Toxicon 57 (2011) 141–147

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

First report about saxitoxins in freshwater fish *Hoplias malabaricus* through trophic exposure

Cesar A. da Silva [a], Eliane T. Oba [b], Wanessa A. Ramsdorf [c], Valéria F. Magalhães [d], Marta M. Cestari [c], Ciro Alberto Oliveira Ribeiro [e], Helena C. Silva de Assis [b,*]

[a] *Programa de Pós-Graduação em Ecologia e Conservação, Universidade Federal do Paraná, Setor de Ciências Biológicas CEP 81531-990, Caixa Postal 19031, CEP 81531-970, Curitiba-Pr, Brazil*
[b] *Departamento de Farmacologia, Universidade Federal do Paraná, Setor de Ciências Biológicas CEP 81531-990, Caixa Postal 19031, CEP 81531-970, Curitiba-Pr, Brazil*
[c] *Departamento de Genética, Universidade Federal do Paraná, Setor de Ciências Biológicas CEP 81531-990, Caixa Postal 19031, CEP 81531-970, Curitiba-Pr, Brazil*
[d] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Ilha do Fundão, Bloco G, CEP 21941-902, Rio de Janeiro, RJ, Brazil*
[e] *Departamento de Biologia Celular, Universidade Federal do Paraná, Setor de Ciências Biológicas CEP 81531-990, Caixa Postal 19031, CEP 81531-970, Curitiba-Pr, Brazil*

ARTICLE INFO

Article history:
Received 26 August 2010
Accepted 29 October 2010
Available online 10 November 2010

Keywords:
Eutrophization
Traíra
Cyanotoxin
Genotocity
Paralytic Shellfish Poison

ABSTRACT

Cyanobacterial waterblooms, such as the saxitoxin (STX) producer *Cylindrospermopsis raciborskii*, have been a worldwide concern in environmental health. However, the bioaccumulation of this neurotoxin in the trophic chain is not completely known. The aim of the present work was to evaluate STX bioaccumulation through chemical analyses and the toxic and trophic effects using biomarkers in the tropical freshwater fish *Hoplias malabaricus*. They were fed once every five days with *Astyanax* sp. before being subjected to intraperitoneal inoculation with STX extract (0.08 μg/100 g) obtained by lysis of toxic *C. raciborskii* strain (T3). After 20 days the brain was collected for acetylcholinesterase (AChE), superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione (GSH), lipoperoxidation (LPO), protein carbonylation (PCO), and comet assay analysis. The muscle was collected for STX chemical analysis. The activities of SOD and concentrations of PCO and LPO increased. The CAT, GST, and GPx activities decreased. Genotoxicity was observed in the experimental group. STX was not detected in muscle samples. Thus, an oxidative stress was observed in the brain, leading to the damage of lipids, proteins, and DNA. The mechanism of action of the neurotoxin in this subchronic exposure suggests an apoptotic cellular process.

1. Introduction

The occurrence of cyanobacterial waterblooms is a worldwide concern in environmental health, and the eutrophication phenomenon due to human activities, mainly waste production, has posed a serious danger to water resources. Species of cyanobacteria such as *Cylindrospermopsis raciborkii* can produce saxitoxins (STX), known as Paralytic Shellfish Poison (PSP), which may also result in serious damages to the freshwater aquatic ecosystem (Clemente et al., 2010).

STX is a water-soluble neurotoxin that blocks sodium (Na^+) channels when applied outside the cell in nanomolecular (1.2×10^{-9} M) concentrations (Hille, 1992). It binds and blocks the inward Na^+ current while leaving the outward potassium (K^+) current unaffected (Cestelle and Catterall, 2000; Hille, 1975), ultimately leading to the hyperpolarization of the cell (Bakke and Horsberg, 2007). However, STX can also bind to calcium (Ca^{+2}) and K^+ channels, interfering with the speed of opening and closing of these channels, which can in turn lead to alteration in the

* Corresponding author. Tel.: +55 41 33611743; fax: +55 41 3266 2042.
E-mail address: helassis@ufpr.br (H.C. Silva de Assis).

0041-0101/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.toxicon.2010.10.015

influx of ions to the cell (Su et al., 2004; Wang et al., 2003). In addition, the Na^+-channel blockage may alter the selective permeability of the membrane and may change the flow of ions, leading to damage to cellular homeostasis (Hille, 1992; Jablonski et al., 2007). Approximately 26 STX analogs have been described, all of them hydrophilic nonprotein toxins with low molecular weights (Ibbelings and Chorus, 2007). Each STX analog shows a different specific toxicity, according to its chemical structure (Oshima, 1995).

Human symptoms caused by STX poisoning start with a burning or tingling sensation on the lips and face within 30 min after exposure, increasing to total numbness. These sensations and effects may reach the extremities and may spread to the fingers and the toes. Other minor symptoms include dizziness, headache, salivation, intense thirst and perspiration, vomiting, diarrhea, and stomach cramps, whereas a lethal dose can kill within hours due to respiratory failure (Llewellyn, 2006). In fish, the symptoms of STX toxicity include loss of equilibrium, swimming on the side or upside down, or gasping at the surface of the water body, and in some cases, death of the individual (White, 1984).

Bioaccumulation of STX has been reported worldwide, mainly in seafood (e.g., shellfish, mussels, lobsters, and marine fish) (Mortensen, 1985; Cembella et al., 2002; Ito et al., 2004; Nogueira et al., 2004; Fernández-Reiriz et al., 2008; Sephton et al., 2007). However, there is little knowledge about bioaccumulation in freshwater fish, which can also be a major public health problem.

The freshwater fish species *Hoplias malabaricus*, for example, shows a large ecological plasticity, with a wide distribution in Brazilian rivers and reservoirs (Hensley and Moody, 1975) and is much appreciated in Brazilian cuisine. During the early stages of its development, it is omnivorous, feeding on microcrustaceans, algae, and insects. In the juvenile and adult stages, it becomes carnivorous, preferentially feeding small fish such as *Astyanax* sp. (Magalhães, 1931). Considering that this species occupies high trophic levels, several studies have shown that it can be used as a bioindicator of environmental contamination as it can accumulate several contaminants within its system (Sundin et al., 1999; Rios et al., 2002).

