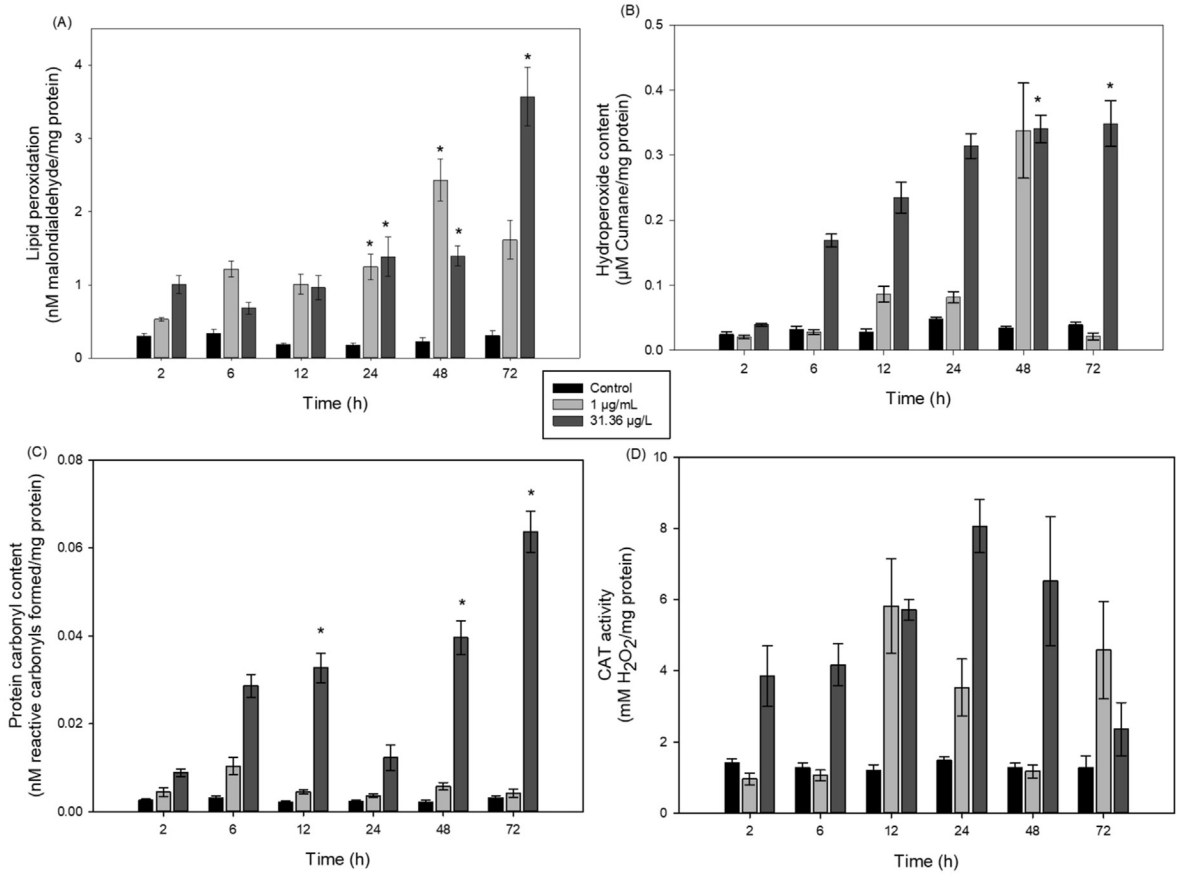

Fig. 4. Biomarkers of oxidative stress: lipid peroxidation (A); hydroperoxide levels (B); carbonyl protein content (C) and catalase enzyme activity, CAT (D) in roots of *A. cepa* exposed to 1 and 31.36 μg L^{-1} of carbamazepine. * indicates the significant difference from its controls by means of the non-parametric ANOVA test, post hoc Dunn test ($p < 0.05$).

proteins (Fig. 4C), the content of reactive carbonyls showed a tendency to increase of almost twice over the control in the low concentration at 6 h of exposure. On the other hand, for the concentration that represents 1/1000 IC_{50}, a significant increase was exhibited at 12, 48 and 72 h of exposure. Finally, regarding activity of the antioxidant enzyme CAT (Fig. 4D) a non-significant increase was found in both concentrations. In the case of the lot of *A. cepa* exposed to 1 μg L^{-1} of CBZ, this change was presented at 12, 24 and 72 h. The maximum increase was three times more than the control at 12 h. For the concentration of 31.36 μg L^{-1} of CBZ, the increase was manifested between the times of 2–48 h, being at a maximum peak of 444% in the activity at 24 h with respect to control group.

Table 2 presents a correlation analysis between MI and the comet assay with oxidative stress biomarkers. It shows that for the concentration of 1 μg L^{-1} of CBZ, there is no correlation between the different biomarkers. On the other hand, the highest concentration shows a correlation between the damage to the genetic material with the levels of HPx, LPx and PCC, as well as with MI ($p < 0.05$). Therefore, an analysis of PCA between the different biomarkers was performed at this concentration. This process finds the greatest possible variability in the normalized data. Each subsequent major component (PC) describes the greatest variation in an orthogonal direction to the previous PC; therefore, each PC is linearly uncorrelated. The plotting of the PCs against each other may reveal groups of lower variance, i.e. they are similar in the parameters that contribute to the variance covered by the PCs plotted (Ballabio, 2015; Driessen et al., 2015). Fig. 5 shows the first two PCs that cover 71.24% of the variation in the data. In these we find that the damage to evaluated DNA by the comet assay is located very close to HPx levels, while the MI is also close to the activity of the CAT enzyme. The numbers represent the individual

Table 2
Pearson's correlation between the different biomarkers evaluated.

Biomarkers		1 μg L^{-1}		31.36 μg L^{-1}	
		Comet assay	MI	Comet assay	MI
Oxidative stress	**LPx**	0.3566	−0.1390	0.5697	−0.0024
	HPx	0.2385	−0.0866	**0.7541**	0.1283
	PCC	0.0628	−0.0720	**0.6136**	−0.0855
	CAT	0.2266	−0.4259	0.0606	0.0616
Cytotoxicity	**MI**	0.1210	–	**0.9340**	–
Exposition	**CBZ***	0.1670	**0.9450**	0.5350	0.4930

Correlation coefficients significant $p < 0.05$ (shown in bold) *At 72 h.

