



A short-term exposure to saxitoxin triggers a multitude of deleterious effects in *Daphnia magna* at levels deemed safe for human health

Albano Pinto^{a,b,*}, Inês P.E. Macário^{a,b}, Sérgio M. Marques^{a,b}, Joana Lourenço^{a,b},
Inês Domingues^{a,b}, Maria João Botelho^{c,d}, Jana Asselman^e, Patrícia Pereira^{a,b}, Joana
L. Pereira^{a,b}

^a CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Portugal

^b Department of Biology, University of Aveiro, Aveiro, Portugal

^c IPMA, Portuguese Institute for the Sea and Atmosphere, Av. Alfredo Magalhães Ramalho 6, 1495-165 Algés, Portugal

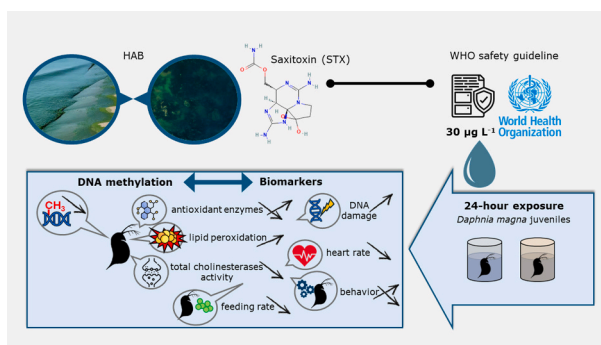
^d CIIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto, Av. General Norton de Matos s/n, 4450-208 Matosinhos, Portugal

^e Blue Growth Research Lab, Ghent University, Bluebridge Building, Ostend Science Park 1, 8400 Ostend, Belgium

HIGHLIGHTS

- A holistic approach was used to assess saxitoxin (STX) effects in *Daphnia magna*.
- Environmental level STX exposure induces sub-lethal effects in *Daphnia magna*.
- Epigenetic, biochemical and behavioral traits could be valid STX biomarkers.
- A STX concentration deemed safe may represent a challenge to aquatic biota.

GRAPHICAL ABSTRACT



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ABSTRACT

Harmful algal blooms and the toxins produced during these events are a human and environmental health concern worldwide. Saxitoxin and its derivatives are potent natural aquatic neurotoxins produced by certain freshwater cyanobacteria and marine algae species during these bloom events. Saxitoxins effects on human health are well studied, however its effects on aquatic biota are still largely unexplored. This work aims at evaluating the effects of a pulse acute exposure (24 h) of the model cladoceran *Daphnia magna* to 30 µg saxitoxin L⁻¹, which corresponds to the safety guideline established by the World Health Organization (WHO) for these toxins in recreational freshwaters. Saxitoxin effects were assessed through a comprehensive array of biochemical (antioxidant enzymes activity and lipid peroxidation), genotoxicity (alkaline comet assay), neurotoxicity (total cholinesterases activity), behavioral (swimming patterns), physiological (feeding rate and heart rate), and epigenetic (total 5-mC DNA methylation) biomarkers. Exposure resulted in decreased feeding rate, heart rate, total cholinesterases activity and catalase activity. Contrarily, other antioxidant enzymes, namely glutathione-S-

* Corresponding author at: CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Portugal.

E-mail address: albanopinto@ua.pt (A. Pinto).

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Oxidative stress biomarkers
Epigenetic biomarkers

transferases and selenium-dependent Glutathione peroxidase had their activity increased, together with lipid peroxidation levels. The enhancement of the antioxidant enzymes was not sufficient to prevent oxidative damage, as underpinned by lipid peroxidation enhancement. Accordingly, average DNA damage level was significantly increased in STX-exposed daphnids. Total DNA 5-mC level was significantly decreased in exposed organisms. Results showed that even a short-term exposure to saxitoxin causes significant effects on critical molecular and cellular pathways and modulates swimming patterns in *D. magna* individuals. This study highlights sub-lethal effects caused by saxitoxin in *D. magna*, suggesting that these toxins may represent a marked challenge to their thriving even at a concentration deemed safe for humans by the WHO.

1. Introduction

Aquatic toxins are potent natural toxins synthesized by some cyanobacteria and marine algae species during harmful cyanobacterial and algal blooms (Botana, 2016; Pinto et al., 2023; Stauffer et al., 2019). Harmful phytoplankton bloom events are a public health concern worldwide since these blooms and the toxins produced during such blooms affect many inland and coastal waters globally, constituting a significant risk to human and environmental health (Altamirano and Sierra-Beltrán, 2008; Huisman et al., 2018; Lovin and Brooks, 2019; Paerl and Otten, 2013). Worryingly, harmful bloom events occurrence and severity is predicted to increase mainly due to ongoing climate change and increasing pollution of aquatic ecosystems (Bláha et al., 2009; Cusick and Sayler, 2013; Stauffer et al., 2019). As a result, aquatic toxins production and occurrence in aquatic ecosystems is also predicted to increase, with some evidence also pointing to the possibility of increased toxicity of some bloom forming species and the production of new toxins (Mutoti et al., 2022; Nicolas et al., 2017).

Among aquatic toxins, the neurotoxin saxitoxin and its derivatives, collectively known as saxitoxins (STXs), emerges as a particularly interesting case study. This widely produced broad family of aquatic neurotoxins that encompasses >50 derivatives of the main STX are produced by both freshwater and marine phytoplankton species (Chorus et al., 2021; Cusick and Sayler, 2013; O'Neill et al., 2016; Pinto et al., 2023; Visciano et al., 2016). A few STXs analogues are directly produced by both cyanobacteria and dinoflagellates, with the others being formed after enzymatic and chemical reactions that typically occur in exposed tissues of aquatic species (Raposo et al., 2020; Vale, 2010; Wiese et al., 2010). In humans, STXs are responsible for the serious Paralytic Shellfish Poisoning (PSP) syndrome that mainly results from the consumption of STXs-contaminated seafood, cephalopods, and fish. Several cases of human PSP intoxication occur every year worldwide (de Carvalho et al., 2019; Ledreux et al., 2010; Murk et al., 2019; O'Neill et al., 2016; Rapala et al., 2005).

The widespread occurrence and the considerable risk posed by STXs led to the establishment of marine monitoring programs by several countries that proved effective at reducing the number of human exposure events (Cusick and Sayler, 2013; Laughrey et al., 2022; Murk et al., 2019; Vale et al., 2008). STXs have also been confirmed or implicated in the deaths of several marine species such as sea birds, whales, and monk seals (Cusick and Sayler, 2013; O'Neill et al., 2016), and also domestic animals (Christensen and Khan, 2020; Pedrosa et al., 2020; Testai et al., 2016). Human and other biota STXs intoxication may also occur through oral exposure by contact and/or ingestion of freshwater (Christensen and Khan, 2020; Metcalf and Codd, 2009; O'Neill et al., 2017; Ramos et al., 2014; Rapala et al., 2005; Smith et al., 2012). Indicatively, STXs-producing cyanobacterial species have been found in many freshwater locations around the world, namely the Arctic, New Zealand, Canada, and Europe, with STXs water concentrations ranging from 0.09 µg L⁻¹ to 1070 µg L⁻¹ (Boyer, 2008; Chorus et al., 2021; Karosienė et al., 2020; Rapala et al., 2005; Smith et al., 2012; Trainer and Hardy, 2015). For freshwaters, the World Health Organization established safe concentration values in drinking (3 µg L⁻¹) and in recreational (30 µg L⁻¹) waters for humans (World Health Organization, 2020).

Saxitoxins main mechanism of toxic action is the selective blockage of voltage-gated Na⁺ and Ca²⁺ channels and act as gating modifiers of K⁺ channels in excitable cells, thus affecting neural impulse generation and ultimately suppressing muscle stimulation resulting in paralysis (Christensen and Khan, 2020; Kao, 1966; Murray et al., 2011). In addition to its widely attributed neurotoxicity and neurological effects, these toxins also possess less understood side mechanisms of toxicity on several human and non-human models, such as genotoxicity (Chen et al., 2020; Costa et al., 2012; Melegari et al., 2015); cytotoxicity related to antioxidant enzymes activity modulation and oxidative stress (Cao et al., 2018; da Silva et al., 2011; Haque et al., 2022; Melegari et al., 2015; Nogueira et al., 2004; Oyaneder-Terrazas et al., 2022; Ramos et al., 2014); reproductive toxicity and teratogenicity (Bif et al., 2013; Oberemm et al., 1999); immunotoxicity (Haque et al., 2022; Mat et al., 2013); and epigenetic toxicity (Perreault et al., 2011). Despite this evidence, a lot remains to be investigated mainly regarding biological effects of STXs other than the neurotoxic effects related to the STXs primary mechanism of toxicity. The effects of STXs have been mostly studied to safeguard human health, while repercussions of exposure to aquatic biota remain poorly understood, despite its importance for the clarification of ecological effects of these toxins in aquatic ecosystems.

