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RESEARCH ARTICLE

Absence of effects of different types of detergents on the cholinesterasic activity and histological markers of mosquitofish (*Gambusia holbrooki*) after a sub-lethal chronic exposure

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Abstract The release of anthropogenic compounds into the aquatic environment has been a particular concern, since some of these substances exhibit biologic activity of different types in non-target species. Among anthropogenic compounds present in the aquatic compartment, detergents are commonly found and may be responsible for physiological modifications in exposed organisms. The impairment of key physiological functions, such as neurotransmission, and tissue damage in some important organs, has been used to assess the effects of several classes of xenobiotics, including detergents, in aquatic organisms. The present study intended to assess the effect of three types of detersive compounds (sodium dodecylsulfate (SDS), benzalkonium chloride (BZC), and Triton X-100 (TX100)) in the acetylcholinesterase activity (AChE) and tissue damage (gills and liver) of *Gambusia holbrooki* after a chronic exposure to realistic levels of these compounds. SDS, BZC, and TX100 did not cause any significant alteration in AChE. Furthermore, no specific gross morphological changes were also observed in the gills and liver of the exposed individuals. It is possible to conclude that, under ecologically relevant conditions of exposure, both tissue damage and cholinesterasic impairment are not toxicological pathways affected by detergents in *G. holbrooki*.

Keywords Sodium dodecylsulfate · Triton X-100 · Benzalkonium chloride · Histological damage · Acetylcholinesterase · Biomarkers · Mosquitofish

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Introduction

Chemical contamination of the aquatic environment by anthropogenic compounds has raised special concerns regarding the effects exerted on exposed organisms. The environmental contamination by specific classes of compounds has been characterized in terms of toxicity effects on biologic systems. Therapeutic agents, cleansing products, and personal care products (namely detergents), are chemical classes of major concern, characterized by growing, indiscriminate and continuous use and biological activity, worldwide dispersed, and for which no effective treatment is usually available (Daughton and Ternes 1999; Halling-Sørensen et al. 1998; Jones et al. 2002; Miao et al. 2002; El-Gawad 2014; Fernández-Serrano et al., 2014). Due to these characteristics and widespread use, these products can be considered as potentially harmful, effective, and environmentally unfriendly compounds (Nunes et al. 2005a, b). For some detergents used as therapeutic agents or co-adjuvants in pharmaceutical formulations, extremely low concentrations were found in the aquatic ecosystems. However, for some compounds, high concentration values have been reported in the aquatic environment (Kümmerer 2001; El-Gawad 2014), allowing to conclude that in general, detergents are one of the most dispersed and abundant classes, due to its use in personal hygiene, pharmaceutical formulations, and industrial purposes (Li 2008).

Sodium dodecylsulfate (SDS) is one of the most common linear alkyl sulfate detergents (Chaturvedi and Kumar 2010), being present in a large number of formulations employed in human routine activities, such as personal hygiene and cosmetics use, including cleansing creams, liquid soaps and shampoos, bubble baths, bath and shower gels and tooth pastes (Sirisattha et al. 2004). It has been found in extremely high concentrations in natural settings, ranging from 0.2 to 10 mg L^{-1} in irrigation fields contaminated with wastewater (Dizer 1990). Benzalkonium chloride (BZC) is a quaternary ammonium cationic detergent, used as a tensioactive and preservative agent essentially in dermocosmetical and pharmaceutical products. Due to its physical-chemical characteristics, it acts on biological membranes being therefore responsible for several toxic phenomena (Okahara and Kawazu 2013; Chang et al. 2015). It is present in ophthalmic pharmaceutical preparations (drops, emulsions, suspensions, and ointments) and also in nasal sprays, and is widely employed as a disinfectant in hospitals and health centers (Marple et al. 2004; Ferk et al. 2007; Gaber et al. 2012). Since it exhibits biocidal activity, it has been thoroughly used not only as a conservative agent in a large number of cosmetic preparations but also as a disinfectant and herbicide in aquaculture facilities (Bartolomé and Sánchez-Fortún 2005). BZC has also been suggested to be a potential substitute for organotins, namely tributyltin paints, since it inhibits the fouling on ship hulls (Beveridge et al. 1998). Due to the fact that it may be extensively used and consequently released in large amounts, especially by ships, fish farms, and from sewage disposal, it has been found in the environment, namely in estuarine, brackish, and salt waters (Bartolomé and Sánchez-Fortún 2005; Martinez-Carballo et al. 2007; Clara et al. 2007). Triton X-100 (TX100) is a non-ionic detergent, commonly used in diverse laboratory procedures, such as extractions of lipid fractions of biomembranes (Liu et al. 2007), biochemical studies requiring dissolution of subcellular components or membranes (Delaunay et al. 2008), biosensor manufacture (Liu et al. 2005), and environmental studies (Cuypers et al. 2002). Besides its uses in laboratory practice, this type of surfactants may be released into the environment as a result of its applications in pulp, paper, and textile industries (Lee 1999).