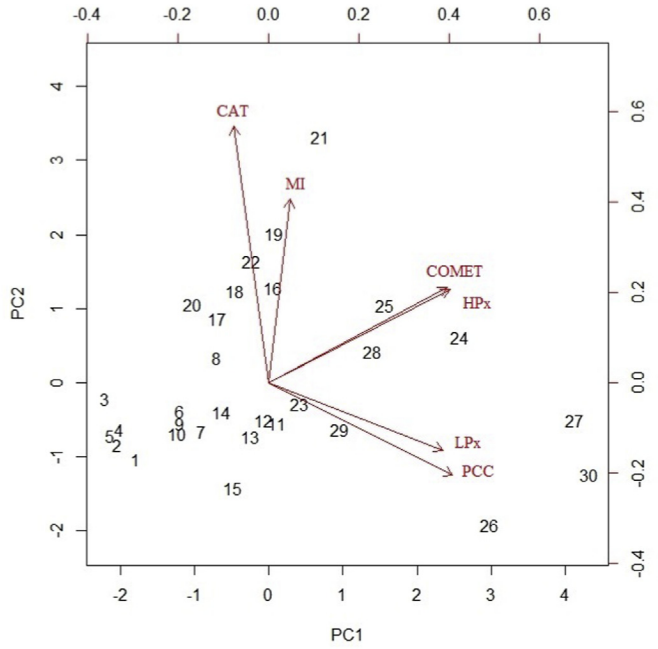

Fig. 5. Principal Component Analyses (PCA) of genotoxic and oxidant response parameters and mitotic index in roots the *A. cepa*. The numbers represent the individual values depending on the exposure time: 1 to 5 corresponds at 2 h; 6 to 10 at 6 h, 11 to 15 at 12 h; 16 to 20 at 24 h; 21 to 25 at 48 h and 26 to 30 at 72 h.

values depending on the time of exposure: 1 to 5 corresponds to 2 h; 6 to 10 at 6 h, 11 to 15 at 12 h; 16 to 20 at 24 h; 21 to 25 at 48 h and 26 to 30 at 72 h. It is observed that the most important changes between the biomarkers of oxidative and DNA damage occurred at 24, 48 and 72 h; while for MI and CAT activity these changes occurred between 24 and 48 h. The comet assay and the levels of HPx, LPx and PCC are grouped around the positive half of the PC1 axis, so oxidation products to biomolecules contribute significantly to the genotoxicity of CBZ. In contrast, the activity of CAT and MI are located around the positive axis of PC2, close to the zero value for PC1.

The concentrations shown in the system coincide with the nominal values. At zero time, 1.004 ± 0.534 μg L^{-1} was quantified for the low concentration and 30.854 ± 1.175 μg L^{-1} for the group exposed to the highest concentration. The CBZ concentration at 72 h in the roots of *A. cepa* was 0.288 ± 0.193 μg/g for the nominal concentration of 1 μg L^{-1} and 2.638 ± 1.260 μg/g for 31.36 μg L^{-1}. When performing the correlation using the damage to the genetic material and the MI at 72 h with the CBZ levels quantified in the roots (Table 2), a correlation around 0.5 in the comet assay for both concentrations was found, whereas regarding MI there was only a high correlation at the low concentration.

4. Discussion

CBZ is an omnipresent pharmaceutical product in the water cycle. It has been detected in effluents even after wastewater treatment and found in river and lake waters, as well as in drinking water (Brezina et al., 2017). It is therefore extremely important to evaluate the possible adverse effects of this contaminant on non-target organisms. By exposing the roots of *A. cepa* to different concentrations of CBZ, a significant reduction in the growth-dependent concentration of *A. cepa* was observed (Table 1). With these data, a IC_{50} of 31.36 mg L^{-1} of the root growth was established. This value is within the reported range for acute toxicity in other species. For example, in *Cyclotella meneghiniana*, Ferrari et al. (2004) found an LC_{50} at 96 h of 31.6 mg L^{-1}; while Cleuvers (2003) reported an EC_{50} of 25.5 mg L^{-1} in *Lemna* minor at 168 h. The ecotoxicological and pharmacological studies suggest that CBZ can act by diverse mechanisms. In mammals there is an inhibition in the sodium, calcium and potassium channels depending on voltage. An alteration of the release of neurotransmitters has also been related to its antiepileptic effect (Heye et al., 2019), as well as to its potential hepatotoxic capacity (Santos et al., 2008). On the other hand, in aquatic organisms such as *Danio rerio, Cyprinus carpio* and *Dugesia tigrina* it has been reported that this compound has several adverse effects, acting as endocrine disruptor, embryotoxic agent and teratogenic, besides producing behavior modifications and oxidative stress in a range of 2−200 mg L^{-1} (Gasca-Pérez et al., 2019; Ramakrishnan and DeSaer, 2011; van den Brandhof and Montforts, 2010). In the case of plants such as *Cucurbita pepo*, an increase in the degrees of chlorosis and necrosis has been observed when the concentrations of CBZ in the soil increased from 1 to 20 mg L^{-1}, as well as a reduction in the number of fruits (Knight et al., 2018).

On the other hand, Jos et al. (2003) studied six ecotoxicological models, among them *A. cepa*, finding that CBZ, in a range between 0.24 and 236.59 mg L^{-1}, produces growth inhibition and cytotoxicity. These authors consider that based on their study they can conclude that CBZ will not produce acute effects in biota since the concentrations tested are higher than those found in the environment. In that sense, this work aims to evaluate the sublethal effects produced by exposure to environmentally relevant concentrations of CBZ on *A. cepa*, using the methodology established by Fiskesjö (1985); whereas differences often occur due to exposure conditions (time, light and temperature) and the variety of *A. cepa* used. Two concentrations were tested, a low concentration (1 μg L^{-1}) that corresponding to the threshold limit value established by the United States to exclude drugs from ecotoxicity testing (Schulman et al., 2002) and a high concentration that represents the 1/1000 IC_{50} (31.36 μg L^{-1}) both concentrations are within the reported range in effluents from treatment plants and water bodies (Ginebreda et al., 2010; Hai et al., 2018; Lester et al., 2013).

A. cepa's MI has been used as a biomarker in environmental monitoring as well as in the evaluation of substances with cytotoxic potential (Bhat et al., 2017). In addition, inhibition of *A. cepa* strain root growth has been associated with the reduction of MI. In this study, roots exposed to both concentrations showed a significant decrease at 12 h in the MI. These results coincide with those observed in mammalian cells, where it has been found that CBZ is cytotoxic mainly due to the production of unstable reactive metabolites such as 2,3-epoxide-carbamazepine and 10,11-epoxide-carbamazepine. This last one is metabolized in unstable and highly reactive quinone species. However, for these metabolites to form they need to be biotransformed by the CYP3A4 system (Ghosh et al., 2015; Vignati et al., 2005). In plants, these same biotransformation products have been identified and as in mammals, the CYP450 enzyme system is responsible for generating them. In several plants − such as carrots, potatoes, tomatoes, cucumbers, among − both the unchanged drug and the epoxy metabolites of CBZ have been identified and quantified (Goldstein et al., 2014; Klampfl, 2019; Malchi et al., 2014; Sauvêtre et al., 2018). In this sense, a high correlation was found between MI and CBZ concentration at 72 h (Table 2), so the effects presented on MI may be attributable to the study drug.