Biomarkers used by various researchers (Walker et al., 1996; Regoli et al., 2005; Rocher et al., 2006; Oliveira Ribeiro et al., 2006; Silva et al., 2009) showed a good response at all levels of biological organization and have been used as important tools to evaluate the effects of organism's exposure to several environmental pollutants, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and heavy metals. Nevertheless, the use of these tools to evaluate the ecotoxicological effects of STXs is still rare.

The aim of the present work was to evaluate the bioaccumulation and the toxic effects of STX in *H. malabaricus* after subchronic and trophic exposure using chemical analyses and biomarkers of environmental contamination.

2. Materials and methods

2.1. STX extraction from C. raciborskii culture

Toxic *C. raciborskii* strain (T3) was cultured in ASM-1 medium (Gorhan et al., 1964), pH 8.0, temperature of 23 °C ± 2, light intensity of 56 μE m^{-2} s^{-1}, and photoperiod of 12 h/12 h. To quantify PSP, the T3 strain was freeze-dried and extracted with 0.5 N acetic acid (Oshima, 1995). The extracts were stirred for 1 h, centrifuged (40,000 × g for 30 min), and the supernatant filtered using 0.45 μm regenerated cellulose filters (Sartorius). The extract was analyzed by high-performance liquid chromatography–postcolumn fluorescence derivatization (HPLC–FLD) (Shimadzu) using a LiChrospher 100 RP-18 reversed-phase analytic column 125 mm × 4 mm × 5 μm (MERCK) (Oshima, 1995).

2.2. Experimental design

Ten individuals per group (experimental STX and control groups) of *H. malabaricus* were fed once every five days with *Astyanax* sp. before being submitted to intraperitoneal inoculation with the lysate of *C. raciborskii* culture containing PSP constituted by 97% STX and 3% by neosaxitoxin (NeoSTX) and gonyautoxin (GTX2). The used dose was 0.08 μg/100 g of *H. malabaricus*, total of four doses. This chosen dose is below that acceptable for human ingestion by Food and Agriculture Organization of the United Nations, which is one daily dose of 800 μg/kg de STXeq. μg/100 g (Chorus and Bartram, 1999). A control group was maintained with fish fed *Astyanax* sp. without PSP; only 0.9% NaCl was administered to this group. After 20 days, the animals were anesthetized and killed by medullar section. The muscle was collected for chemical analysis of STX by HPLC, and the brain was collected for analysis of biochemical biomarkers, such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx), glutathione reduced (GSH), lipoperoxidation (LPO), protein carbonylation (PCO), acetylcholinesterase (AChE), and genotoxic test (comet assay).

2.3. Determination of STX in fish muscle tissue

Approximately 10 g of fish muscle were used to determine the PSP contents in each group. The samples were homogenized in HCl (0.1 N) and centrifuged at 10,000 × g at 19 °C for 10 min. The supernatants were filtered with cellulose filters before HPLC analysis.

Analyses of STX, NeoSTX, and GTX in fish muscle tissue were carried out by HPLC with postcolumn oxidation using a fluorescence detector, as described by Oshima (1995). STX standards from National Research Council (NRC-Canada), were run before and after sample analyses. The detection limits for the compounds were STX, 0.89 ng/L; NeoSTX, 2.33 ng/L; GTX 1, 1.03 ng/L; GTX 2, 0.72 ng/L; GTX 3, 0.21 ng/L; GTX 4, 0.24 ng/L; and GTX 5, 0.01 ng/L.

The concentrations of each toxin analog determined by chromatographic analyses were converted to equivalents of STX (STXeq) by comparing the toxicity of each variant with the toxicity of STX (Hall et al., 1990).

2.4. Biochemical biomarkers

Samples of brain were homogenized in phosphate buffer (0.1 M, pH 7.0) and centrifuged at 10,000 × g for 20 min at 4 °C.

The supernatants were used to estimate the activities of the enzymes SOD, CAT, GST, GPx, and AChE, and to estimate the concentrations of GSH, LPO and products of PCO.

The activity of SOD was assayed by measuring its ability to inhibit the reduction of nitroblue tetrazolium (NBT), which was determined by the method described by Crouch et al. (1981). CAT activity was measured at 240 nm on the basis of the method described by Aebi (1984). GST activity was measured at 340 nm by the method described by Keen et al. (1976), GPx activity was measured at 340 nm (Sies et al., 1979), and GSH concentration was measured at 415 nm (Sedlak and Lindsay, 1968).

The analysis of LPO was carried out using the ferrous oxidation–xylenol orange assay at 570 nm (Jiang et al., 1992), PCO analysis was conducted at 360 nm by derivatization of the protein carbonyl groups with 2,4-dinitrophenol hydrazine to yield dinitrophenyl hydrazones (Levine et al., 1994; Quinlan and Gutteridge, 2000), and AChE activity was measured spectrophotometrically at 415 nm by the method of Ellman et al. (1961), modified for use in the microplate assay by Silva de Assis (1998).

Protein concentration was determined using Bradford's method (Bradford, 1976), with bovine serum albumin as the standard.

2.5. Comet assay

The comet assay was carried out as described by Singh et al. (1988), modified by Ferraro et al. (2004). A fragment of the brain was homogenized in fetal bovine serum; 20 μL of the homogenate were diluted in 120 μL of low-melting agarose (LMA) and placed on a slide covered by normal agarose. The slides were placed in lysis solution stock solution: NaCl (2.5 M), ethylenediaminetetraacetic acid (EDTA; 100 mM), tris(hydroxymethyl)aminomethane (Tris; 10 mM), NaOH (0.8%), and N-lauroyl sarcocinate (1%); working lysis solution: Triton X-100 (1%), dimethyl sulfoxide (DMSO) (10% in lysis stock solution) for 24 h at 4 °C. Then, the slides were immersed in a solution of NaOH (10 N) and EDTA (200 mM), pH > 13, for 20 min to cause DNA denaturation and were subjected to electrophoresis at 300 mA/25 V for 25 min. After neutralization in 0.4 M Tris, pH 7.5, and fixation in ethanol for 10 min, the slides were stained with 0.02 g/ml ethidium bromide, and the DNA strand breaks were scored using a Leica DMLS2 epifluorescence microscope at a magnification of 400× For each brain slide, 100 cells were visually analyzed by the method of Collins et al. (1997) and scored visually as belonging to one of five classes—from undamaged (0) to maximally damaged (4)—predefined with reference to the tail intensity. The score of the comets for a group could range from 0 (completely undamaged = 100 cells × 0) to 400 (maximum damage = 100 cells × 4).