In this way, the model freshwater cladoceran *Daphnia magna* was selected as test organism in the present study. This is a keystone freshwater species with a pivotal position in food webs. Its sensitivity towards multiple environmental contaminants has been demonstrated, and its fast and productive parthenogenic reproduction cycle that also allows for the exclusion of genetic variability explains why *D. magna* is widely utilized in ecotoxicology studies (Altshuler et al., 2011; Bownik and Pawlik-Skowrońska, 2019; Herrera et al., 2015; Trijau et al., 2018). *Daphnia* sp. vertebrate-like myogenic heart, fully sequenced genome and known epigenetic responses to environmental cues also makes them ideal for cardiotoxicity, genetic and epigenetic studies, with possible uses as a model species for human health research (Colbourne et al., 2011; Harris et al., 2012; Jeremias et al., 2018; Pirtle et al., 2018; Santoso et al., 2020; Spicer, 2001; Trijau et al., 2018). In addition, *D. magna* has been emerging as a relevant model species in neurodegeneration and neurological studies (Campos et al., 2016; Gómez-Canela et al., 2019; Rivetti et al., 2019), namely involving aquatic neurotoxins (Bownik and Pawlik-Skowrońska, 2019; Brooke-Jones et al., 2018). These invertebrates are phytoplankton grazers that feed on various bacteria, microalgae, unicellular and filamentous cyanobacteria and protozoans; representing primary recipients of cyanobacterial toxins, including saxitoxins, in freshwater ecosystems (Bownik and Pawlik-Skowrońska, 2019; Herrera et al., 2015; Pinto et al., 2023; Suarez-Ulloa et al., 2015; Vilar et al., 2021). Despite STXs metabolism not being entirely understood in *Daphnia*, sub-lethal effects can be hypothesized following previous findings in other model species and in vitro-cultured cells, all of which highlighting the relevance of integrating several different types of biomarker responses to understand the full spectrum of STXs toxicity towards aquatic biota (Cao et al., 2018; Chen et al., 2020; Haque et al., 2022; Melegari et al., 2015; Nogueira et al., 2004; Oyaneder-Terrazas et al., 2022; Perreault et al., 2011; Ramos et al., 2014; Silva de Assis et al., 2013). This biomarker-based approach is already widely used for a variety of other environmental contaminants, also with systemic toxic effects, like pharmaceuticals

(Dionísio et al., 2020; Nunes et al., 2006) and metals (Jemec et al., 2008; Nunes et al., 2015; Ré et al., 2021).

This study aimed at systematically evaluating the effects of a short-term exposure (24 h) to a concentration ($30 \mu\text{g L}^{-1}$) of STX corresponding to the WHO safety level for recreational waters (World Health Organization, 2020), and thereby theoretically safe for inhabitant wildlife (Mehinto et al., 2021), with a wide array of biomarkers. The selected biomarkers included: (i) sub-cellular biomarkers, namely the activity of antioxidant enzymes (Catalase, Glutathione S-Transferases, Glutathione peroxidases and Glutathione reductase) that make up the first line of the defense towards oxidate stress in eukaryotes (Barata et al., 2005; Haque et al., 2022; Ighodaro and Akinloye, 2018; Nogueira et al., 2004; Nunes et al., 2015; Oliveira et al., 2015; Oyaneder-Terrazas et al., 2022), and thiobarbituric acid reactive substances formation as an indication of lipid peroxidation and thus oxidative damage; (ii) DNA damage level assessed through the alkaline comet assay, to measure DNA strand breaks acting as a genotoxicity biomarker (Cordelli et al., 2021); (iii) total cholinesterases (ChEs) activity, directly reporting on impairment of key neurotransmission pathways, which are affected by STX; (iv) behavioral biomarkers, encompassing total swimming activity and swimming preferences, that assess behavioral alterations induced by STX; (v) two vital physiological biomarkers, *Daphnia* post-exposure feeding rate and heart rate, sensitive to a variety of environmental contaminants including aquatic toxins (Bownik and Pawlik-Skowrońska, 2019; Ferrão-Filho and da Silva, 2020); and (vi) total 5-mC DNA methylation, which is an useful early-warning putative biomarker of exposure and effect since it is the first molecular response to the stress caused by environmental contaminants, regulating gene expression directly impacting phenotypic responses (Asselman et al., 2017; Jeremias et al., 2021, 2018; Pinto et al., 2023). To the best of our knowledge, there are no studies to date using such a diverse biomarker battery that allows for a holistic assessment on the hypothesis that STXs promote noxious ecotoxicological effects in zooplankters at levels that are used as a safety reference for human health.

2. Material and methods

2.1. Test species and culturing conditions

Monoclonal bulk cultures of *Daphnia magna* (clone BEAK) were cultured in ASTM hard water medium (ASTM, 1980) enriched with vitamins and supplemented with *Ascomyllum nodosum* extract (Baird et al., 1989; Elendt and Bias, 1990). Cultures were maintained under a temperature of $20 \pm 2^\circ\text{C}$ and a 16 h/8 h light/dark photoperiod. Culture medium was renewed, and organisms were fed three times a week with concentrated suspensions of *Raphidocelis subcapitata* (3×10^5 cells mL^{-1}), cyclically cultured in Woods Hole MBL medium (Stein, 1973).

2.2. Saxitoxin and concentration determination

Saxitoxin was obtained from CIFGA Standards (Spain) in its hydrochloride form ($\geq 99\%$) (Saxitoxin dihydrochloride; CAS: 35554-08-6). A stock solution of saxitoxin was prepared in ultrapure water and then diluted to the final test concentration ($30 \mu\text{g L}^{-1}$) in ASTM, immediately before each assay. The confirmation of STX concentration in ASTM was carried out by HPLC-FLD according to the official AOAC method (Lawrence et al., 2005) with modifications applied to water samples. Toxin oxidation procedure, chromatographic conditions and details of the method are described in Costa et al. (2016), and the instrumental detection limit for STX was $1.2 \mu\text{g L}^{-1}$. The measured STX concentration ($\mu\text{g L}^{-1}$) after the end of exposure showed only a 20 % variation from the intended nominal concentration of $30 \mu\text{g L}^{-1}$ (mean = 24.1; SD = 0.84; $n = 3$); the nominal concentration is used throughout for clarity purposes and consistency with previous sections.

2.3. *Daphnia magna* exposure and sample processing

Exposures were performed in agreement with standard protocols (ASTM, 1980; OECD, 2004), under the same temperature and photo-period conditions described for bulk cultures. All experiments were initiated with *D. magna* juveniles with 4 days, born between the 3rd and 5th broods from a healthy parent bulk culture and grown from birth until 4 days of age in the same conditions as the bulk cultures. *D. magna* female juveniles with 4 days were used in these assays to collect enough biomass for all biomarker determinations. Acute exposures of 24 h, simulating an environmental pulse of $30 \mu\text{g L}^{-1}$ STX were then performed. Organisms were exposed in 1000 mL glass vessels containing 700 mL of the test solution. One hundred daphnids were randomly assigned to each test vessel, with five replicates for the control (blank ASTM medium) and for the STX treatment. During the exposure period, daphnids were not fed and physical and chemical parameters were measured to ensure compliance with the adopted guideline (OECD, 2004): average temperature was $20.97 \pm 0.21^\circ\text{C}$; average pH was 7.91 ± 0.21 and 7.96 ± 0.12 for the control and STX treatment, respectively; average dissolved oxygen was $9.04 \pm 0.17 \text{ mg L}^{-1}$ and $8.94 \pm 0.26 \text{ mg L}^{-1}$, for the control and STX treatment, respectively.

At the end of exposure period, the daphnids were randomly isolated from the test medium. Part was immediately observed for heart rate assessment (see Section 2.6), processed for DNA damage determination (see Section 2.7), monitored for behavioral analysis (see Section 2.8), and transferred for feeding rate assessment (see Section 2.9). The remaining organisms were transferred to microtubes according to the requirements of downstream analyses (Sections 2.4, 2.5 and 2.10), then stored at -80°C until analysis.

2.4. Assessment of antioxidant enzymes activity and lipid peroxidation

Samples (40 organisms per replicate) were thawed and homogenized using a rotary tissue homogenizer at 14,000 rpm in 2.2 mL ice-cold phosphate buffer (50 mM, pH 7.0) with 0.1 % Triton X-100. Samples were kept on ice during homogenization. Homogenized samples were centrifuged at $15,000 \times g$ for 10 min at 4°C and supernatants were divided into aliquots, which were used for the different enzymatic and lipid peroxidation determinations, as well as protein quantification. Supernatants were frozen and stored at -80°C until biomarkers quantification. Total soluble protein concentrations of the samples were determined according to the spectrophotometric (595 nm) method of Bradford (1976), adapted to microplate use, using γ -globulin (1 mg mL^{-1}) as a standard, allowing the normalization of enzyme activities. Spectrophotometric measurements were made in a Biotek Synergy H1 Microplate Reader (Gen5 Software 3.05).

Catalase (CAT) activity was assayed following Aebi (1984), based on the degradation rate of hydrogen peroxide (H_2O_2) monitored at 240 nm (ϵ of H_2O_2 at 240 nm = $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$) for 3 min at 10 s intervals. The results were expressed as nanomoles of H_2O_2 consumed per minute, per milligram of protein. Glutathione S-Transferases (GSTs) activity was determined according to Habig et al. (1974), considering that GSTs catalyze the conjugation of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione, forming a thioether that can be followed by the increment of absorbance at 340 nm (ϵ of CDBN conjugate at 340 nm = $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) for 5 min at 20 s intervals. GSTs enzymatic activity was expressed as nanomoles of thioether produced per minute, per milligram of protein. Glutathione peroxidases (GPx) activity was determined as described by Flohé and Günzler (1984). Glutathione peroxidase enzymes participate in the oxidation of reduced glutathione (GSH) and hydrogen peroxide (H_2O_2) to oxidized glutathione (GSSG) and H_2O . The GPx mediated oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) was monitored at 340 nm (ϵ of NADPH at 340 nm = $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$) for 5 min at 20 s intervals, using two different enzyme substrates: H_2O_2 for the determination of selenium-dependent GPx (Se-dependent GPx); and cumene hydroperoxide

(C₉H₁₂O₂) used to assess total GPx activity. Enzymatic activities were expressed as nanomoles of H₂O₂ or C₉H₁₂O₂ per minute, per milligram of protein. Glutathione reductase (Gred) was determined by the procedure described by [Carlberg and Mannervik \(1985\)](#), following the decrease of absorbance due to NADPH oxidation measured at 340 nm (ϵ of NADPH at 340 nm = 6.2 mM⁻¹ cm⁻¹) for 5 min at 20 s intervals. The results were expressed as nanomoles of NADPH oxidized per minute, per milligram of protein.