On the other hand, in the group exposed to the nominal concentration of 31.36 μg L^{-1} of CBZ, a significant increase in MI was found at 48 h. This effect matchess with that observed in Vero cells

exposed to doses higher than 100 Mm of CBZ. Here they found that the relative number of cells in the phases of mitosis presented an increase in MI due to the accumulation of cells in prometaphase/metaphase and a corresponding decrease in the number of cells submitted to anaphase at 9 h. Subsequently, a decrease in MI was observed due to the increase in the frequency of cells in apoptosis at 18 and 24 h caused by the sustained mitotic blockade (Pérez et al., 2008). In the present work, a similar behavior was presented, since a decrease of the MI was observed at 72 h with respect to the levels found at 48 h after the increase. For example, Türkoğlu (2012) observed that by exposing the roots of *A. cepa* to the pesticides chlorfenvinphos and fenbuconazole, there was a reduction in mitotic activity. This was accompanied by a decrease in DNA content and an increase in DNA damage, as shown in Table 2. A high correlation was found between MI and damage to genetic material, particularly in the nominal concentration of 31.36 μg L^{-1}.

Genotoxicity is defined as damage to genetic material produced by a chemical. In this case, genotoxic damage was evaluated by means of the comet assay, a technique that allows the detection of DNA lesions, including single and double DNA strand breaks (SSBs and DSBs) and also alkali-labile sites (Tice et al., 2000). Due to the simplicity of the assay, its high sensitivity in a short time and the small amount of biological material used, it has been an important tool for the determination of genotoxicity in ecotoxicological studies (de Lapuente et al., 2015). However, although the popularity in animal cell studies is wide, few studies are conducted in plants despite the fact that many of these are in constant contact with a wide variety of contaminants through various media, such as air, soil or water (Santos et al., 2015; Türkoğlu, 2012). Besides, there are studies on the cyto and genotoxicity of CBZ in various models, these mainly establish the concentration-response relationships (Jos et al., 2003; Juhel et al., 2017; Ofoegbu et al., 2019), however the time-course relationships is another important aspect to understand the toxic characteristics of xenobiotics (Zhang et al., 2018). By exposing *A. cepa* root cells to the two concentrations of CBZ, a significant increase in DNA damage was observed, depending on the time and drug concentration. In particular, a significant time-dependent increase was shown starting at 12 h in the highest CBZ concentration, with a maximum increase of 68% with respect to the control at 48 h. In the case of the low concentration, only a significant increase of 12% was observed at 48 h. Two different behaviours were observed as a function of exposure time to CBZ; for the higher concentration a linear increase was observed as time increased, suggesting that DNA damage is continuously accumulating with time of exposure. On the other hand, in organisms exposed to the lowest concentration of CBZ, damage increased at the beginning (48 h) but then decreased, suggesting that DNA damage is induced early, but is repaired or cell death is induced. These results coincide with that reported by Rocco et al. (2011). He found a significant increase in DNA damage in *Danio rerio* erythrocytes exposed to 0.31 μg L^{-1} of the same drug during 15 days, with the maximum damage being found after 3 days. In general, DNA damage can be classified into two subtypes, complex DNA damage and simple DNA damage. A simple lesion is usually isolated DNA damage that cannot directly or indirectly induce double DNA strand breaks (DSB) due to cellular DNA repair mechanisms, this lesion included simple breaks and modification of isolated bases, which can be quickly repaired and are usually not biologically relevant. Whereas the complex DNA damage occurs when there are clustered lesions in the DNA (separated by 1–10 bp), which often occurs in the form of DSB and other forms of damage that can be converted to DSBs during repair. This complex DNA damage is much more difficult to repair so it tends to accumulate, as seen in the concentration of 31.36 μg L^{-1}, and is generally considered biologically relevant (Zhang et al., 2018). The mechanism by which CBZ is genotoxic is still not well established, on the one hand, it has been demonstrated that its epoxy metabolite has mutagenic character due to its ability to bind to macromolecules. It has also been demonstrated *in vitro* that CBZ alone produces increased DNA damage determined by the comet assay, chromosomal aberrations, exchange of sister chromatids and micronuclei (Awara et al., 1998; Çelik, 2006; Kardoost et al., 2019). Some authors mention that damage to genetic material may be caused by CBZ's ability to decrease folate concentration, causing alterations in nucleotides and DNA synthesis (Çelik, 2006; Kardoost et al., 2019).

On the other hand, there are reports on the capacity of CBZ to generate oxidative stress in diverse organisms (Aliyu et al., 2017; Freitas et al., 2015b; Gasca-Pérez et al., 2019; Talari et al., 2019). This shows the consequences of the increase of reactive spices and the modification of the activity of antioxidant enzymes can affect different biomolecules, including genetic material. In this sense, in the present investigation it was determined if CBZ is capable of producing oxidative damage in the cells of the roots of *A. cepa* and its relation with the biomarkers of cyto and genotoxicity. For this purpose, different biomarkers of oxidative stress were evaluated, such as the degree of lipoperoxidation, the content of hydroperoxides and oxidized proteins, as well as the activity of the CAT enzyme. The degree of LPx was determined based on the malondialdehyde content (MDA), and it was found that it increased significantly in a time dependent way in the roots exposed to both concentrations. An increase in the MDA content and therefore in the LPx grade, indicates that there was damage to cell membranes due to the peroxidation of polyunsaturated fatty acids, resulting in the accumulation of reactive oxygen species (ROS) and oxidative stress (Montillet et al., 2005). There is evidence that CBZ can generate oxidative stress due to the formation of intermediate epoxies and sand oxides ([O]CBZ), as demonstrated by Santos et al. (2008). He observed that [O]CBZ are capable of producing an increase in MDA levels, with a depletion of mitochondrial antioxidant defenses in rat liver. This imbalance may result in an overproduction of ROS – as shown by Eghbal et al. (2013) – when high levels of MDA are found in rat hepatocytes that coincide with the increase of ROS.