2.6. Statistical analysis

The normality test preceded data analysis. The biological parameters were analyzed using the unpaired Students' *t*-test. The comet assay results were analyzed using the Mann–Whitney test. All tests were regarded as statistically significant when $p < 0.05$.

3. Results

During the experiments any behavioral alterations or fish death was not observed in both experimental and control groups.

The chemical analyses did not detect STX, NeoSTX, and GTX (1,2,3,4,5) in fish muscle.

In the fish brain, the specific activity of SOD increased in the STX group in relation to the control group ($p < 0.005$; Fig. 1A), suggesting the generation of free radicals, whereas, the specific activity of CAT, GST, and GPx decreased in the STX group in relation to the control group ($p < 0.01$; $p < 0.01$; $p < 0.001$; Fig. 1B–D, respectively), suggesting inhibition of the antioxidant system of brain cells in the group exposed to STX. Meanwhile, the concentration of GSH and the AChE activity did not show any significant differences between the groups ($p > 0.05$).

In addition, the LPO process, expressed as the concentration of hydroperoxides, and the PCO process, expressed as the concentration of dinitrophenyl hydrazones, increased in the STX group ($p < 0.0001$; Fig. 2A, B, respectively). Increased damages were observed in terms of the DNA (comet assay), with most of the incidences being for the 3rd and 4th levels ($p < 0.0001$; Fig. 3), suggesting the occurrence of oxidative stress, genotoxic damage and apoptotic processes in fish exposed to STX.

4. Discussion

In this work, the absence of STX in the muscle of *H. malabaricus* after exposure is probably due to the STX being converted to other analogs, which were not analyzed, or due to the low tested dose. Bioaccumulation is commonly found for lipophilic toxicants such as PCBs but is less probable for hydrophilic compounds such as STX (Ibbelings and Chorus, 2007). However, STX bioaccumulation in the muscles of aquatic organisms has already been reported (Asakawa et al., 2004; Jang and Yotsu-Yamashita, 2006; Ibbelings and Chorus, 2007), suggesting a potential risk for humans. STX and its analogs were found in the muscles of *Geophagus brasiliensis* due to a permanent bloom of toxic *C. raciborskii* (Clemente et al., 2010). In addition, PSP bioaccumulation in the tissues of aquatic organisms has been reported during cyanobacterial bloom (Montoya et al., 1998; Nogueira et al., 2004; Pereira et al., 2004; Ibbelings and Chorus, 2007; Linares et al., 2009).

Although White (1984), White et al. (1989) reported that fish are not able to accumulate STX in sufficiently high levels to cause damage to human health, other authors described that these toxins can be concentrated in fish through the food chain (Kao, 1993; Oikawa et al., 2002; Kwong et al., 2006; Sephton et al., 2007). Several researchers reported STX in the digestive system and the gills of aquatic organisms (Watson-Wright et al., 1991; Haya et al., 1994; Desbiens and Cembella, 1997; Cembella et al., 2002; Jang and Yotsu-Yamashita, 2006; Sephton et al., 2007). In addition, *Salmo salar* exposed to STX had increased activities of the cytochrome-P450 enzymes, indicating that STX can be detoxicated (Gubbins et al., 2000), but the possibility of biodegradation of STX in *H. malabaricus* was not evaluated in the present work.