Supernatants were used to quantify the extension of lipid peroxidation, following the methodology described by [Buege and Aust \(1978\)](#). Briefly, this method is based on the reaction of lipid peroxidation by-products, such as malondialdehyde (MDA), with 2-thiobarbituric acid (TBA) that forms thiobarbituric acid reactive substances (TBARS). The amount of TBARS was evaluated at 535 nm (ϵ of TBARS at 535 nm = 1.56 × 10⁶ M⁻¹ cm⁻¹), and results were expressed as nanomoles of MDA equivalents per milligram of protein.

2.5. Assessment of total cholinesterases activity

Samples for the quantification of total ChEs activity (10 organisms per replicate) were homogenized in 400 µL ice-cold phosphate buffer (0.1 M, pH 7.2). Homogenates were subsequently centrifuged at 3300 ×g for 3 min at 4 °C and the recovered supernatants frozen until at -80 °C until enzymatic determinations. The activity of *D. magna* unspecific cholinesterases ([Diamantino et al., 2003](#)) was determined by the method of [Ellman et al. \(1961\)](#), adapted to microplate as described and optimized for *D. magna* by [Guilhermino et al. \(1996\)](#). This method consists of monitoring the changes in absorbance at 412 nm (Biotek Synergy H1 Microplate Reader with Gen5 Software 3.05) due to the formation of a conjugate between thiocholine and dithionitrobenzoic acid (DTNB) (ϵ of DTNB at 412 = 13.6 mM⁻¹ cm⁻¹). The activity of the enzyme was expressed as nanomoles of the complex formed per minute, per milligram of protein.

2.6. Heart rate assessment

Immediately after the end of the exposure, 10 daphnids per replicate were recovered from the test media and heart rate determinations were made. Briefly, each single animal was placed in a microscopic slide, the excess of medium was aspirated to immobilize the daphnid, and using an Olympus CKX41 inverted microscope a video was recorded at 60 frames per second for >1 min using a microscope-mounted digital camera. The heart rate activity was measured by a frame-by-frame method using VLC media player by playing single frames of the video (1 min) at a resolution of 60 frames per second; separate phases of heart contractions were counted and recorded as beats per minute (bpm). All heart rate videos were made within 2 min of the transfer of the *D. magna* to each slide, and the animals were manipulated with the utmost care to avoid causing any handling related stress that could influence the obtained results. Room temperature was maintained stable during measurements.

2.7. DNA damage evaluation

After the end of the exposure 10 daphnids per replicate were isolated from the exposure media and the DNA damage was evaluated through the alkaline comet assay, as described by [Reis et al. \(2018\)](#). Briefly, five replicates per treatment with 10 daphnids each were gently macerated in phosphate-buffered saline (PBS), 10 % (v/v) dimethyl sulfoxide and 20 µM ethylene diamine tetra-acetic acid disodium salt. Samples were then centrifuged, and the obtained pellet was mixed with low melting point agarose at 37 °C, then placed on top of slides pre-coated with 1 % normal melting point agarose. Slides were immersed in a lysing solution (2.5 M NaCl + 100 mM EDTA + 10 mM Tris-HCl + 1 % DMSO + 10 % TritonX-100) for one hour at 4 °C, protected from light. After lysis, slides were placed in an alkaline buffer (0.3 M NaOH and 1 mM EDTA, pH 13) for 15 min for the denaturation and unwinding of the DNA, followed by

electrophoresis (10 min at 0.7 V/cm, 300 mA). Slides were then neutralized in Tris-HCl (0.4 M), dehydrated using absolute ethanol and stored in the dark until observation. The whole assay was conducted under yellow light, to prevent UV-induced DNA damage. Before observation, slides were stained with ethidium bromide (20 µg mL⁻¹). DNA damage was visually scored under a fluorescence microscope (Leica DM6 B; amplification 400×) on a 0 to 4 scale as described in [Duthie and Collins \(1997\)](#) and [Reis et al. \(2018\)](#). Per slide, 100 cells were scored and a value in arbitrary units of DNA damage was calculated by sum of the value of each class multiplied by the number of cells in each category, divided by total cells viewed. To avoid bias on the results, the observations were blind, i.e., scored without knowledge of the origin of the slide, and always by the same person.

2.8. Post-exposure behavioral analysis

After exposure, 10 daphnids per replicate were transferred to 24 well plates with 2 mL of clean ASTM medium, allocating a single organism per well, to ensure that the minimum volume of medium per organism stipulated by standard protocol is met ([OECD, 2004](#)). Each plate was composed of one replicate per treatment, i.e., 10 control organisms and 10 exposed organisms, totaling 20 wells with daphnids. Behavior analysis started after five minutes of acclimatization to new conditions. Movement of the animals was tracked employing ZebraBox (Viewpoint, Lyon, France) tracking system, using a 25 frames per second infrared camera over a period of 10 min, and following the protocol described in [Dionísio et al. \(2020\)](#) and [Sousa and Nunes \(2020\)](#), with slight adaptations. Owing to *Daphnia magna* high responsiveness to light ([Effertz and von Elert, 2014](#); [Ringelberg, 1964](#)), movement was stimulated by alternating between one cycle of dark and light periods (300D:300 L seconds). Light intensity was set at 50 % in the ZebraBox. At the end of each period (300 s) data for total swimming distance (mm) and total swimming time (seconds) was calculated. To evaluate swimming patterns, swimming activity was studied in two distinct areas of each well: an inner circle (with approximately 12.2 mm of ray) and an outer ring (with approximately 4 mm). The tendency to swim near the edges of the well was calculated by dividing the distance moved in the outer area by the total swimming distance and multiplying by 100 (%Dout), as described in [Santos et al. \(2023\)](#) and [Schnörr et al. \(2012\)](#).

2.9. Post-exposure feeding inhibition

Post-exposure feeding rate was analyzed following the procedure described by [McWilliam and Baird \(2002\)](#). Immediately after the end of the exposure period, five daphnids per replicate were transferred to new glass vessels containing 50 mL of clean ASTM with food (*Raphidocelis subcapitata*, at a concentration of 3 × 10⁵ cells mL⁻¹). The assay consisted of eight blank replicates (ASTM medium and food, but no daphnids) considered to remove the variability of possible algal growth/death during the assay period, and five replicates with 5 daphnids per treatment (control and STX exposed). Before the addition of the daphnids to the test vessels, the absorbance was measured at 440 nm (UV-1800 spectrophotometer, Shimadzu Corporation, Japan), and used to estimate algal cell density based on a previously established calibration curve. After 4 h in total darkness to avoid algal growth and under the same temperature conditions used for bulk cultures, daphnids were removed, the vials vigorously shaken, and absorbance read again in all test vessels. Individual feeding rates (number of algal cells ingested per animal per hour) were determined for each replicate by following the change in algal cell density at the beginning of the assay and after 4 h, according to [Allen et al. \(1995\)](#).

2.10. Assessment of total DNA methylation as an epigenetic biomarker

Samples for the determination of total 5-methylcytosine (5-mC) levels (10 organisms) were thawed and genomic DNA (gDNA) extraction

was performed using the MasterPure Complete DNA and RNA Purification Kit (Epicenter, Madison, WI, USA), according to the instructions of the manufacturer. DNA extraction was performed in three of the five replicates (chosen randomly) for each treatment, followed by gDNA quality assessment using a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an electrophoresis gel run to evaluate gDNA integrity. Samples with a 260/230 ratio above 1.7 and a 260/280 ratio between 1.7 and 2.1 were further assessed for gDNA concentration using a Qubit 3.0 Fluorometer (Invitrogen). Then, DNA extracts were used for quantification of total 5-mC methylation using the Methylflash™ Global DNA Methylation (5-mC) ELISA Easy Kit (Colorimetric) (Epigentek, Farmingdale, NY, USA), following the manufacturers protocol. The plate was read at 450 nm (Biotek Synergy H1 Microplate Reader with Gen5 Software 3.05) and the absolute percentage of 5-mC methylated gDNA was calculated from the optical density (OD) using the slope of a standard curve. Results were expressed in total 5-mC percentage per total DNA content.

2.11. Data analysis

For each biomarker dataset, parameters of normality (Shapiro–Wilk test) and homogeneity of variances (Levene's equal variance test) were verified. The biomarkers parameters were analyzed with an Independent Samples *t*-test (or Mann-Whitney *U* test when *t*-test assumptions were not met) to discriminate significant differences between control and exposed treatments. All statistical analysis was performed using IBM SPSS statistical software package version 27 and Microsoft® Excel. The adopted level of significance was 0.05.