Another by-product that usually forms in LPx are lipidic hydroperoxides. In the roots of *A. cepa* an increase in its levels was found, particularly in the highest concentration where the increase was significant after 48 h. Other authors have reported similar results. For example, Gasca-Pérez et al. (2019) found that in various tissues of the common carp exposed to a concentration of 2 mg L^{-1} of CBZ, there was a time dependent increase in HPx levels from 12 to 48 h of exposure. On the other hand, Aycicek and Iscan (2007) showed that in epileptic children treated with a dose of 20–30 mg/kg/day of CBZ, there was an increase in the hydroperoxide content of up to 94% with respect to the control group. This is attributed to the excessive generation of drug metabolites that increases the levels of free radicals in the body, so they are responsible for the adverse effects of this anticonvulsant. LPx is usually one of the main parameters demonstrating the involvement of RL reactions in cell damage and the production of ROS that exceed antioxidant defenses. The result is a complex process involving RL reactions in biological membranes, forming lipid hydroperoxides that break down the double bonds of unsaturated fatty acids that destroy membrane lipids (Oliveira et al., 2015). In addition to the damage generated in the membranes, the resulting LPx products may be responsible for the damage generated in the DNA. This can be seen in the correlation analysis, where biomarkers related to oxidative damage in lipids present a high Pearson coefficient with the damage to genetic material. When reviewing the PC analysis,

these biomarkers are grouped around the positive half of the PC1 axis, so that the oxidation products of this biomolecule contribute significantly to the genotoxicity of CBZ. This behavior was also presented in the reactive carbonyls produced by protein oxidation.

The PCC level is a biomarker of oxidative protein damage. It is generally accepted because it is considered a reliable marker with respect to its appearance at a relatively early stage and greater stability, with a long half-life (Dalle-Donne et al., 2003). In the roots of *A. cepa* exposed to 31.36 μg L^{-1} of CBZ, a significant increase was found at 12, 48 and 72 h, while for the low concentration there was only a tendency to increase after 6 h of exposure. In this sense, the results found coincide with what was reported by Abdelhafidh et al. (2018) in *Ruditapes decussatus* exposed to CBZ concentrations similar to those of this work (20.35 and 32.66 μg L^{-1}). These caused an increase of PCC in the gills of the clam. It is known that one of the mechanisms of the main metabolite of CBZ, the [O]CBZ, is its ability to escape from the microsomal microenvironment and modify soluble proteins (Yip et al., 2017), which would explain the results obtained. On the other hand, PCC formation triggers conformational changes in proteins, decreasing the catalytic activity of enzymes and resulting in greater susceptibility of molecules to the action of protease (Abdelhafidh et al., 2018; Dalle-Donne et al., 2003).

When an overproduction of RL occurs, the plants, like the rest of the organisms, have to counteract the inevitable damages caused by these in order to maintain the homeostasis. Among the main enzymatic antioxidants, CAT is distinguished by its ability to metabolize H_2O_2 generated by stress and to control the cell concentration of this compound. This plays an important role not only in the metabolism and defense of plants, but also as a messenger involved in processes of growth and normal development of these (Anjum et al., 2016; Chioti and Zervoudakis, 2017). In both concentrations there was a tendency to increase, observing a maximum peak at 12 h in the nominal concentration of 1 μg L^{-1} and in the high concentration at 24 h. The increase in CAT has also been reported by other authors such as Abdelhafidh et al. (2018) who demonstrated that CAT activity in gills of *Ruditapes decussatus* increased after exposure to 20.35 and 32.66 μg L^{-1} of CBZ. Although the levels presented seem high, a high variability was observed in each of the exposure times. This is due to a different activity profile among several isoenzymes of the CAT, since a bifunctionality of this enzyme has been identified in the roots of the plants. This enzyme not only counteracts oxidative stress, but CAT isoenzymes are associated with root respiration, providing energy for growth, absorption and ion transport (Chioti and Zervoudakis, 2017). Thus, cytotoxicity would be influenced by the activity of this enzyme, as can be observed in PC analysis where enzyme activity and MI are close together in the positive quadrant of PC2. This suggests that the profile of MI is related to CAT activity, where the decrease in MI coincides with the peak of enzyme activity at 12 h. In the case of higher concentration, the decrease in enzyme activity coincides with the increase in MI.

The Environmental Risk Assessment Guideline for Medicinal Products for Human Use (2006) by the European Medicines Agency (EMA) recommends an environmental risk assessment for products placed on the market before that year (Ågerstrand et al., 2015). However, these recommendations have not been fully complied with today. In the case of Mexico, the regulation of these pollutants is restricted, limited only to the protection and destruction of packaging, expired medicine and domestic waste (De Loera -González et al., 2016). This means there is still a large gap in the evaluation of the effects of these compounds on non-target organisms (Heye et al., 2019; Holm et al., 2013).

In this sense, this paper contributes to understand the mechanism by which CBZ produces adverse effects in plants, as we demonstrated this compound is able to penetrate the roots and access the meristematic cells to generate an imbalance in the generation of ROS and antioxidant systems, as well as damage to biomolecules such as lipids and proteins. In addition, damage to genetic material occurs as a result of oxidative stress, depending on the concentrations of CBZ to which the test organisms were exposed. Two types of damage were observed; simple damage that can be easily repaired or eliminated by cell death, so the MI was reduced, or complex damage that remains and may not be repaired so it accumulates over time. These changes can result in major damage affecting the health of organisms, as well as their survival, which can impact at the population, community and ecosystem levels.

Although plants differ from animals, apparently in their metabolism, development and cell structure, the response to cyto-genotoxic agents in plant and mammalian test systems follows similar basic rules. There are various reports on the correlation between plant bioassays with other systems, for example, the EPA Gene-Tox database indicates that there is a 91% concordance between the results obtained with plant bioassays, included *A. cepa*, and those obtained with mammalian systems and other biological models (Geras'kin et al., 2011; Grant, 1982; Leme and Marin-Morales, 2009; Rank and Nielsen, 1994). Therefore, the information obtained with this model can be used as a warning for other test systems and thus be able to take the necessary action on decisions regarding the release and restriction of this compound to the environment.

5. Conclusions

Carbamazepine is an emerging contaminant that has been detected in a variety of water bodies. This causes that in recent years, its potential adverse effects on a variety of organisms have been studied. However, few have focused on understanding the mechanism by which CBZ produces such damage. The present work demonstrated the cytotoxic and genotoxic capacity of this drug in the roots of *A. cepa*, finding that this effect is related to the generation of oxidative stress, in particular with damage to genetic material. The results of the present study clearly indicate that bioassays with sensitive plants such as *A. cepa* are useful and complementary tools to evaluate the environmental impact of emerging contaminants.

Declaration of competing interest

The authors declare that they have no current or potential competing financial interests.