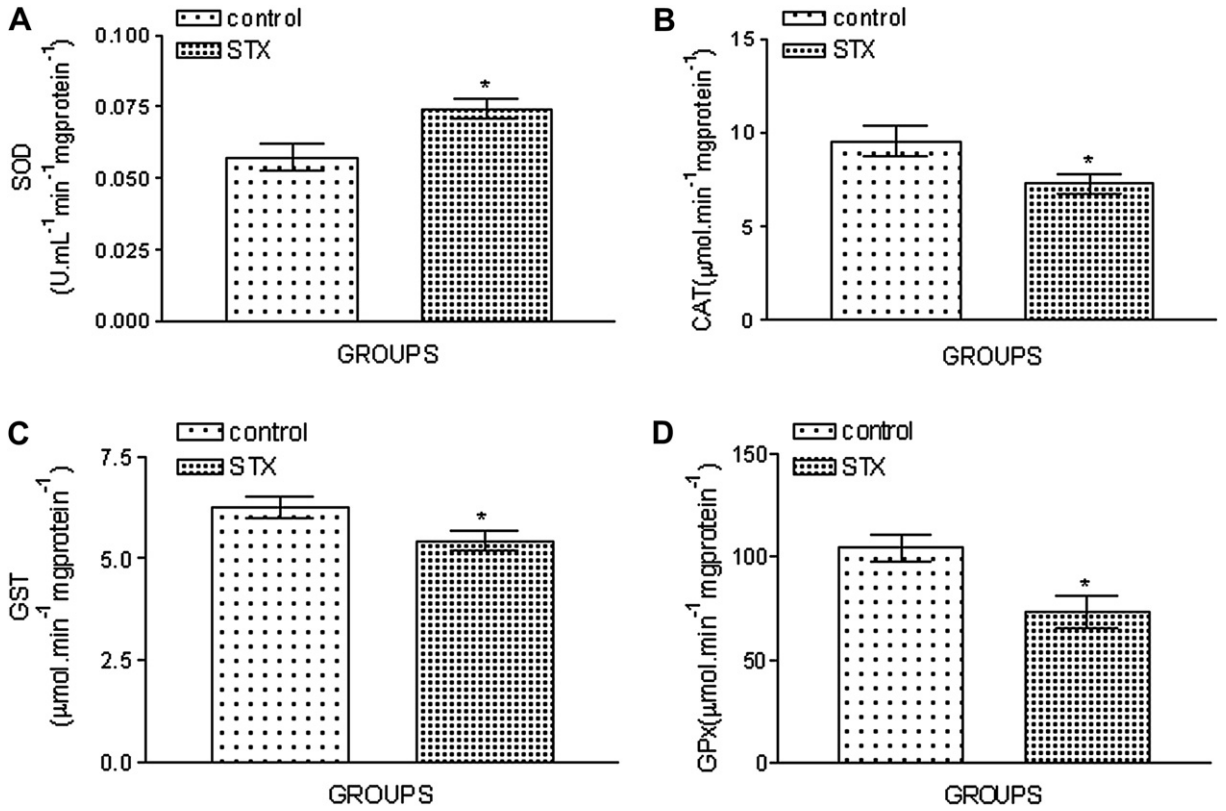

Fig. 1. Biochemical biomarkers evaluated in *H. malabaricus* exposed to the STX extract: (A) Specific activity of Superoxide dismutase (SOD; $*p < 0.005$); (B) Specific activity of catalase (CAT; $*p < 0.01$); (C) Specific activity of Glutathione S-transferase (GST; $*p < 0.01$); (D) Specific activity of Glutathione peroxidase (GPx; $*p < 0.001$). The results are expressed as mean values ± standard error. $*p$ values obtained by Students' *t*-test.

In this study, the specific activity of SOD increased, suggesting a generation of superoxide radicals ($O_2^{-}\bullet$). Reactive oxygen species (ROS), such as $O_2^{-}\bullet$, can be produced during the process of xenobiotic detoxification or due to the changing voltages in cellular membranes (Hayes et al., 1997). Moreover, transition metals such as Fe_2^{+} can catalyze the formation of hydroxyl ($OH^{-}\bullet$) radicals from hydrogen peroxide (H_2O_2) by reacting with the $O_2^{-}\bullet$ previously produced; the hydroxyl ion, in turn, is able to damage the structure of macromolecules such as DNA, proteins, carbohydrates, and lipids (Halliwell and Gutteridge, 2000). SOD is reported to be an indicator of oxidative stress and is an important enzyme of the antioxidant system of the cell against free-radical damage (Hayes et al., 1997; Halliwell and Gutteridge, 2000).

CAT is the most important enzyme that catalyzes the degradation of H_2O_2 produced during both oxidative stress and dismutation processes by SOD (Hayes et al., 1997; Halliwell and Gutteridge, 2000; Regoli et al., 2005). In this experimental exposure of *H. malabaricus* to STX the specific activity of CAT decreased. This inhibition can lead to cellular damages.

Several authors previously described the importance of GST, GPx, and GSH in preventing cellular damages (George,

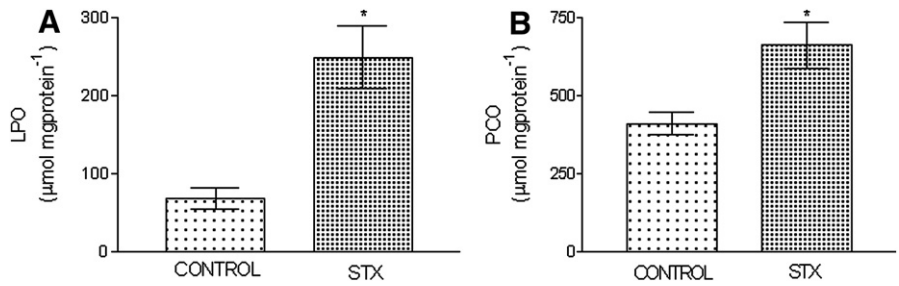

Fig. 2. Biochemical biomarkers evaluated in *H. malabaricus* exposed to the STX extract: (A) Concentration of hydroperoxides (LPO; $*p < 0.0001$). (B) Concentration of dinitrophenyl hydrazones (PCO; $*p < 0.0001$); the results are expressed as mean values ± standard error. $*p$ values obtained by Students' *t*-test.

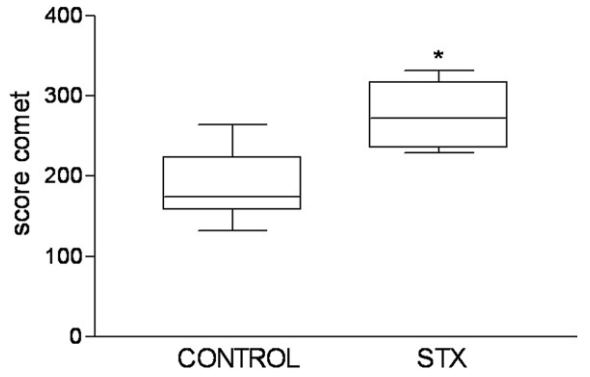

Fig. 3. DNA damages (comet assay) evaluated in *H. malabaricus* exposed to the STX extract. The results are expressed as Median/Quartile 1 (25%) and 3 (75%). *$p < 0.0001$, obtained by Mann–Whitney test.

1993; Pompella et al. 2003, Silva et al., 2009). Increase in the specific activity of GST was found in *Salmo salar* liver, suggesting that metabolites of STX can be conjugated in the liver (Gubbins et al., 2000). On other hand, in a study of clams exposed to STXs, a higher GST activity was observed in the gills than in the hepatopancreas (Choi et al., 2006). It showed that some organs in different organisms present antagonic results after exposure to STXs. In addition, the conjugation reaction of reactive substances released from the biodegradation processes involving GST is important for excretion of xenobiotics as inert compounds (George, 1993; Hermes-Lima, 2004). In this study, the decrease in GST activity suggests that GST is not able to conjugate the possible metabolites or reactive substances because STX exposure in *H. malabaricus* causes damage to macromolecules.