The study of effects of detersive compounds has shown that several key enzymatic functions, such as the activity of acetylcholinesterase (AChE), could function as indicators of toxicity consequent to exposure to detergents. Besides its classical role in biomonitoring studies, in which AChE was used for the assessment of exposure to organophosphates and carbamates pesticides (Oliveira et al. 2007; Vioque-Fernández et al. 2007; Nunes 2011), this particular cholinesterasic form was shown to be responsive to detersive action (Guilhermino et al. 2000; Arduini et al. 2006) and to other compounds, such as metals (Labrot et al. 1996) and complex mixtures (Payne et al. 1996). Besides the evaluation of biochemical parameters, histopathological biomarkers can be used in environmental screening, since it allows examining specific target organs, such as gills and liver, which are responsible for vital functions, such as respiration, excretion, accumulation, and biotransformation of xenobiotics in fish (Wood and Soivio 1991; Olsson et al. 1996). Exposure to chemical contaminants can cause a number of damages and injuries in different fish organs suitable for histological assessment in searching for cells and tissue damages (Bernet et al. 1999).

In order to study the potential effects concerning the impairment of neurotransmission in organisms exposed to detergents, the present study involved the selection of three chemically distinct compounds, with common detersive properties (SDS, BZC, and TX100). Furthermore, the effects of the selected compounds were also assessed at the tissue level, through the qualitative analysis of the histological alterations on fish gills and liver. More than just a simple evaluation of ecotoxicological effects, one of the aims of this study was to clarify the putative mechanisms of toxic action elicited by different types of detergents, using the above-mentioned tools.

Material and methods

Capture of test organisms

Gambusia holbrooki, also known as mosquitofish, is a worldwide spread Poeciliidae fish that, due to its invasive nature and high adaptability to adverse conditions, is found in all hydrographic basins of the Iberian Peninsula (Cabral and Marques 1999). It is easy to capture, can be reared under laboratory-controlled conditions, and is adapted to several testing protocols (Nunes et al. 2008).

Fish were captured in Pateira de Fermentelos (40° 34 48″ N, 8° 31 12″ W), a natural lake found in the central region of Portugal within the hydrographic basins of the rivers Cértima and Águeda (Ahmad et al. 2006). Fish were captured with hand nets and, after capture, individuals (males and sexually immature females) with size comprised between 2.0 and 2.5 cm were kept alive; all other individuals were immediately discarded. The selected individuals, to be used in the subsequent testing, were transported in the natural medium found in the lake to the laboratory facilities. Here, they were kept under laboratory-controlled conditions (dechlorinated tap water, temperature 20 °C, photoperiod 16 h L^{-1}: 8 h day^{-1}, continuous aeration) for 1 month before toxicity tests. Animals were fed daily ad libitum with commercially available fish food (Sera Vipan ®).

Chemicals

Benzalkonium chloride (BZC), Triton X-100 (TX100), acetylthiocholine iodide, 5,5 -dithio-bis-γ-nitrobenzoic acid

(DTNB), and γ-bovine globulins were purchased from SIGMA (USA). Bradford reagent was purchased from Bio-Rad UK. SDS 99 % pure was purchased from Merck Germany.