CRediT authorship contribution statement

Sandra García-Medina: Conceptualization, Writing - original draft, Formal analysis. **Marcela Galar-Martínez:** Resources, Methodology, Writing - review & editing. **Leobardo Manuel Gómez-Oliván:** Writing - review & editing. **Rosalía María del Consuelo Torres-Bezaury:** Investigation, Visualization. **Hariz Islas-Flores:** Data curation, Visualization. **Eloy Gasca-Pérez:** Data curation, Investigation.

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References

Abdelhafidh, K., Ali, M., Hassen, K., Badreddine, S., Jaume, A., Sandra, P., Ethel, E., Damià, B., Hamouda, B., Ezzeddine, M., 2018. Uptake and metabolism of carbamazepine (CBZ) by clam Ruditapes decussatus and its effects in biochemical responses. Xenobiotica 48, 727–733. https://doi.org/10.1080/00498254.2017.1354268.

Ågerstrand, M., Berg, C., Björlenius, B., Breitholtz, M., Brunström, B., Fick, J., Gunnarsson, L., Larsson, D.G.J., Sumpter, J.P., Tysklind, M., Rudén, C., 2015. Improving environmental risk assessment of human pharmaceuticals. Environ. Sci. Technol. 49, 5336–5345. https://doi.org/10.1021/acs.est.5b00302.

Aliyu, M., Saleh, M., Alhassan, A., Zuberu, J., Adamu, B., Iliya, B., Emmanuel, N., 2017. Evaluation of the effect of co-administration of resveratrol and vitamin E on carbamazepine-induced oxidative stress in male adult wistar rats. J. African Assoc. Physiol. Sci. 5, 93–100.

Anjum, N.A., Sharma, P., Gill, S.S., Hasanuzzaman, M., Khan, E.A., Kachhap, K., Mohamed, A.A., Thangavel, P., Devi, G.D., Vasudhevan, P., Sofo, A., Khan, N.A., Misra, A.N., Lukatkin, A.S., Singh, H.P., Pereira, E., Tuteja, N., 2016. Catalase and ascorbate peroxidase—representative H2O2-detoxifying heme enzymes in plants. Environ. Sci. Pollut. Res. 23, 19002–19029. https://doi.org/10.1007/s11356-016-7309-6.

Athanásio, C.G., Prá, D., Rieger, A., 2014. Water quality of urban streams: the Allium cepa seeds/seedlings test as a tool for surface water monitoring. Sci. World J. https://doi.org/10.1155/2014/391367, 2014.

Awara, W.M., El-Gohary, M., El-Nabi, S.H., Fadel, W.A., 1998. In vivo and in vitro evaluation of the mutagenic potential of carbamazepine: does melatonin have anti-mutagenic activity? Toxicology 125, 45–52. https://doi.org/10.1016/S0300-483X(97)00160-1.

Aycicek, A., Iscan, A., 2007. The effects of carbamazepine, valproic acid and phenobarbital on the oxidative and antioxidative balance in epileptic children. Eur. Neurol. 57, 65–69. https://doi.org/10.1159/000098053.

Bahlmann, A., Carvalho, J.J., Weller, M.G., Panne, U., Schneider, R.J., 2012. Immunoassays as high-throughput tools: monitoring spatial and temporal variations of carbamazepine, caffeine and cetirizine in surface and wastewaters. Chemosphere 89, 1278–1286. https://doi.org/10.1016/j.chemosphere.2012.05.020.

Ballabio, D., 2015. A MATLAB toolbox for Principal Component Analysis and unsupervised exploration of data structure. Chemometr. Intell. Lab. Syst. 149, 1–9. https://doi.org/10.1016/j.chemolab.2015.10.003.

Bhat, S.A., Singh, J., Singh, K., Vig, A.P., 2017. Genotoxicity monitoring of industrial wastes using plant bioassays and management through vermitechnology: a review. Agric. Nat. Resour. 51, 325–337. https://doi.org/10.1016/j.anres.2017.11.002.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3.

Brezina, E., Prasse, C., Meyer, J., Mückter, H., Ternes, T.A., 2017. Investigation and risk evaluation of the occurrence of carbamazepine, oxcarbazepine, their human metabolites and transformation products in the urban water cycle. Environ. Pollut. https://doi.org/10.1016/j.envpol.2016.10.106.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. Methods Enzymol. https://doi.org/10.1016/S0076-6879(78)52032-6.

Burcham, P.C., 2007. Modified protein carbonyl assay detects oxidised membrane proteins: a new tool for assessing drug- and chemically-induced oxidative cell injury. J. Pharmacol. Toxicol. Methods 56, 18–22. https://doi.org/10.1016/j.vascn.2006.02.015.

Calisto, V., Domingues, M.R.M., Erny, G.L., Esteves, V.I., 2011. Direct photodegradation of carbamazepine followed by micellar electrokinetic chromatography and mass spectrometry. Water Res. https://doi.org/10.1016/j.watres.2010.10.037.

Cariño-Cortés, R., Álvarez-González, I., Martino-Roaro, L., Madrigal-Bujaidar, E., 2010. Effect of naringin on the DNA damage induced by daunorubicin in mouse hepatocytes and cardiocytes. Biol. Pharm. Bull. 33, 697–701. https://doi.org/10.1248/bpb.33.697.

Çelik, A., 2006. The assesment of genotoxicity of carbamazepine using cytokinesis-block (CB) micronucleus assay in cultured human blood lymphocytes. Drug Chem. Toxicol. 29, 227–236. https://doi.org/10.1080/01480540600566832.

Chioti, V., Zervoudakis, G., 2017. Is root catalase a bifunctional catalase-peroxidase? Antioxidants 6, 39. https://doi.org/10.3390/antiox6020039.

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.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38. https://doi.org/10.1016/S0009-8981(03)00003-2.

de Lapuente, J., LourenÃ§o, J., Mendo, S.A., BorrÃ s, M., Martins, M.G., Costa, P.M., Pacheco, M., 2015. The Comet Assay and its applications in the field of ecotoxicology: a mature tool that continues to expand its perspectives. Front. Genet. 6 https://doi.org/10.3389/fgene.2015.00180.

De Loera -González, M.A., Hugo Sánchez-Rodríguez, S., Castro-Pastrana, L.I., Flores-De La Torre, J.A., López-Luna, A., 2016. Ecofarmacovigilancia. Rev. CENIC Ciencias Biol.

Driessen, M.D., Mues, S., Vennemann, A., Hellack, B., Bannuscher, A., Vimalakanthan, V., Riebeling, C., Ossig, R., Wiemann, M., Schnekenburger, J., Kuhlbusch, T.A.J., Renard, B., Luch, A., Haase, A., 2015. Proteomic analysis of protein carbonylation: a useful tool to unravel nanoparticle toxicity mechanisms. Part. Fibre Toxicol. 12, 36. https://doi.org/10.1186/s12989-015-0108-2.