The index “integrated biomarker response version 2” (IBRv2) was determined using the results of all biomarkers to provide a holistic overview of the stress caused by saxitoxin in *D. magna*. The IBRv2 was calculated as described in Sanchez et al. (2013). Briefly, each biomarker individual data (Xi) was divided by the control mean value (X0), followed by a log transformation to reduce variance ($Y_i = \log(X_i / X_0)$). Then, data were standardized ($Z_i = (Y_i - \bar{Y}) / s$, being $\bar{Y}$ the general mean, and s the standard deviation); finally, the biomarker deviation index (A) was calculated subtracting Z0 (the Z value for the control treatment) to the Zi value of the exposed treatment ($A = Z_i - Z_0$), and the A value was represented in a star plot. The IBRv2 value is the sum of the absolute value deviation of each biomarker relative to the control (A values) ($IBRv2 = \sum |A|$). As a general stress index IBRv2 provides a simple interpretation of the level of stress that an organism is subjected: the higher its value, the higher the stress caused by the toxicant.

3. Results & discussion

Saxitoxin imbalances antioxidant defenses in *D. magna* and triggers oxidative damage to critical molecules, as well as changes in physiological parameters, all preceded by oxidative stress onset and DNA methylation changes, as detailed below in groups paired per biological endpoint/biomarker. Neither the concentration of STX tested herein nor higher concentrations ($30\text{--}100 \mu\text{g L}^{-1}$) tested in trials (data not shown, but available openly from a repository – see the corresponding section at the end of the paper) caused immobilization (proxy for death) in daphnids, likely due to an acquired tolerance to phytoplanktonic toxins comparable to what has been described for other filter-feeding organisms, like copepods (Abdulhussain et al., 2020; Roncalli et al., 2016) and clams (Bricelj et al., 2005). Despite this, our obtained results pointed out significant changes of several molecular and physiological sub-lethal parameters in *D. magna*. The deleterious effects triggered by a short-term (24 h) exposure can be explained by STX chemical characteristics and its primary mechanism of action. Since STX is an alkaloid with a small molecular weight it can rapidly cross biological membranes and bind to voltage-gated channels (Bordner et al., 1975; Dos Reis et al., 2023; Ferrão-Filho et al., 2010; Handy et al., 2013; Wiese et al., 2010), therefore the toxic effects appeared in the crustaceans soon after the

exposure. The used exposure period mimics a possible pulse exposure that may occur in a freshwater ecosystem, with toxins being released into the water especially when a harmful bloom event ages and cell lysis occurs (Christensen and Khan, 2020; Rodgers et al., 2018). Although phytoplankton blooms could last for several days in natural ecosystems, once released into the water, STX and other aquatic toxins generally have relatively short persistence (half-life) under normal light conditions, due to their photosensitivity (Christensen and Khan, 2020; Funari and Testai, 2008; Testai et al., 2016). Additionally, concentration of solubilized toxins rapidly decreases owing to degradation at elevated pH values, dilution, wind mixing and adsorption in freshwater systems (Christensen and Khan, 2020; Funari and Testai, 2008; Jørgensen et al., 2022).

3.1. Effects of STX in *D. magna* oxidative stress balance and DNA integrity

In aerobic cells, reactive oxygen species (ROS) generated by normal or affected metabolic processes are removed or inactivated and kept in balanced quantities by antioxidant defenses, therefore the interplay between these radicals and the activity of antioxidant defenses is of the utmost importance for living organisms (Ayala et al., 2014; Haque et al., 2022; Ighodaro and Akinloye, 2018; Liang et al., 2011).

Data obtained from the biochemical biomarkers showed a significant decrease of CAT activity in exposed *D. magna* juveniles (Independent Samples *t*-test, $t(22) = -5.572$, $p < 0.001$; Fig. 1A). This enzyme is present in the peroxisome and prevents the formation of excessive H_2O_2 , being responsible by its decomposition once it is formed (Halliwell, 1974). The inhibition of CAT activity leaves organisms with an impaired antioxidant metabolism. A similar response of CAT activity inhibition was described in *Chlamydomonas reinhardtii* algae, a possible ecological target of STX in aquatic environments, after exposure to $0.11\text{--}0.89 \mu\text{g L}^{-1}$ of the purified toxin (Melegari et al., 2012), as well as following a chronic exposure to $1 \mu\text{g L}^{-1}$ of purified STX in *Danio rerio* larvae (Haque et al., 2022). However, not all species exhibited this response, with the oyster *Crassostrea gigas* and the scallop *Chlamys farreri* showing an increase of CAT activity in response to $20 \mu\text{g L}^{-1}$ STX exposure (Cao et al., 2018); *Daphnia similis* also showed an increase of CAT activity after exposure to mixtures of microcystin- and saxitoxins-producing cyanobacteria ($38.7\text{--}309.5 \text{ ng STX equivalents L}^{-1}$) (Ferrão-Filho et al., 2017). The conflicting results may be explained by different sensitivities of the various species to STX, by local adaptation to deal with particular toxins frequency patterns, and by variation in exposure conditions/designs among studies, e.g., testing STX-producing phytoplankton or complex cyanobacterial extracts containing toxins mixtures vs. purified aquatic toxins (Pinto et al., 2023). The exact mechanism by which STX is capable of inhibiting CAT activity is not clear, but the occurrence of oxidative stress and the consequent rise of the superoxide anion cellular levels may lead to reduced CAT enzymatic activity as antioxidant enzymes are sensitive to damage by ROS (Bagnyukova et al., 2006). Additionally, oxidative stress-related changes to the epigenetic landscape can also be responsible for fundamental changes in expression of genes coding for antioxidant enzymes, potentially leading to depleted cellular levels of these enzymes and therefore decreasing enzymatic activity, a relationship already established for other xenobiotics (Kim et al., 2018; Liu et al., 2019; Niu et al., 2015).

Previous studies have shown that the loss of CAT activity can be compensated with increased GSTs and GPx activity (Bagnyukova et al., 2006, 2005; Clemente et al., 2010; Kim et al., 2010; Van der Oost et al., 2003). Accordingly, effects on *D. magna* glutathione metabolism were noticed upon STX exposure. Our results showed a significant increase in the activity of GSTs (Mann-Whitney test, $U = 46$, $p = 0.010$; Fig. 1B), as well as of the Se-dependent GPx activity (Mann-Whitney test, $U = 13$, $p = 0.027$; Fig. 1C), with a mild, nonsignificant increase in activity verified in the case of total GPx (Mann-Whitney test, $U = 25.5$, $p = 0.185$; Fig. 1D).

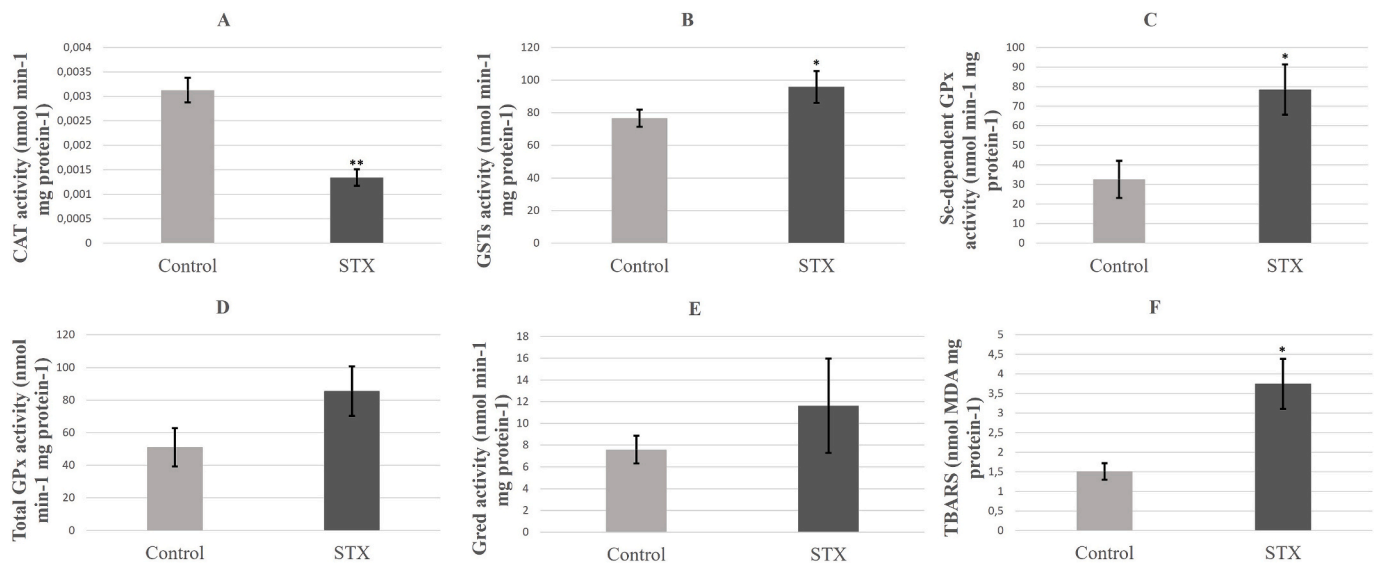


Fig. 1. Catalase (A), Glutathione S-transferases (B), Selenium-dependent Glutathione peroxidases (C), total Glutathione peroxidases (D), Glutathione reductases (E) activities; and TBARS levels (F) in *D. magna* exposed to 30 $\mu\text{g L}^{-1}$ of saxitoxin (STX, in black) and control organisms (Control, in grey). Error bars correspond to standard error of the mean values; * represents statistically differences (Independent samples *t*-test or Mann-Whitney *U* test, $p < 0.05$), ** represents statistically differences (Independent samples *t*-test or Mann-Whitney *U* test, $p < 0.001$) between the exposed and control organisms.