Exposure to the detergents

In vivo studies were performed through exposure of fish to sub-lethal concentrations of SDS, BZC, and TX100, for a period of 28 days, generally following the recommendations of the OECD 215 guideline (OECD 2000). Ranges of concentrations used in this study were chosen according to previously calculated lethal concentration $(LC)_{50}$ values, available in the literature. Nunes et al. (2005a) calculated a 96-h LC_{50} for SDS with *G. holbrooki* of 15.1 mg L^{-1}. The work by Buhl and Hamilton (2000) allowed calculating a 96-h LC_{50} for SDS with rainbow trout (*Oncorhynchus mykiss*) of 24.9 mg L^{-1}. The review by Cserháti et al. (2002) also summarized LC_{50} values for SDS, with distinct aquatic organisms, such as crabs (*Callinectes sapidus*; $LC_{50} = 9.8$ mg L^{-1}), grass shrimp (*Palaemonetes* spp.; $LC_{50} = 34$ mg L^{-1}), and misids ($LC_{50} = 48$ mg L^{-1}). SDS was tested in the following nominal concentrations: 0.05, 0.10, 0.20, 0.40, and 0.80 mg L^{-1}, which were at least two orders of magnitude below previously calculated LC_{50} values for aquatic organisms.

The compilation prepared by Mayer and Ellersieck evidenced a 96-h LC_{50} of 11.5 mg L^{-1} for rainbow trout exposed to benzalkonium chloride (Mayer and Ellersieck 1986). Nominal concentrations of this toxicant were 0.025, 0.050, 0.100, 0.200, and 0.400 mg L^{-1}, which were also at least two orders of magnitude below previously calculated LC_{50} values, for aquatic organisms.

According to the compilation by Crompton (2007), Triton X-100 acute toxicity towards aquatic organisms occurs in the range of the 10 to 100 mg L^{-1}; data presented by the chemical manufacturer General Electric Healthcare also indicate a toxicity from 4.5 to 6 mg L^{-1} for *Pimephales promelas*, and from 12 to 531 mg L^{-1} for *Lepomis macrochirus* (GE Healthcare 2006). The selected nominal levels of exposure were 0.00025, 0.0005, 0.0010, 0.0020, and 0.0040 mg L^{-1} for TX100, which were three to four orders of magnitude below previously published lethality data. This choice was justified by the low use of this substance, which is not comparable to either SDS or BZC. By testing low doses of TX100, we intended to obtain ecologically realistic data.

Each assay had an independent control (non-exposed fish). Fish (with size comprised between 2.0 and 2.5 cm, and weight of 0.140 ± 0.03 g) were individually exposed in 200 mL of dechlorinated tap water. Ten replicates were used per treatment (10 individually exposed fish). Abiotic conditions were controlled during the exposure period (photoperiod 16 h L^{-1}; 8 h day^{-1}, temperature of 20 ± 1 °C, continuous aeration). Food was supplied ad libitum during exposure, once every 2 days. Media were replaced twice every week. Exposure apparatuses were composed of plastic containers, previously thoroughly rinsed with distilled water. Parameters such as mortality, pH, temperature, and dissolved oxygen were monitored during exposure for test validation purposes. After exposure, fish were processed for the determination of acetylcholinesterasic activity and observation of histological alterations.

The use of test organisms was previously sanctioned by the Ethical Committee of the institution where the work was carried out. This work took into consideration the Portuguese animal welfare testing regulations (DL 113/2013).

Determination of acetylcholinesterasic activity

After the end of exposure, five animals per treatment were anesthetized by immersion in an ice-water (4 °C) bath (Wilson et al. 2009), euthanized by decapitation, and head tissues were homogenized in ice-cold phosphate buffer (0.1 M, pH = 7.2). Homogenized tissues were centrifuged at 3300 G for 3 min and supernatants were used for enzymatic determinations. Data published by Nunes et al. (2005b) showed that the main cholinesterasic form in the head tissue of *G. holbrooki* was acetylcholinesterase. The activity of AChE was determined by the method of Ellman et al. (1961) adapted to microplate, but using 0.050 mL of fish head homogenate and 0.250 mL of the reaction mixture. Protein concentration in the samples was determined according to the method of Bradford (1976) adapted to microplate, in order to express enzymatic activities as function of the protein content of the analyzed samples.