The GPx is an enzyme that reduces a variety of peroxides to their corresponding alcohols, whereas CAT uses one molecule of H_2O_2 as the donor for the reduction of another H_2O_2 molecule, because peroxidases use other reductants as hydroperoxides (Van der Oost et al. 2003). GPx plays an especially important role in protecting membranes from damage due to LPO, and the major detoxification function of GPx is the termination of free radical–chain propagation by quick reduction to yield further radicals (Lauterburg et al., 1983). Increased GPx activity was observed in experiments with fish exposed to paraquat, PCBs, and hexachloro benzene (HCB) contaminated food, whereas a decrease was observed after 3-methylcholanthrene (3 MC) exposure (Van der Oost et al. 2003). The GPx decrease in *H. malabaricus* after STX exposure suggests that the integrity of the cell membranes was compromised, which is associated with an increase in the levels of PCO and LPO processes.

The GSH is also described as an important molecule for protection against cellular damage (Pompella et al., 2003), but in this experiment, its status was not altered. It is possible that GSH is not as sensitive a biomarker as are CAT, GPx, and GST for STX exposure at the concentration tested in this experiment.

The deficiency in the antioxidant system found in this work may have led to an increase in the hydroperoxide concentration, thus promoting LPO which can lead to the loss of cell-membrane integrity (Hayes et al., 1997; Regoli et al., 2005) and the carbonylation of proteins, which can provoke cellular damage by forming adducts of proteins, concurrent with the generation of organic reactive species leading to oxidative stress.

Alterations in the selective permeability of the membrane due to STX exposure can lead to intracellular dysfunction (Hille, 1992), which can decrease the antioxidant enzymes. In some cases, other substances, such as dichlorovinyl cysteine (DCVC), aminotriazole, and 4,6-dinitro-o-cresol (DNOC), are able to inhibit the antioxidant system (Bagnyukova et al., 2005; Van der Oost et al., 2003). Although the mechanism of the inhibition of antioxidant activity is still unknown, some of these substances can inhibit the transcription of specific genes, resulting in the decrease of mRNA levels, which can reflect in lower activities of detoxification-specific enzymes (George, 1993; Halliwell and Gutteridge, 2000; Hayes et al., 1997), such as those described in this work.

In addition, the comet assay showed that STX can be a genotoxic substance that can lead to neurodegeneration (Thompson, 2008). Moreover, the damages found in this study due to DNA fragmentation are an important result because may induce cellular death by apoptotic processes (Fairbairn et al., 1995), and oxidative stress can lead to neurodegenerative disorders and provoke neuron pathology (Coyle and Puttfarcken, 1993).

In spite of the fact that STX shows reversible effects (Hille, 1992; Lefebvre et al., 2004; Llewellyn, 2006), more studies with chronic exposure are needed, because the blockage of Na^+ influx by STX provokes alterations in the balance of ions, which perturbs the intracellular homeostasis, and the presence of ROS, as observed in the present work, can lead to cellular apoptotic processes (Butterfield et al., 1994; Nobel et al., 2000; Calamita et al., 2005; Bortner and Cidlowski, 2007; Jablonski et al., 2007; Xuan et al., 2009). Studies *in vitro* with this species are being carried out to elucidate the effects of STX on cell death.

Finally, organophosphates and carbamates are known to inhibit AChE activity, but other substances, such as PAHs and metals, can modify the enzyme activity (Akaishi et al., 2004; Galgani et al., 1992; Martınez-Tabche et al., 1997; Oliveira Ribeiro et al., 2006). In this experiment, the enzyme was not altered, although the Na^+ channel could be blocked by STX (Hille, 1975), indicating that the chemical synapses continue functioning. Moreover, the pollutants, which are not enzyme-specific, are required at high concentrations to cause enzyme inhibition (Sturm et al., 1999).

In summary, the results found in this work suggest that STXs provoke damage to the brain cells of *H. malabaricus*, which can lead to ecological damage because this species is important to maintain the balance in the aquatic ecosystem as it occupies high trophic levels. Moreover, a further study with different doses of STXs is highly recommended because *H. malabaricus* is a freshwater fish widely consumed in Brazil and could represent an important vehicle of STX transfer to humans when exposed to cyanobacterial blooms.

5. Conclusion

Evaluation of biochemical and genotoxical biomarkers has been used as an efficient tool to investigate the effects of subchronic and trophic exposure to STX. The results suggest

that the damage to lipids, proteins, and DNA is due to a decline in the antioxidant status, which during more chronic exposure at times of permanent blooms of cyanobacteria, could lead to damages such as apoptosis; however, this process should be investigated by more *in vivo* and *in vitro* studies. Therefore, it is not possible to conclude whether STX is bioaccumulated or not because although the inoculated STXs have not been detected in fish muscle, they could have suffered biodegradation or bioaccumulation in other organs or may have been converted into its analogs. In addition, more studies should be carried out to evaluate the risk to public health caused by humans feeding on freshwater fish.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This research was financially supported by CNPq, Brazil. The Carlos Chagas Institute, University of Rio de Janeiro, provided support for the chemical analyses of STX.