The enhancement of GSTs activity suggests that conjugation with reduced glutathione (GSH) may be a major pathway of STX detoxification. A similar enzymatic response of GSTs accompanied by a decrease in reduced GSH levels was also reported by two different studies in the neotropical fish *Geophagus brasiliensis* exposed to 5.15–50.78 ng STX equivalents L^{-1} in a concentration dependent manner (Calado et al., 2020; Clemente et al., 2010); the same was recorded in STX-exposed bivalve species *Crassostrea gigas* and *Chlamys farreri* (Cao et al., 2018), in *Moina micrura* exposed to STX and microcystin-producing cyanobacterial cells containing 22.1–85.9 ng STX equivalents L^{-1} , however this effect may be due to a synergistic relationship between the components of the complex cyanobacterial cell mixture (Ferrão-Filho et al., 2017), and in *C. reinhardtii* algae cells exposed to STX (Melegari et al., 2012), further substantiating the potential role of this antioxidant pathway in STX detoxification by the aquatic biota and the occurrence of conjugation of STX with reduced GSH.

Glutathione peroxidase, similarly to CAT, is responsible for the decomposition of H_2O_2 (Ighodaro and Akinloye, 2018; Liang et al., 2011). Our results showed that Se-dependent GPx activity was increased in STX-exposed organisms. Similar responses in GPx activity have been reported upon exposure to multiple STX analogues (36.7–86.5 $\mu\text{g STX equivalents/L}$) in the bivalves *Mytilus chilensis* and *Ameghinomya antiqua*, and in the gastropod *Concholepas concholepas* (Oyaneder-Terrazas et al., 2022). Accordingly, following exposure to 0.08 $\mu\text{g STX/100 g}$, the freshwater fish *Hoplias malabaricus* showed elevated GPx activity in liver tissues compared to control organisms (Silva de Assis et al., 2013); elevated liver GPx activity was also recorded in STX exposed zebrafish (Zhang et al., 2015). Taken together this evidence seems to indicate that the activation of Se-dependent GPx is a compensatory response to the lack of CAT activity. Increased GPx activity is likely to be a resistance mechanism against toxin-induced oxidative stress and lipid peroxidation (Zhang et al., 2015).

Contrarily to what could be expected considering the trend found for GSTs activity, the activity of Gred, an enzyme responsible for catalyzing the reaction of transforming oxidized GSH back to reduced GSH, was not significantly altered (Mann-Whitney test, $U = 14$, $p = 0.589$; Fig. 1E). The variability observed for Gred activity can potentially be attributed to technical caveats, since enzymatic determinations are obtained from whole organisms, thus using a pool of cells with different roles regarding antioxidant defenses may hinder the understanding of the enzymatic

responses. Despite this, the lack of a full activation of Gred may have a biological explanation, such as a sufficient availability of reduced GSH levels in cells. The STX exposure period of 24 h could be insufficient to fully deplete the reserves of reduced GSH and therefore induce a significant increase in Gred activity. However, if STX indeed prevents the full activation of Gred, this may lead to decreased cellular levels of reduced GSH, an essential player in the reactions catalyzed by the two other enzymes involved in this metabolism (GSTs and GPx, using reduced GSH as the primary cofactor), and an important direct ROS scavenger (Barker et al., 1996; Liang et al., 2011; Ramos et al., 2014; Zhang et al., 2015). The decrease in reduced GSH reserves may then compromise the efficiency of the glutathione antioxidant system in the long term. For example, Chen et al. (2020) reported damage to the antioxidant system of exposed zebrafish embryos coupled with a significant depletion in reduced GSH levels, indicating on the adverse effects to the glutathione metabolism caused by STX (14.96–29.92 $\mu\text{g L}^{-1}$). However, reduced GSH levels were not measured to fully understand if STX is indeed inhibiting Gred activity and compromising the availability of reduced GSH.

Enzymatic activity data indicates that significant alterations were observed for glutathione-related enzymes involved in the metabolism of GSH (such as GSTs and Se-dependent GPx), evidencing that STX metabolism is mediated via this intracellular ROS scavenger and showing the sensitivity of these biomarkers. Accordingly, *Daphnia* was found to be able to metabolize various toxicants, including the aquatic toxin microcystin, using the glutathione detoxification system, in particular GSTs enzymes that catalyze the conjugation of these toxins with reduced GSH (Lyu et al., 2016).

The disturbance of the fragile equilibrium between ROS and antioxidant defenses often results in uncontrolled oxidative stress, culminating in molecular damage, especially to DNA, cellular lipids, and proteins (Barzilai and Yamamoto, 2004; Gonzalez-Hunt et al., 2018; Song et al., 2020). In fact, the amount of TBARS was significantly increased by STX (Mann-Whitney test, $U = 0$, $p = 0.009$; Fig. 1F), suggesting that lipid peroxidation occurred in *D. magna* after short-term exposure to STX. Lipid peroxidation is one of the most direct effects of the accumulation of free radicals in cells (Ayala et al., 2014), hence its long-established use as an oxidative stress biomarker to indirectly assess the existence of excessive ROS and oxidative damage in cells (Jesus et al., 2023; Lykkesfeldt, 2007; Pires et al., 2021; Yavuzer et al., 2016).

The observed increase of TBARS clearly confirms that STX acts as an exogenous source of oxidative stress, that the *D. magna* global antioxidant defense was compromised after exposure, and that glutathione-related enzymes were not able to fully compensate the loss of CAT activity. Cao et al. (2018) described a similar oxidative damage scenario in two marine bivalve species exposed to concentrations of STX comparable to the ones used in this study. Additionally, lipid peroxidation and cellular abnormalities were described in fish exposed to STXs-producing cyanobacteria, confirming the ability of these neurotoxins to cause oxidative stress scenarios and necrosis in exposed aquatic biota (Clemente et al., 2010). Similar results were observed in zebrafish embryos exposed to STX, that showed extensive lipid peroxidation, DNA damage and increased apoptotic rates due to oxidative stress (Chen et al., 2020).

Having in consideration the previously described oxidative stress scenario caused by STX, the significantly increased DNA damage assessed by the Comet assay (Independent Samples *t*-test, $t(8) = -6.762$, $p < 0.001$) observed in exposed organisms (mean = 185; SD = 5.96; DNA damage score in arbitrary units) compared to the control (mean = 115; SD = 8.57; DNA damage score in arbitrary units) is not surprising. The average DNA damage level obtained for control daphnids is in line with corresponding results obtained in Parrella et al. (2015) or Reis et al. (2018), reinforcing the evidence that the tested concentration of STX induces significant DNA damage to daphnids. Consistently, STX was found to cause DNA damage and decreased cell viability in exposed freshwater fish species (Chen et al., 2020; Haque et al., 2022), and in in vitro cultured cells (Melegari et al., 2015; da Silva et al., 2014) at concentrations bellow the safety guideline (0.11–29.92 $\mu\text{g L}^{-1}$). Despite this, the exact mechanisms through which STX induces DNA fragmentation is not completely known, but we hypothesize that DNA damage is caused indirectly by oxidative damage onset. This claim is supported by the clear link between oxidative stress and DNA damage (Gonzalez-Hunt et al., 2018; Kryston et al., 2011), and by a similar indirect mechanism already described for other aquatic toxins (Žegura et al., 2008). There is also the possibility that crucial genes encoding DNA damage repair enzymes are silenced due to altered DNA methylation patterns (see Section 3.3), or that DNA damage is mediated by cell cycle processes, such as apoptosis, which is known to be caused by STX through a still elusive caspases-related process (da Silva et al., 2014). Additionally, the occurrence of DNA damage can lead to increased genomic instability and the rise of chromosomal alterations, as described by Costa et al. (2012) in the white seabream (*Diplodus sargus*) exposed to dinoflagellate extracts containing STX (1.60 $\mu\text{g STXeq kg}^{-1}$ body weight). In addition to the possible perturbation in proteome stability (Huiting and Bergink, 2020), genomic instability can lead to the need to divert extra metabolic energy to high energy-dependent DNA and chromosomal repair pathways (Cucchi et al., 2021; MacFadyen et al., 2004; Szewczuk et al., 2020), and potentially leave exposed biota with compromised fitness.

Accordingly, total protein values (mg mL^{-1}) were found to be significantly decreased (Independent Samples *t*-test, $t(8) = 2.778$, $p = 0.024$) in exposed *D. magna* (mean = 0.1474; SD = 0.01) when compared to control organisms (mean = 0.1807; SD = 0.02), which seems to consolidate the hypothesis of an oxidative damage scenario caused by STX. Decrease of protein levels may be the result of oxidative stress-related protein fragmentation and degradation (Dasuri et al., 2013; Gómez-Oliván et al., 2014; Reinheckel et al., 2000). Another study using similar STX concentrations (1–100 $\mu\text{g L}^{-1}$) described significant oxidative damage of proteins in exposed *Caenorhabditis elegans* nematodes (Wu et al., 2023). Extensive DNA damage, particularly bulky chemical-induced and UV-induced DNA lesions, are known to cause severe blocks to the elongating transcription machinery therefore affecting gene expression and protein synthesis rates (Heine et al., 2008; Lagerwerf et al., 2011). Another explanation for total protein content decrease in exposed organisms is the increase of intracellular MDA levels, that is known to inhibit protein synthesis and cause DNA adducts (Dasuri et al., 2013; Halliwell, 1974; Marnett, 1999). STX-induced

dysregulation of ribosomal-associated genes, potentially due to an altered epigenetic landscape, can also help explain the observed decrease in total protein content, as evidenced in *Daphnia pulex* fed with cyanobacteria producing microcystin (Asselman et al., 2012).