Eghbal, M.A., Taziki, S., Sattari, M.R., 2013. Protective role of melatonin and taurine against carbamazepine-induced toxicity in freshly isolated rat hepatocytes. Int. J. Morphol. 31, 1081–1089. https://doi.org/10.4067/S0717-95022013000300049.

Fenet, H., Mathieu, O., Mahjoub, O., Li, Z., Hillaire-Buys, D., Casellas, C., Gomez, E., 2012. Carbamazepine, carbamazepine epoxide and dihydroxycarbamazepine sorption to soil and occurrence in a wastewater reuse site in Tunisia. Chemosphere 88, 49–54. https://doi.org/10.1016/j.chemosphere.2012.02.050.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment?. In: Environmental Toxicology and Chemistry https://doi.org/10.1897/03-246.

Fiskesjö, G., 1988. The Allium test — an alternative in environmental studies: the relative toxicity of metal ions. Mutat. Res. Mol. Mech. Mutagen. 197, 243–260. https://doi.org/10.1016/0027-5107(88)90096-6.

Fiskesjö, G., 1985. The Allium test as a standard in environmental monitoring. Hereditas 102, 99–112. https://doi.org/10.1111/j.1601-5223.1985.tb00471.x.

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: new data on single and combined long-term effects of carbamazepine on Scrobicularia plana. Sci. Total Environ. 541, 977–985. https://doi.org/10.1016/j.scitotenv.2015.09.138.

Freitas, R., Almeida, Â., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Soares, A.M.V.M., Figueira, E., 2015a. How life history influences the responses of the clam Scrobicularia plana to the combined impacts of carbamazepine and pH decrease. Environ. Pollut. 202, 205–214. https://doi.org/10.1016/j.envpol.2015.03.023.

Freitas, R., Almeida, Â., Pires, A., Velez, C., Calisto, V., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2015b. The effects of carbamazepine on macroinvertebrate species: comparing bivalves and polychaetes biochemical responses. Water Res. 85, 137–147. https://doi.org/10.1016/j.watres.2015.08.003.

García-Medina, S., Angélica Núñez-Betancourt, J., Lucero García-Medina, A., Galar-Martínez, M., Neri-Cruz, N., Islas-Flores, H., Manuel Gómez-Oliván, L., 2013. The relationship of cytotoxic and genotoxic damage with blood aluminum levels and oxidative stress induced by this metal in common carp (Cyprinus carpio) erythrocytes. Ecotoxicol. Environ. Saf. 96, 191–197. https://doi.org/10.1016/j.ecoenv.2013.06.010.

García-Medina, S., Razo-Estrada, C., Galar-Martinez, M., Cortéz-Barberena, E., Gómez-Oliván, L.M., Álvarez-González, I., Madrigal-Bujaidar, E., 2011. Genotoxic and cytotoxic effects induced by aluminum in the lymphocytes of the common carp (Cyprinus carpio). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 153, 113–118. https://doi.org/10.1016/j.cbpc.2010.09.005.

Gasca-Pérez, E., Galar-Martínez, M., García-Medina, S., Pérez-Coyotl, I.A., Ruiz-Lara, K., Cano-Viveros, S., Pérez-Pastén Borja, R., Gómez-Oliván, L.M., 2019. Short-term exposure to carbamazepine causes oxidative stress on common carp (Cyprinus carpio). Environ. Toxicol. Pharmacol. 66, 96–103. https://doi.org/10.1016/j.etap.2018.12.017.

Geras'kin, S., Evseeva, T., Oudalova, A., 2011. Plants as a tool for the environmental health assessment. In: Encyclopedia of Environmental Health. Elsevier, pp. 571–579. https://doi.org/10.1016/B978-0-444-52272-6.00612-7.

Ghosh, C., Hossain, M., Spriggs, A., Ghosh, A., Grant, G.A., Marchi, N., Perucca, E., Janigro, D., 2015. Sertraline-induced potentiation of the CYP3A4-dependent neurotoxicity of carbamazepine: an in vitro study. Epilepsia 56, 439–449. https://doi.org/10.1111/epi.12923.

Ginebreda, A., Muñoz, I., de Alda, M.L., Brix, R., López-Doval, J., Barceló, D., 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.

Glassmeyer, S.T., Furlong, E.T., Kolpin, D.W., Batt, A.L., Benson, R., Boone, J.S., Conerly, O., Donohue, M.J., King, D.N., Kostich, M.S., Mash, H.E., Pfaller, S.L., Schenck, K.M., Simmons, J.E., Varughese, E.A., Vesper, S.J., Villegas, E.N., Wilson, V.S., 2017. Nationwide reconnaissance of contaminants of emerging concern in source and treated drinking waters of the United States. Sci. Total Environ. 581–582, 909–922. https://doi.org/10.1016/j.scitotenv.2016.12.004.

Goldstein, M., Shenker, M., Chefetz, B., 2014. Insights into the uptake processes of wastewater-borne pharmaceuticals by vegetables. Environ. Sci. Technol. 48, 5593–5600. https://doi.org/10.1021/es5008615.

Grant, W.F., 1982. Chromosome aberration assays in Allium. A report of the U.S. Environmental protection agency gene-tox program. Mutat. Res. 99, 273–291. https://doi.org/10.1016/0165-1110(82)90046-x.

Gupta, A.K., Ahmad, M., 2012. Assessment of cytotoxic and genotoxic potential of refinery waste effluent using plant, animal and bacterial systems. J. Hazard. Mater. 201– 202, 92–99. https://doi.org/10.1016/j.jhazmat.2011.11.044.

Hai, F., Yang, S., Asif, M., Sencadas, V., Shawkat, S., Sanderson-Smith, M., Gorman, J., Xu, Z.-Q., Yamamoto, K., 2018. Carbamazepine as a possible anthropogenic marker in water: occurrences, toxicological effects, regulations and removal by wastewater treatment technologies. Water 10, 107. https://doi.org/10.3390/w10020107.

Herrero, O., Pérez Martín, J.M., Fernández Freire, P., Carvajal López, L., Peropadre, A., Hazen, M.J., 2012. Toxicological evaluation of three contaminants of emerging concern by use of the Allium cepa test. Mutat. Res. Toxicol. Environ. Mutagen. 743, 20–24. https://doi.org/10.1016/j.mrgentox.2011.12.028.

Heye, K., Wiebusch, J., Becker, J., Rongstock, L., Bröder, K., Wick, A., Schulte-Oehlmann, U., Oehlmann, J., 2019. Ecotoxicological characterization of the antiepileptic drug carbamazepine using eight aquatic species: baseline study for future higher tier tests. J. Environ. Sci. Heal. Part A 54, 441–451. https://doi.org/10.1080/10934529.2018.1562819.