References

Aebi, H., 1984. Catalase in vitro. Meth. Enzymol. 105, 121–126.
Akaishi, F.M., Silva de Assis, H.C., Jakobi, S.C.G., Eiras-Stofella, D.R., St-Jean, S.D., Courtenay, S.C., Lima, E.F., Wagener, A.L.R., Scofield, A.L., Oliveira Ribeiro, C.A., 2004. Morphological and neurotoxicological findings in tropical freshwater fish (*Astyanax* sp.) after waterborne and acute exposure to water soluble fraction (wsf) of crude oil. Arch. Environ. Contam. Toxicol. 46, 244–253.
Bagnyukova, T.V., Storey, K.B., Lushchak, V.I., 2005. Adaptive response of antioxidant enzymes to catalase inhibition by aminotriazole in goldfish liver and kidney. Comp. Biochem. Physiol. 142, 335–341.
Bakke, M.J., Horsberg, T.E., 2007. Effects of algal-produced neurotoxins on metabolic activity in telencephalon, optic tectum and cerebellum of Atlantic salmon (*Salmo salar*). Aquat. Toxicol. 85, 96–103.
Bortner, C.D., Cidlowski, J.A., 2007. Cell shrinkage and monovalent cation fluxes: role in apoptosis. Arch. Biochem. Biophys. 462, 176–188.
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.
Butterfield, D.A., Hensley, K., Harris, M., Mattson, M., Carney, J., 1994. Betaamyloid peptide free radical fragments initiate synaptosomal lipoperoxidation in a sequence-specific fashion: implications to Alzheimer's disease. Biochem. Biophys. Res. Commun. 200, 710–715.
Calamita, G., Ferri, D., Gena, P., Liquori, G.E., Cavalier, A., Thomas, D., Svelto, M., 2005. The inner mitochondrial membrane has aquaporin-8 water channels and is highly permeable to water. J. Biol. Chem. 280, 17149–17153.
Cembella, A.D., Quilliam, M.A., Lewis, N.I., Bauder, A.G., Dell' Aversano, C., Thomas, K., Jellet, J., Cusack, R.R., 2002. The toxigenic marine dinoflagellate *Alexandrium tomarense* as the probable cause of mortality of caged salmon in Nova Scotia. Harmful Algae 1, 313–325.
Cestelle, S., Catterall, W.A., 2000. Molecular mechanisms of neurotoxin action on voltage-gated sodium channels. Biochimie 82, 883–892.
Choi, N.M.C., Yeung, L.W.Y., Siu, W.H.L., So, I.M.K., Jack, R.W., Hsieh, D.P.H., Wu, R.S.S., Lam, P.K.S., 2006. Relationships between tissue concentrations of paralytic shellfish toxins and antioxidative responses of clams, *Ruditapes philippinarum*. Mar. Pollut. Bull. 52, 572–578.
Chorus, I., Bartram, J., 1999. Toxic Cyanobacteria in Water – A Guide to Their Public Health Consequences, Monitoring and Management. E & FN Spon, London and New York.
Clemente, Z., Busato, R.H., Oliveira Ribeiro, C.A., Cestari, M.M., Ramsdorf, W.A., Magalhães, V.F., Wosiack, A.C., Silva de Assis, H.C., 2010. Analyses of paralytic shellfish toxins and biomarkers in a Southern Brazilian Reservoir. Toxicon 55, 396–406.
Collins, A., Dusinská, M., Franklin, M., Somorovská, M., Petrovská, H., Duthie, S., Fillion, L., Panayiotidis, M., Raslová, K., Vaughan, N., 1997. Comet assay in human biomonitoring studies: reliability, validation and applications. Environ. Mol. Mutagen. 30, 139–146.
Coyle, J.T., Puttfarcken, P., 1993. Oxidative stress, glutamate, and neurodegenerative disorders. Science 262 (5134), 689–695.
Crouch, R.K., Gandy, S.C., Kimsey, G., 1981. The inhibition of islet superoxide dismutase by diabetogenic drugs. Diabetes 30, 235–241.
Desbiens, M., Cembella, A.D., 1997. Retention and possible transformation of paralytic shellfish toxins in lobster (*Homarus americanus*). Bull. Aquacul. Assoc. Can. 97, 75–77.
Ellman, G.L., Courtney, K.D., Andreas, V.J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
Fairbairn, D.W., Olive, L.P., O'Neill, K.L., 1995. The comet assay: a comprehensive review. Rev. Gen. Toxicol. 339, 37–59.
Fernández-Reiriz, M.J., Navarro, J.M., Contreras, A.M., Labarta, U., 2008. Trophic interactions between the toxic dinoflagellate *Alexandrium catenella* and *Mytilus chilensis*: feeding and digestive behaviour to long-term exposure. Aquat. Toxicol. 87, 245–251.
Ferraro, M.V.M., Fenocchio, A.S., Cestari, M.M., Mantovani, M.S., Lemos, P. M.M., 2004. Genetic damage induced by trophic doses of lead in the neotropical fish *Hoplias malabaricus* (Characiformes, Erythrinidae) as revealed by the comet assay and chromosomal aberrations. Gen. Mol. Biol. 27 (2), 270–274.
Galgani, F., Bocquené, G., Cadiou, Y., 1992. Evidence of variation 425 in cholinesterase activity in fish along a pollution gradient in the North Sea. Mar. Ecol. Prog. Ser. 91, 72–82.
George, S.G., 1993. Enzymology and molecular biology of phaseII xenobiotic-conjugating enzymes in fish. In: Malins, C.D., Ostrander, G.R. (Eds.), Aquatic Toxicology: Molecular. Biochemical and Cellular Perspective, Boca Raton, London, pp. 37–85.
Gorhan, P.R., Maclachlav, J.R., Hammer, V.T., Kim, W.K., 1964. Isolation and culture of toxic strains of *Anabaena flos-aquae* (Lyngb.) de Bréb. Verhandlungen der Internationalen Vereinigung fur Theoretische und Angewandte. Limnologie 15, 796–804.
Gubbins, M.J., Eddy, F.B., Gallacher, S., Stagg, R.M., 2000. Paralytic shellfish poisoning toxins induce xenobiotic metabolising enzymes in Atlantic salmon (*Salmo salar*). Mar. Environ. Res. 50, 479–483.
Hall, S., Stricharz, G., Moczydlowski, E., Ravindran, A., Reichardt, P.B., 1990. The saxitoxins: sources, chemistry and pharmacology. In: Hall, S., Stricharz, G. (Eds.), Marine Toxins: Origin, Structure and Molecular Pharmacology, vol. 418. American Chemical Society Symposium Series, Washington, DC, pp. 29–65.
Halliwell, B., Gutteridge, J.C., 2000. Free Radicals in Biology and Medicine, third ed. Oxford, New York.
Haya, K., Oshima, Y., Young-Lai, W.W., 1994. Profile of paralytic shellfish poisoning toxins in lobsters during uptake and depuration. In: Forbes, J.R. (Ed.). Proceedings of the fourth Canadian Workshop on Harmful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 2016, 17.
Hayes, M.A., Timmins, A.C., Yau, E.H., Palazzo, M., Watson, D., Hinds, C.J., 1997. Oxygen transport patterns in patients with sepsis syndrome or septic shock: influence of treatment and relationship to outcome. Crit. Care. Med. 25, 926–936.
Hensley, D.A., Moody, D.P., 1975. Occurrence and possible establishment of *Hoplias malabaricus* (Characoidei, Erythrinidae) in Florida. Fla. Scient 38, 122–128.
Hermes-Lima, M., 2004. Oxygen in biology and biochemistry: role of free radicals. In: Storey, K.B. (Ed.), Functional Metabolism: Regulation and Adaptation. Wiley-Liss, Hoboken, NJ, pp. 319–368.
Hille, B., 1975. Receptor for tetrodotoxin and saxitoxin—structural hypothesis. Biophys 15, 615–619.
Hille, B., 1992. Ionic Channels of Excitable Membranes, second ed. Sinauer Associates Inc. Publishers, Sunderland, Massachusetts, EUA. 605.
Ibbelings, B.W., Chorus, I., 2007. Accumulation of cyanobacterial toxins in freshwater "seafood" and its consequences for public health: a review. Environ. Pollut. 150, 177–192.
Ito, K., Asakawa, M., Beppu, R., Takayama, H., Miyazawa, K., 2004. PSP-toxicification of the carnivorous gastropod *Rapana venosa* inhabiting the estuary of Nikoh River, Hiroshima Bay, Hiroshima Prefecture. Japan. Mar. Poll. Bull. 48, 1116–1121.
Jablonski, E.M., Mattocks, M.A., Sokolov, E., Koniaris, L.G., Francis, M., Hughes, F.M., Fausto, N., Pierce, R.H., McKillop, I.H., 2007. Decreased aquaporin expression leads to increased resistance to apoptosis in hepatocellular carcinoma. Cancer Lett. 250, 36–46.
Jang, J., Yotsu-Yamashita, M., 2006. Distribution of tetrodotoxin, saxitoxin, and their analogs among tissues of the puffer fish *Fugu pardalis*. Toxicon 48, 980–987.