3.2. Saxitoxin neurotoxicity and related post-exposure phenotypic effects

Excessive ROS in cells can lead to the oxidation and loss of enzymes activity that regulate key physiological processes such as those related to neurotransmission. In this context, cholinesterases can be oxidized, and therefore inactivated, after exposure to a multitude of pro-oxidative compounds (Abdollahi et al., 2004; Daniel et al., 2019; Oliveira et al., 2015; Özcan Oruç et al., 2006). Consistently, we found *D. magna* total ChEs activity significantly inhibited in exposed organisms (Independent Samples *t*-test, $t(8) = -3.384$, $p = 0.010$; Fig. 2A), suggesting the potential inhibition of these enzymes' activity by STX-induced oxidative damage. Similar responses were reported in adult zebrafish exposed to cyanobacterial extracts containing three STX analogues (9.52 ng STX equivalents mg^{-1} dry cell weight) (Zhang et al., 2013). Human neural cell lines (SH-SY5Y) exposed to cyanobacterial extracts containing STX (10 $\mu\text{g L}^{-1}$) also registered a significant impairment in AChE activity and increased apoptosis rate, showcasing the strong neurotoxicity exerted by STX to neuronal cells even at concentrations considered safe to humans (Constante et al., 2022).

Organisms with decreased ChEs activity may show signs of muscle paralysis, especially in the respiratory apparatus (in vertebrates), and altered behavior patterns owing to excessive accumulation of acetylcholine in the synaptic cleft and subsequent cholinergic overstimulation (Carvalho et al., 2003; Özcan Oruç et al., 2006; Pope et al., 2005; Silva et al., 2019; Xuereb et al., 2009). Accordingly, inhibition of ChEs activity impairs neurotransmission, which is generally followed by a decrease of locomotor activity and impaired physiological parameters in *D. magna* and other aquatic biota as reported by different authors (Beauvais et al., 2000; Carvalho et al., 2003; Ferrão-Filho and da Silva, 2020; Ren et al., 2017, 2015; Silva et al., 2019). The results obtained in this study also support this outcome, with exposed daphnids showing significantly reduced total swimming time during the light period when compared to control organisms (Independent Samples *t*-test, $t(98) = 2.021$, $p = 0.046$; Fig. 2B). In the absence of the light stimulus, a slight decrease was observed (yet not confirmed statistically: Mann-Whitney test, $U = 1108.5$, $p = 0.329$) in total swimming time of exposed organisms compared to control individuals. Altogether, these results suggest that STX impairs the swimming capacity of *D. magna* and modulates their ability to respond to an external stimulus known to elicit a strong phototactic response in this species (Effertz and von Elert, 2014; Ringelberg, 1964). Adult zebrafish exposed to cyanobacterial extracts containing three STX analogues (9.52 ng STX equivalents mg^{-1} dry cell weight) exhibited similar locomotor and behavioral effects, including paralysis and reduced touch response, in combination with AChE inhibition in brain tissues (Zhang et al., 2013). Consistently, Lefebvre et al. (2005) reported rapid onset of STX-induced paralysis in Pacific herring larvae, with significant reductions in spontaneous and touch-activated swimming behavior. Despite the most obvious link between STX toxicity and reduced swimming time is its strong neurotoxic potential, the indirect effect of other STX-induced physiological effects cannot be disregarded, especially having in consideration the results obtained concerning heart rate (see below).

While total swim distance tended to or confirmedly decreased (see above), exposed daphnids did not show significant alterations concerning distance covered in the periphery of the assay wells during the dark period (Independent Samples *t*-test, $t(98) = 1.889$, $p = 0.061$), but during the light period, exposed *Daphnia* swam significantly more in the periphery of the well (mean = 56.8 %; SD = 1.43 %; Fig. 2C) compared to the control individuals (mean = 52 %; SD = 1.3 %; Fig. 2C) (Mann-Whitney test, $U = 788$, $p = 0.001$; Fig. 2C). This suggests that, although STX does not change the normal phototactic behavior showcased by

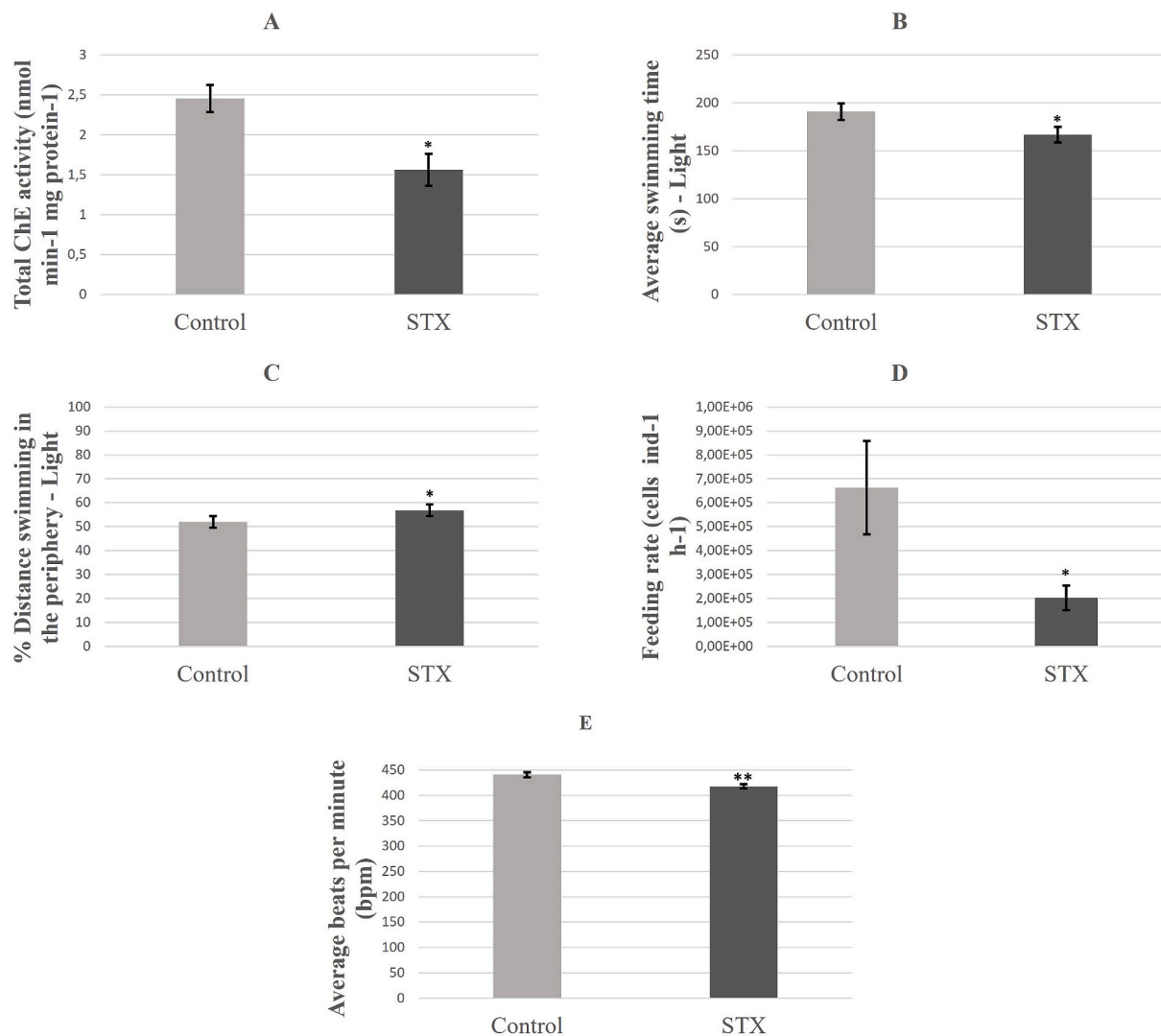


Fig. 2. Total unspecific cholinesterases activity (A), Average swimming time in the light period (B), Percentage of distance covered swimming in the periphery of the well (C); Feeding rate (D); and Heart rate (E) in *D. magna* exposed to 30 $\mu\text{g L}^{-1}$ of saxitoxin (STX, in black) and control organisms (Control, in grey). Error bars correspond to standard error of the mean values; * represents statistically differences (Independent samples *t*-test or Mann-Whitney U test, $p < 0.05$), ** represents statistically differences (Independent samples *t*-test or Mann-Whitney U test, $p < 0.001$) between the exposed and control organisms.

control *Daphnia* due to the light stimulus (swimming to the periphery of the well), it modulates *D. magna* response to light. This response by exposed organisms can also be interpreted as a sign of increased stress/anxiety due to the neurotoxicity exerted by STX (Schnörr et al., 2012). Similar responses were recorded in other species exposed to aquatic toxins and cholinesterase inhibiting compounds. For example, adult zebrafish exposed to cyanobacterial extracts containing three STX analogues (9.52 ng STX equivalents mg^{-1} dry cell weight) exhibited altered behavior patterns (increased preference to a particular area of the test vessel) putatively linked to impaired motor function and hypoxia caused by toxin-induced altered neurotransmission (Zhang et al., 2013). Juvenile totoabas (*Totoaba macdonaldi*) and striped bass (*Morone saxatilis*) juveniles exposed to domoic acid (0.8–6.4 $\mu\text{g DA g}^{-1}$) exhibited altered behavioral patterns, such as increased preference to a specific area of the test arena, reduced response to light/dark stimulus, increased paralysis, and impaired anti-predator response (Beltrán-Solís et al., 2023). Zebrafish (*Danio rerio*) larvae exposed to caffeine (20–67 mg L^{-1}) showcased a trend to increase movement at the edges of the wells, a behavior known as thigmotaxis that is a marker of stress (Schnörr et al., 2012), and a decrease in AChE activity (Santos et al., 2023). *Daphnia magna* exposed to Chlorpyrifos-oxon, which is a strong inhibitor of

AChE, showed increased response to light stimulus, therefore showcasing a significant alteration in their behavioral patterns (Bedrossiantz et al., 2020).