Holm, G., Snape, J.R., Murray-Smith, R., Talbot, J., Taylor, D., Sörme, P., 2013. Implementing ecopharmacovigilance in practice: challenges and potential opportunities. Drug Saf. 36, 533–546. https://doi.org/10.1007/s40264-013-0049-3.

Jiang, Z.-Y., Hunt, J.V., Wolff, S.P., 1992. Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. Anal. Biochem. 202, 384–389. https://doi.org/10.1016/0003-2697(92)90122-N.

Jos, A., Repetto, G., Rios, J., Hazen, M., Molero, M., del Peso, A., Salguero, M., Fernández-Freire, P., Pérez-Martın, J., Cameán, A., 2003. Ecotoxicological evaluation of carbamazepine using six different model systems with eighteen endpoints. Toxicol. Vitro 17, 525–532. https://doi.org/10.1016/S0887-2333(03)00119-X.

Juhel, G., Bayen, S., Goh, C., Lee, W.K., Kelly, B.C., 2017. Use of a suite of biomarkers to assess the effects of carbamazepine, bisphenol A, atrazine, and their mixtures on green mussels, Perna viridis. Environ. Toxicol. Chem. 36, 429–441. https://doi.org/10.1002/etc.3556.

Kardoost, M., Hajizadeh-Saffar, E., Ghorbanian, M.T., Ghezelayagh, Z., Pooshang Bagheri, K., Behdani, M., Habibi-Anbouhi, M., 2019. Genotoxicity assessment of antiepileptic drugs (AEDs) in human embryonic stem cells. Epilepsy Res. 158, 106232. https://doi.org/10.1016/j.eplepsyres.2019.106232.

Klampfl, C.W., 2019. Metabolization of pharmaceuticals by plants after uptake from water and soil: a review. TrAC Trends Anal. Chem. (Reference Ed.) 111, 13–26. https://doi.org/10.1016/j.trac.2018.11.042.

Knight, E.R., Carter, L.J., McLaughlin, M.J., 2018. Bioaccumulation, uptake, and toxicity of carbamazepine in soil-plant systems. Environ. Toxicol. Chem. 37, 1122–1130. https://doi.org/10.1002/etc.4053.

Lanier, C., Manier, N., Cuny, D., Deram, A., 2015. The comet assay in higher terrestrial plant model: review and evolutionary trends. Environ. Pollut. 207, 6–20. https://doi.org/10.1016/j.envpol.2015.08.020.

Leme, D.M., Marin-Morales, M.A., 2009. Allium cepa test in environmental monitoring: a review on its application. Mutat. Res. Mutat. Res. 682, 71–81. https://doi.org/10.1016/j.mrrev.2009.06.002.

Lester, Y., Mamane, H., Zucker, I., Avisar, D., 2013. Treating wastewater from a pharmaceutical formulation facility by biological process and ozone. Water Res. 47, 4349–4356. https://doi.org/10.1016/j.watres.2013.04.059.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. [37] Carbonyl assays for determination of oxidatively modified proteins. In: Methods in Enzymology, pp. 346–357. https://doi.org/10.1016/S0076-6879(94)33040-9.

Li, W.C., 2014. Occurrence, sources, and fate of pharmaceuticals in aquatic environment and soil. Environ. Pollut. 187, 193–201. https://doi.org/10.1016/j.envpol.2014.01.015.

Liman, R., 2013. Genotoxic effects of Bismuth (III) oxide nanoparticles by Allium and Comet assay. Chemosphere 93, 269–273. https://doi.org/10.1016/j.chemosphere.2013.04.076.

Liu, X., Lee, J., Ji, K., Takeda, S., Choi, K., 2012. Potentials and mechanisms of genotoxicity of six pharmaceuticals frequently detected in freshwater environment. Toxicol. Lett. 211, 70–76. https://doi.org/10.1016/j.toxlet.2012.03.003.

Malchi, T., Maor, Y., Tadmor, G., Shenker, M., Chefetz, B., 2014. Irrigation of root vegetables with treated wastewater: evaluating uptake of pharmaceuticals and the associated human health risks. Environ. Sci. Technol. 48, 9325–9333. https://doi.org/10.1021/es5017894.

Montillet, J.-L., Chamnongpol, S., Rustérucci, C., Dat, J., van de Cotte, B., Agnel, J.-P., Battesti, C., Inzé, D., Van Breusegem, F., Triantaphylidès, C., 2005. Fatty acid hydroperoxides and H 2 O 2 in the execution of hypersensitive cell death in tobacco leaves. Plant Physiol. 138, 1516–1526. https://doi.org/10.1104/pp.105.059907.

Ofoegbu, P.U., Lourenço, J., Mendo, S., Soares, A.M.V.M., Pestana, J.L.T., 2019. Effects of low concentrations of psychiatric drugs (carbamazepine and fluoxetine) on the freshwater planarian, Schmidtea mediterranea. Chemosphere 217, 542–549. https://doi.org/10.1016/j.chemosphere.2018.10.198.

Oliveira, M., Cardoso, D.N., Soares, A.M.V.M., Loureiro, S., 2015. Effects of short-term exposure to fluoxetine and carbamazepine to the collembolan Folsomia candida. Chemosphere 120, 86–91. https://doi.org/10.1016/j.chemosphere.2014.06.038.

Parvez, S., Raisuddin, S., 2005. Protein carbonyls: novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish Channa punctata (Bloch). Environ. Toxicol. Pharmacol. 20, 112–117. https://doi.org/10.1016/j.etap.2004.11.002.

Pérez, M.J.M., Fernández, F.P., Labrador, V., Hazen, M.J., 2008. Carbamazepine induces mitotic arrest in mammalian Vero cells. Mutat. Res. Mol. Mech. Mutagen. 637, 124–133. https://doi.org/10.1016/j.mrfmmm.2007.07.014.

Petrie, B., Barden, R., Kasprzyk-Hordern, B., 2015. A review on emerging contaminants in wastewaters and the environment: current knowledge, understudied areas and recommendations for future monitoring. Water Res. 72, 3–27. https://doi.org/10.1016/j.watres.2014.08.053.

Petrovic, M., Radjenovic, J., Postigo, C., Kuster, M., Farre, M., Alda, M.L., Barceló, D., 2008. Emerging contaminants in waste waters: sources and occurrence. In: Emerging Contaminants from Industrial and Municipal Waste. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–35. https://doi.org/10.1007/698_5_106.