Jiang, Z.Y., Hunt, J.V., Wolf, 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.

Kao, C.Y., 1993. Paralytic shellfish poisoning. In: Falconer, I.R. (Ed.), Algal Toxins in Seafood and Drinking Water. Academic Press, San Diego, CA, USA, pp. 75–86.

Keen, J.H., Habig, W.H., Jakoby, W.B., 1976. Mechanism for several activities of the glutathione-S-transferase. J. Biol. Chem. 251, 6183–6188.

Kwong, R.W.M., Wang, W., Lam, P.K.S., Yu, P.K.N., 2006. The uptake, distribution and elimination of paralytic shellfish toxins in mussels and fish exposed to toxic dinoflagellates. Aquat. Toxicol. 80, 82–91.

Lauterburg, B.H., Smith, C.V., Hughes, H., Mitchell, J.R., 1983. Determinants of hepatic glutathione turnover: toxicological significance. In: Lamble, J.W. (Ed.), Drug Metabolism and Distribution. Elsevier Biomedical Press, Amsterdam, Netherlands, pp. 166–180.

Lefebvre, K.A., Trainer, V.L., Scholz, N.L., 2004. Morphological abnormalities and sensorimotor deficits in larval fish exposed to dissolved saxitoxin. Aquat. Toxicol. 66, 159–170.

Levine, R.L., Williams, J.A., Stadtman, E.P., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Meth. Enzymol. 233, 346–357.

Linares, J.P., Ochoa, J.L., Martínez, A.G., 2009. Retention and tissue damage of PSP and NSP toxins in shrimp: is cultured shrimp a potential vector of toxins to human population? Toxicon 53, 185–195.

Llewellyn, L.E., 2006. Saxitoxin, a toxic marine natural that targets a multitude of receptors. Royal Soc. Chem. 23, 200–222.

Magalhães, A.C., 1931. Monografia brasileira de peixes fluviais. Graphicars, São Paulo. 100p.

Martınez-Tabche, L., Ramírez, B.M., Germán-Faz, C., Galar, C.I., Madrigal, O. M., Ulloa, G.V., Orozco, F.M., 1997. Toxic effect of sodium dodecilbencensulphonate, lead, petroleum and their mixtures on the activity of acetylcholinesterase of *Moina macrocopa* in vitro. Environ. Toxicol. Water Qual. 12, 1–5.

Mortensen, A.M., 1985. Massive Fish Mortalities in the Faroe Islands Caused by a *Gonyaulax excavata* Red Tide. In: Anderson, D.A., White, A.W., Baden, D.G. (Eds.). Toxic Dinoflagellates, New York, pp. 165–170.

Nobel, C.S.I., Aronson, J.K., van den Dobbelsteen, D.J., Slater, A.F.G., 2000. inhibition of Na+/K+-ATPase may be one mechanism contributing to potassium efflux and cell shrinkage in CD95-induced apoptosis. Apoptosis 5, 153–163.

Nogueira, I.C.G., Pereira, P., Dias, E., Pflugmacher, S., Wiegand, C., Franca, S. , Vasconcelos, V.M., 2004. Accumulation of paralytic shellfish toxins (PST) from the cyanobacterium *Aphanizomenon issatschenkoi* by the cloderan *Daphnia magna*. Toxicon 44, 773–780.

Oikawa, H., Fujita, T., Satomi, M., Suzuki, T., Kotni, Y., Yano, Y., 2002. Accumulation of paralytic shellfish poisoning toxins in the edible shore crab *Telmessus acutidens*. Toxicon 40, 1593–1599.

Oliveira Ribeiro, C.A., Filipak Neto, F., Mela, M., Silva, P.H., Randi, M.A.F., Rabitto, I.S., Alves Costa, J.R.M., Pelletier, E., 2006. Hematological findings in neotropical fish *Hoplias malabaricus* exposed to subchronic and dietary doses of methylmercury, inorganic lead, and tributyltin chloride. Environ. Res. 101, 74–80.