Overall, the behavioral results obtained seem to show that STX leaves daphnids with altered behavioral patterns even after the exposure is ceased. This might have a significant ecological impact at the population level, for example by affecting migration patterns and rendering individuals more vulnerable to predators and/or harming the search for food. Additionally, multiple studies showed that other ChEs inhibiting drugs significantly compromised *D. magna* feeding efficiency by reducing daphnids thoracic limb activity, provided that feeding and thoracic limbs movement are heavily correlated in *Daphnia* species (Christensen et al., 2005; Dionísio et al., 2020; Ren et al., 2017; Reynaldi et al., 2006; Rocha et al., 2014). Consistently, STX elicited significant post-exposure feeding inhibition in exposed animals when compared to control organisms (Mann-Whitney test, $U = 1$, $p = 0.016$; Fig. 2D). Similar feeding inhibition, accompanied by thoracic appendage beat rate inhibition, was reported by Haney et al. (1995) in *Daphnia carinata* exposed to cyanobacterial extracts containing STX (200 ng ml^{-1}). The observed feeding impairment may result in lesser amount of nutrients being ingested, leading to less metabolic energy being available or to a

compromise in its allocation to different physiological compartments. Further analysis of the STX-exposed organisms energetic metabolism status (for example, glycogen content) should be conducted to better understand the possible consequences of an STX exposure event at the energetic budget level.

Another likely consequence of ChEs activity inhibition is heart rate reduction, as suggested by our results. Namely, the number of heart beats per minute was significantly reduced in exposed daphnids compared to control (Independent Samples *t*-test, $t(98) = -3.475$, $p < 0.001$; Fig. 2E). The relationship between reduced ChEs activity, acetylcholine accumulation (known as cholinergic crisis) and reduced heart rate is well established in vertebrates (Moss et al., 2018; Ohbe et al., 2018; Pope et al., 2005). The same seems to be true in *D. magna* (unlike in other crustaceans) having a myogenic heart with various similarities to the hearts of mammals and mollusks (Bownik and Pawlik-Skowrońska, 2019; McMahon, 2001; Santoso et al., 2020; Spicer, 2001). Therefore, daphnids heart rate is modulated by inhibitory cholinergic neurons, meaning that *D. magna* heart responds to acetylcholine accumulation (as a consequence of decreased ChE activity) by decreasing number and strength of beating similarly to what happens in vertebrate species (He et al., 2023; Kaas et al., 2009; McMahon, 2001; Spicer, 2001). *Daphnia* heart is responsible for circulating the hemolymph, and its beating rate is indeed easily altered by physical and chemical disturbances (Bownik and Pawlik-Skowrońska, 2019; Ferrão-Filho and da Silva, 2020; McMahon, 2001), therefore serving as a valuable physiological biomarker linkable to neurotoxicity. The herein recorded average heart rate in control organisms (mean = 441; SD = 34.77; Fig. 2E) was comparable to the records found for different *Daphnia* species in other studies (Bownik and Pawlik-Skowrońska, 2019; Ferrão-Filho and da Silva, 2020; He et al., 2023; Santoso et al., 2020), which supports confidence in the results.

Saxitoxins were already shown to cause inactivation of nerves function and muscle relaxation, which in turn can trigger hypotension, reduced heart rate, paralysis, and subsequent death through failure of the respiratory and/or cardiovascular system (Lefebvre et al., 2005, 2004). These adverse effects on cardiovascular performance were reported in STX-exposed *D. similis* ($79.8 \text{ ng STX L}^{-1}$) (Ferrão-Filho and da Silva, 2020); *Danio rerio* embryos ($14.96\text{--}29.92 \text{ } \mu\text{g L}^{-1}$) (Chen et al., 2020); and *Danio rerio* larvae ($372 \pm 69 \text{ } \mu\text{g STX equivalents/L}$) (Lefebvre et al., 2004). Other aquatic neurotoxins, such as anatoxin-a, were also shown to reduce *Daphnia* sp. heart rate due to changes in neurotransmitters stimulation of the heart muscle, although through a different mechanism than STX (Bownik and Pawlik-Skowrońska, 2019). Whereas purified anatoxin-a ($2.5\text{--}50 \text{ } \mu\text{g mL}^{-1}$) mimics acetylcholine and induces the overstimulation of the cholinergic system (Bownik and Pawlik-Skowrońska, 2019), it can be postulated that STX alters daphnids heart rate indirectly due to changes in ChEs bioactivity and subsequent acetylcholine accumulation, leading to excessive cholinergic stimulation and the reduction of the heart rate.

However, the blockage of the Na^+ , Ca^{2+} and hERG K^+ channels in the nerve axon membranes and cardiac myocytes caused by STX could also be an alternative explanation to the recorded heart rate reduction. This mechanism would prevent the passage of these ions through the cell membrane, therefore blocking the transfer of the nerve impulse in skeletal and cardiac muscles (Chen et al., 2020; Murray et al., 2011; O'Neill et al., 2016). Additionally, *D. magna* juveniles exposed to cylindrospermopsins ($1.5\text{--}1000 \text{ } \mu\text{g L}^{-1}$) were found to have Gpr1, muscarinic acetylcholine receptor and ECM-receptor interaction genes down-regulated, which should play crucial roles in the repression and abnormal cardiovascular function (He et al., 2023). This transcriptomic response can also be happening in our study as a response to STX exposure, especially having in mind the results obtained concerning total DNA methylation (see Section 3.3), a possibility that should be further explored.

Changes to daphnids heart rate coupled with behavioral changes due to altered neurotransmission pathways result in compromised

swimming ability, reduced capacity to respond to environmental stimuli and impaired feeding efficiency, potentially compromising organisms' overall fitness. The neurotoxic and the related physiological effects of STXs on *D. magna* may have an impact on its survival and reproduction. Drawing a parallel with insecticides with similar primary mechanism of toxicity such as pyrethroids (sodium channel modulators), a vast number of studies described the profound negative impacts on *D. magna* survivability and other critical life history parameters (Felten et al., 2020; Rasmussen et al., 2013; Toumi et al., 2013).

3.3. Saxitoxin-induced general epigenetic changes

DNA methylation is one of the main epigenetic mechanisms, predominantly found in invertebrates at CpG sites within exons and introns, i.e., gene bodies of certain genes (Keller et al., 2016; Kvist et al., 2018; Lindeman et al., 2019). Increased DNA methylation in humans is normally correlated with the decrease in gene expression, whereas in daphnids and other invertebrates, a positive correlation between increased gene expression and higher methylation levels has been shown (Kvist et al., 2018; Song et al., 2020). In the present study, the average level of global 5-mC DNA methylation (mean = 1.11 %; SD = 0.198; Fig. 3) in control organisms was within the range previously reported for this species (Asselman et al., 2015; Lindeman et al., 2019; Thaulow et al., 2020). Organisms exposed to STX showed a significant decrease in total 5-mC DNA methylation levels (Mann-Whitney test, $U = 2$, $p = 0.028$; Fig. 3), indicating an epigenetic response to the toxin exposure. The observed reduction in total 5-mC DNA methylation levels in exposed organisms probably elicits a short-term gene expression inhibition, which is consistent and may correlate with the responses found for the other biomarkers used in the present work. For example, the Keap1/Nrf2 signaling pathway, which is modulated through complex epigenetic regulation is a key cellular defense system known to be capable of stimulate/repress the genetic expression of antioxidant enzymes (Guo et al., 2015). Another interesting example can be the genes involved in DNA double-strand break damage repair in *Daphnia* sp., for example *Rad50* and *MRE11* (Gomes et al., 2018), that if down-regulated can affect DNA damage repair and potentially lead to the here reported DNA fragmentation. Importantly, the (re)establishment of DNA methylation marks upon exposure ceasing requires a great amount of energy (Bultman, 2017; Donohoe and Bultman, 2012; Harshman and Zera, 2007; Song et al., 2020; Ulrey et al., 2005), which may result in further impairment of physiological parameters such as growth, heart rate,

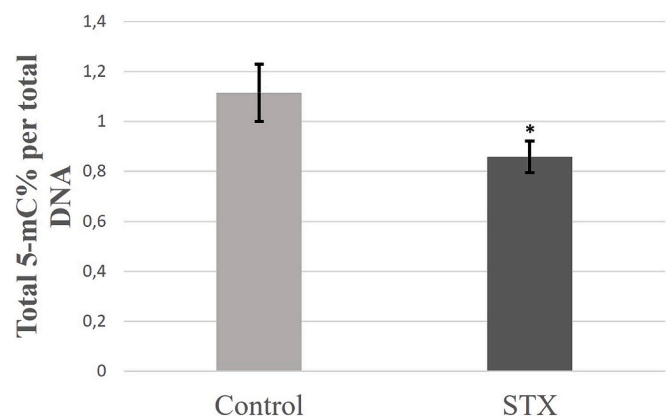


Fig. 3. Total 5-methylcytosine (5-mC) DNA methylation levels in *D. magna* exposed to $30 \text{ } \mu\text{g L}^{-1}$ of saxitoxin (STX, in black) and control organisms (Control, in grey). Error bars correspond to standard error of the mean values; * represents statistically differences (Independent samples *t*-test or Mann-Whitney U test, $p < 0.05$), ** represents statistically differences (Independent samples *t*-test or Mann-Whitney U test, $p < 0.001$) between the exposed and control organisms.

reproduction and lifespan; in likely scenarios of pulse long-term exposures, these indirect effects of STX should contribute to increase the severity of effects in the biota.