Radi, R., Turrens, J.F., Chang, L.Y., Bush, K.M., Crapo, J.D., Freeman, B.A., 1991. Detection of catalase in rat heart mitochondria. J. Biol. Chem. 266, 22028–22034.

Ramakrishnan, L., DeSaer, C., 2011. Carbamazepine inhibits distinct chemoconvulsant-induced seizure-like activity in Dugesia tigrina. Pharmacol. Biochem. Behav. 99, 665–670. https://doi.org/10.1016/j.pbb.2011.06.003.

Rank, J., Nielsen, M.H., 1994. Evaluation of the Allium anaphase-telophase test in relation to genotoxicity screening of industrial wastewater. Mutat. Res. Mutagen. Relat. Subj. 312, 17–24. https://doi.org/10.1016/0165-1161(94)90004-3.

Rocco, L., Izzo, A., Zito, G., Peluso, C., 2011. Genotoxicity in zebrafish (Danio rerio) exposed to two pharmacological products from an impacted Italian river. J. Environ. Anal. Toxicol. https://doi.org/10.4172/2161-0525.1000103, 01.

Santos, N.A.G., Medina, W.S.G., Martins, N.M., Rodrigues, M.A.C., Curti, C., Santos, A.C., 2008. Involvement of oxidative stress in the hepatotoxicity induced by aromatic antiepileptic drugs. Toxicol. Vitro 22, 1820–1824. https://doi.org/10.1016/j.tiv.2008.08.004.

Santos, C.L.V., Pourrut, B., Ferreira de Oliveira, J.M.P., 2015. The use of comet assay in plant toxicology: recent advances. Front. Genet. 6 https://doi.org/10.3389/fgene.2015.00216.

Sauvêtre, A., May, R., Harpaintner, R., Poschenrieder, C., Schröder, P., 2018. Metabolism of carbamazepine in plant roots and endophytic rhizobacteria isolated from Phragmites australis. J. Hazard Mater. 342, 85–95. https://doi.org/10.1016/j.jhazmat.2017.08.006.

Schulman, L.J., Sargent, E.V., Naumann, B.D., Faria, E.C., Dolan, D.G., Wargo, J.P., 2002. A human health risk assessment of pharmaceuticals in the aquatic environment. Hum. Ecol. Risk Assess. 8, 657–680. https://doi.org/10.1080/20028091057141.

Talari, H.R., Bahrami, M., Ardestani, A.T., Bahmani, F., Famili, P., Asemi, Z., 2019. Effects of folate supplementation on carotid intima-media thickness, biomarkers of inflammation, and oxidative stress in carbamazepine-treated epileptic children. Int. J. Prev. Med. 10, 1–14. https://doi.org/10.4103/ijpvm.IJPVM_152_18.

Tanoue, R., Nomiyama, K., Nakamura, H., Kim, J.-W., Isobe, T., Shinohara, R., Kunisue, T., Tanabe, S., 2015. Uptake and tissue distribution of pharmaceuticals and personal care products in wild fish from treated-wastewater-impacted streams. Environ. Sci. Technol. 49, 11649–11658. https://doi.org/10.1021/acs.est.5b02478.

Ternes, T.A., 1998. Occurrence of drugs in German sewage treatment plants and rivers1Dedicated to Professor Dr. Klaus Haberer on the occasion of his 70th birthday.1. Water Res. 32, 3245–3260. https://doi.org/10.1016/S0043-1354(98)00099-2.

Theodorakis, C.W., Swartz, C.D., Rogers, W.J., Bickham, J.W., Donnelly, K.C., Adams, S.M., 2000. Relationship between genotoxicity, mutagenicity, and fish community structure in a contaminated stream. J. Aquatic Ecosyst. Stress Recovery 7, 131–143. https://doi.org/10.1023/A:1009971330138.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.-C., Sasaki, Y.F., 2000. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 35, 206–221. https://doi.org/10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J.

Türkoğlu, Ş., 2012. Determination of genotoxic effects of chlorfenvinphos and fenbuconazole in Allium cepa root cells by mitotic activity, chromosome aberration, DNA content, and comet assay. Pestic. Biochem. Physiol. 103, 224–230. https://doi.org/10.1016/j.pestbp.2012.06.001.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178–189. https://doi.org/10.1016/j.ecoenv.2005.03.013.

Valdés, M.E., Huerta, B., Wunderlin, D.A., Bistoni, M.A., Barceló, D., Rodriguez-Mozaz, S., 2016. Bioaccumulation and bioconcentration of carbamazepine and other pharmaceuticals in fish under field and controlled laboratory experiments. Evidences of carbamazepine metabolization by fish. Sci. Total Environ. 557 (558), 58–67. https://doi.org/10.1016/j.scitotenv.2016.03.045.

van den Brandhof, E.-J., Montforts, M., 2010. Fish embryo toxicity of carbamazepine, diclofenac and metoprolol. Ecotoxicol. Environ. Saf. 73, 1862–1866. https://doi.org/10.1016/j.ecoenv.2010.08.031.

Vignati, L., Turlizzi, E., Monaci, S., Grossi, P., Kanter, R. De, Monshouwer, M., 2005. An in vitro approach to detect metabolite toxicity due to CYP3A4-dependent bioactivation of xenobiotics. Toxicology 216, 154–167. https://doi.org/10.1016/j.tox.2005.08.003.

Xie, Z., Lu, G., Liu, J., Yan, Z., Ma, B., Zhang, Z., Chen, W., 2015. Occurrence, bioaccumulation, and trophic magnification of pharmaceutically active compounds in Taihu Lake, China. Chemosphere 138, 140–147. https://doi.org/10.1016/j.chemosphere.2015.05.086.

Yip, V.L.M., Meng, X., Maggs, J.L., Jenkins, R.E., Marlot, P.T., Marson, A.G., Park, B.K., Pirmohamed, M., 2017. Mass spectrometric characterization of circulating covalent protein adducts derived from epoxide metabolites of carbamazepine in patients. Chem. Res. Toxicol. 30, 1419–1435. https://doi.org/10.1021/acs.chemrestox.7b00063.

Zhang, S., Chen, H., Wang, A., Liu, Y., Hou, H., Hu, Q., 2018. Genotoxicity analysis of five particle matter toxicants from cigarette smoke based on γH2AX assay combined with Hill/Two-component model. Environ. Toxicol. Pharmacol. 58, 131–140. https://doi.org/10.1016/j.etap.2018.01.003.

Zhang, Y., Geißen, S.-U., Gal, C., 2008. Carbamazepine and diclofenac: removal in wastewater treatment plants and occurrence in water bodies. Chemosphere 73, 1151–1161. https://doi.org/10.1016/j.chemosphere.2008.07.086.