Oshima, Y., 1995. Post-column derivatization HPLC methods for paralytic shellfish toxins. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on Harmful Marine Microalgae, 33. IOC Manuals and Guides, pp. 81–94.

Pereira, P., Dias, E., Franca, S., Pereira, E., Carolino, M., Vasconcelos, V., 2004. Accumulation and depuration of cyanobacterial paralytic shellfish toxins by the freshwater mussel *Anodonta cygnea*. Aquat. Toxicol. 68, 339–350.

Pompella, A., Visvikis, A., Paolicchi, A., De Tata, V., Casini, A.F., 2003. The changing faces of glutathione, a cellular protagonist. Biochem. Pharmacology 66, 1499–1503.

Quinlan, G.J., Gutteridge, J.M.C., 2000. Carbonyl assay for oxidative damage to proteins. In: Taniguchi, N., Gutteridge, J.M.C. (Eds.), Experimental Protocols for Reactive Oxygen and Nitrogen Species. Oxford University, New York, pp. 257–258.

Regoli, F., Nigro, M., Benedetti, M., Fattorini, D., Gorbi, S., 2005. Antioxidant efficiency in early life stages of the antarctic silverfish, *Pleuragramma antarcticum*: responsiveness to pro-oxidant conditions of platelet ice and chemical exposure. Aquat. Toxicol. 75, 43–52.

Rios, F.S., Kalinin, A.L., Rantin, F.T., 2002. The effects of long-term food deprivation on respiration e hematology of the neotropical fish Hoplias malabaricus. J. Fish. Biol. 61, 85–95.

Rocher, B., Le Goff, J., Peluhet, L., Briand, M., Manduzio, H., Gallois, J., Devier, H., Geffard, O., Gricourt, L., Augagneur, S., Budzinski, H., Pottier, D., Andre, V., Lebailly, P., Cachot, J., 2006. Genotoxicant accumulation and cellular defense activation in bivalves chronically exposed to waterborne contaminants from the SeineRiver. Aquat. Toxicol. 79, 65–77.

Sedlak, J., Lindsay, R.H., 1968. Estimation of total bound and nonprotein sulphydril groups in tissues with Ellman's reagent analytical. Biochemistry 25, 192–205.

Sephton, D.H, Haya, K., Martin, J.L., Legresley, M.M., Page, F.H., 2007. Paralytic shellfish toxins in zooplankton, mussels, lobsters and caged atlantic salmon, Salmo salar, during a bloom of Alexandrium fundyense of Grand Manan Island, in the Bay of Fundy. Harmful Algae 5, 745–758.

Sies, H., Koch, O.R., Martino, E., Boveris, A., 1979. Increased biliary glutathione disulfide release in chronically ethanol-treated rats. FEBS. Lett. 103, 287–290.

Silva de Assis, H.C, 1998. Der Einsatz von Biomarkern zur summarischen Erfassung vom Gewässerverschmutzungen. Thesis, Berlin Technical University, Germany.

Silva, C.A., Oliveira Ribeiro, C.A., Katsumiti, A., Araujo, M.L.P., Zandona, E. M., Costa Silva, G.P., Maschio, J., Roche, H., Silva de Assis, H.C., 2009. Evaluation of waterborne exposure to oil spill 5 years after an accident in Southern Brazil. Ecotoxicol. Environ. Saf. 72, 400–409.

Singh, N.P., McCoy, M.T., Tice, R.R., Sch, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell. Res. 175 (1), 184–191.

Sturm, A., Silva de Assis, H.C., Hansen, P.D., 1999. Cholinesterases of marine teleost fish: enzymological characterization and potential use in the monitoring of neurotoxic contamination. Mar. Environ. Res. 47, 389–398.

Su, Z., Sheets, M., Ishida, H., Li, F., Barry, H., 2004. Saxitoxin blocks L-Type I_{Ca}. J. Pharmacol. Exp. Ther. 308, 324–329.

Sundin, L.I., Reis, S.G., Kalinin, A.L., Rantin, F.T., Milson, W.K., 1999. Cardiovascular and respiratory reflexes: the tropical fish, Traíra (*Hoplias malabaricus*). O_2 chemosresponses. Respir. Physiol. 116, 181–199.

Thompson, L.M., 2008. Neurodegeneration: a question of balance. Nature 452 (7188), 707–708.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Walker, C.H., Hopkin, S.P., Sibly, R.M., Peakal, D.B., 1996. Principles of Ecotoxicology. Taylor & Francis, London.

Wang, J., Salata, J.J., Bennett, P.B., 2003. Saxitoxin is a Gating Modifier of hERG K^+ channels. J. Gen. Physiol. 121, 583–598.

Watson-Wright, W., Gillis, M., Smyth, C., Trueman, S., McGuire, A., Moore, W., McLachlan, D., Sims, G., 1991. Monitoring of PSP in hepatopancreas of lobster from Atlantic Canada. In: Gordon, D.C. (Ed.). Proceedings of the Second Canadian Workshop on Hamful Marine Algae. Can. Tech. Rep. Fish. Aquat. Sci. 1799, 27-28.

White, A.W., 1984. Paralytic shellfish toxins and finfish. In: Ragelis, E.P. (Ed.), Seafood Toxins, American Chemical Society, Washington, DC, pp. 171–180.

White, A.W., Fukuhara, O., Anraku, M., 1989. Mortality of fish larvae from eating toxic dinoflagellates or zooplankton containing dinoflagellate toxins. In: Okaichi, T., Anderson, D.M., Nemoto, T. (Eds.), Red Tides: Biology, Environmental Science and Toxicology. Elsevier, New York, USA, pp. 395–398.

Xuan, Y., Yuguang, W., Minghui, Y., Qingqing, W., Qiande, L., Zengchun, M., Boli, Z., Yue, G., 2009. Investigating the *in vitro* metabolism of veratridine: characterization of metabolites and involved cytochrome P450 isoforms. J. Chromat. B. 877, 141–148.