The observed decrease in total 5-mC DNA methylation may be explained by changes in DNA methyltransferases activity and/or alterations in the methionine cycle affecting the availability of methyl donors, in a similar way to changes in the methylome caused by other environmental contaminants (Angrish et al., 2018; Athanasio et al., 2018; Cuiping et al., 2023; Donkena et al., 2010; Guo et al., 2021; Harris et al., 2012; Huang et al., 2022; Lindeman et al., 2019; Šrut, 2021). Changes in DNA methyltransferases activity may be a direct result of oxidative stress that inhibits the expression and biological activity of these enzymes, critical to maintaining existing and establishing de novo DNA methylation marks (Donkena et al., 2010; Huang et al., 2022; Šrut, 2021). Considering our results regarding oxidative stress, it is reasonable to hypothesize that the observed DNA hypomethylation results from the indirect interference in DNA methyltransferases activity caused by excessive ROS accumulation in cells. Another possible explanation to the observed reduced DNA methylation could be alterations in the methionine cycle caused by STX, leading to the depletion of the primary methyl donor required for the epigenetic modifications of DNA S-adenosylhomocysteine (SAM), as reported for other environmental contaminants (Angrish et al., 2018; Ulrey et al., 2005). The reduction in total protein content in exposed organisms when compared to control organisms seems also to support a reduced protein synthesis rate as an outcome of the loss in DNA methylation. Considering the results obtained herein and reported by others, sequencing-based approaches to identify genes differentially methylated and the corresponding changes in gene expression patterns caused by STX should be considered in the near future.

A holistic view of the response of the analyzed biomarkers to STX in

D. magna is provided by the IBRv2 results diagram (Fig. 4). Through the analysis of the IBRv2 star plot it is possible to simultaneously observe the variation of the several biomarkers used between control (blue line) and exposed (orange line). The calculated IBRv2 value (Marques et al., 2016; Sanchez et al., 2013) is also presented (IBRv2 = 13.21). The IBRv2 results clearly highlight that a STX concentration deemed safe for humans by the WHO can induce remarkable overall stress on *D. magna*. This approach provides a simple tool for the visualization of biological effects by integrating different biomarker signals allowing for a quick analysis of a toxicant effect on tested biomarkers (Sanchez et al., 2013), and potentially aiding in the identification of effective biomarkers to detect and evaluate STX environment contamination. Our results seem to indicate that CAT activity, lipid peroxidation (TBARS), total 5-mC, DNA damage and total ChEs could be effective biomarkers to address to STX effects. In addition, behavioral traits may also be considered as useful parameters to determine STX effects in exposed organisms.

4. Conclusions

Our results underpinned the extensive deleterious effects of STX in *D. magna*, spanning from oxidative stress, enzymatic inhibition, DNA damage, changes in neurotransmission and related physiological and behavioral impairment, as well as epigenetic dysregulation. These adverse effects caused by a short pulse of the toxin meeting a widely accepted safety guideline (below $30 \mu\text{g L}^{-1}$) constitute a significant challenge to *D. magna*, likely translating into population thriving difficulties in the long term. Based on the evidence obtained, it could be concluded that these toxins should pose a credible hazard to the aquatic biota even at a concentration deemed safe for humans by the WHO. Additionally, the obtained results further confirm fast and prolific mechanisms of toxicity of STX, which extend beyond simply inhibiting

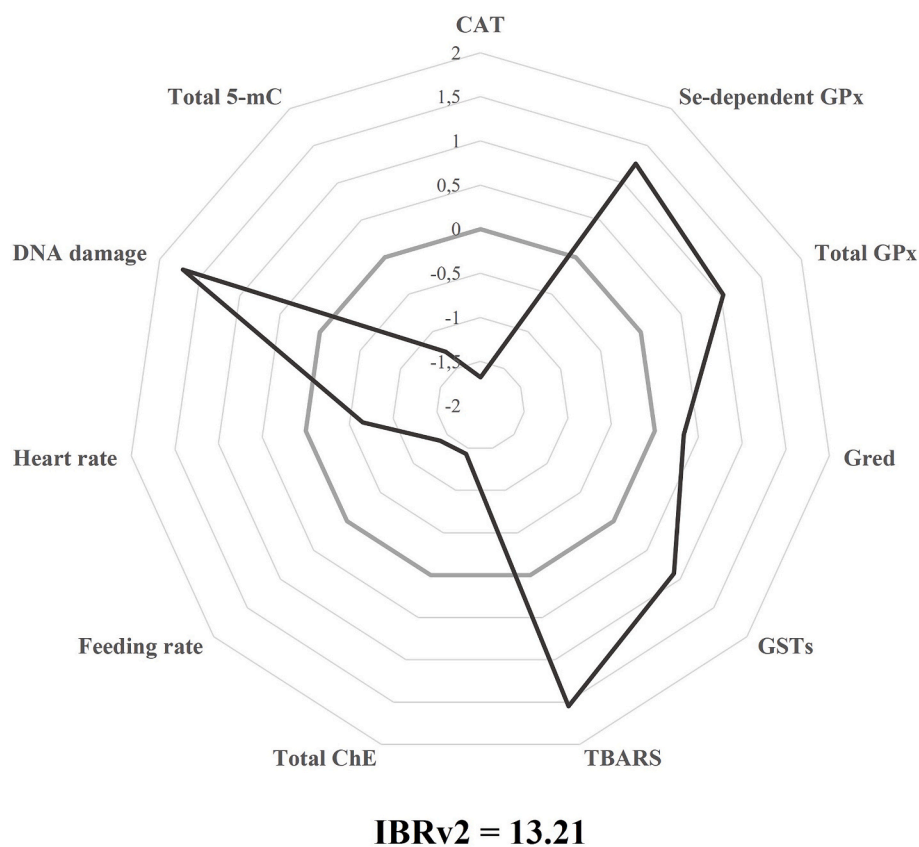


Fig. 4. Integrated biomarker response (IBRv2) value of *Daphnia magna* individuals after 24 h exposure to saxitoxin at $30 \mu\text{g L}^{-1}$. In the star plot, the black line represents the IBRv2 index of STX-exposed organisms that is presented relatively to the control organisms (grey line; 0). Values above 0 indicate biomarker induction, and values below 0 indicate biomarker inhibition.

neuronal transmission. This work will hopefully stimulate further research concerning the effects of STX and other aquatic neurotoxins on aquatic life using environmentally relevant test concentrations and exposure designs. It also contributes to the establishment of much needed Adverse Outcome Pathways linking epigenetic changes, that probably function as the molecular initiating events, to downstream subcellular and individual phenotypic changes induced by saxitoxins toxicity.

Abbreviations

5-mC	5-methylcytosine
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
ChEs	total cholinesterases
DTNB	dithionitrobenzoic acid
GPx	Glutathione peroxidases
Gred	Glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GSTs	Glutathione S-Transferases
H ₂ O ₂	hydrogen peroxide
IBRv2	integrated biomarker response version 2
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
PSP	Paralytic Shellfish Poisoning syndrome
ROS	reactive oxygen species
SAM	S-adenosylhomocysteine
Se-dependent GPx	selenium-dependent GPx
STX	saxitoxin
TBA	2-thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
WHO	World Health Organization

CRediT authorship contribution statement

Albano Pinto: Writing – original draft, Visualization, Investigation, Formal analysis. **Inês P.E. Macário:** Visualization, Investigation, Formal analysis. **Sérgio M. Marques:** Investigation, Formal analysis. **Joana Lourenço:** Investigation, Formal analysis. **Inês Domingues:** Investigation, Formal analysis. **Maria João Botelho:** Writing – review & editing, Investigation, Formal analysis. **Jana Asselman:** Writing – review & editing, Supervision, Funding acquisition. **Patrícia Pereira:** Writing – review & editing, Supervision, Conceptualization. **Joana L. Pereira:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Dataset on effects of saxitoxin (24-h exposure) of *Daphnia magna* at the individual, sub-cellular and molecular levels (Original data) (DUNAs - Repositório de Dados de Investigação da Universidade de Aveiro)

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