Histological evaluation

The organs (gill and liver) of five individuals per treatment were fixed in Bouin solution (24 h); decalcified (12 h, only for gills); dehydrated through a graded series of alcohols (70, 80, 90, and 100 %); cleared with xylene; embedded in paraffin wax (56–58 °C); and sectioned (5–7 μm) using a manual microtome (Reichert-Jung 2030). Sections were stained with hematoxylin-eosin, mounted with DPX in glass slides, and examined at ×100 and ×400 by light microscopy (Olympus CX41). Micrographs were taken using a digital camera (Olympus SC30). Identification of the histological alterations in fish was based on standard protocols (Takashima and Hibiya 1995; Jagoe et al. 1996) and prevalence of histopathological findings in gills and liver were recorded.

Statistical analysis

After testing for normality and homogeneity of variances, acetylcholinesterasic activity data were compared by one-way analysis of variance, followed (if needed) by a Dunnet

multi-comparison test to discriminate differences of treatments in relation to the control treatments. Chi-square analysis was used to test differences in the prevalence of histological alterations between treatments within each experiment. The adopted level of significance (α) was 0.05. Data were presented as mean and respective standard error. Statistical analyses were performed with the software Sigmaplot 11.

Results

The chronic exposure of *G. holbrooki* to BZC did not cause any significant alteration in AChE activity ($F = 2.95$; d.f. = 5.24; $p > 0.05$; Fig. 1a). However, it was noticeably a non-significant, albeit evident, rise in the cholinesterasic activity of exposed organisms. Acetylcholinesterasic activity of animals exposed to SDS was also not altered when compared to control ($F = 1.88$; d.f. = 5.24; $p > 0.05$; Fig. 1b). A similar finding was obtained after exposure of organisms to TX100 ($F = 0.8$; d.f. = 5.24; $p > 0.05$; Fig. 1c).

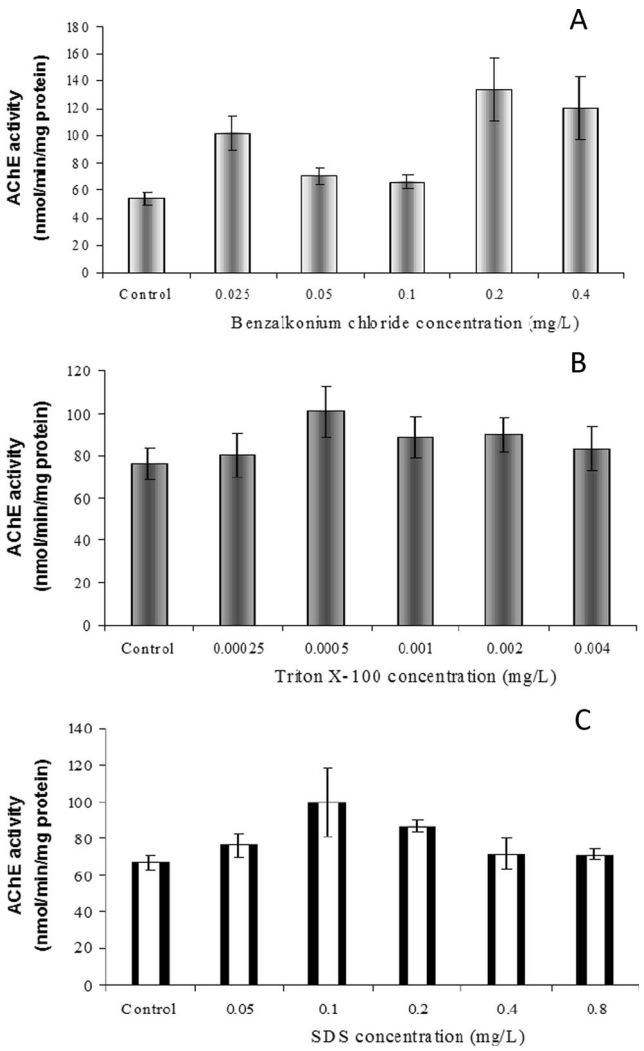

Fig. 1 Chronic effects of sub-lethal concentrations of BZC (**a**), SDS (**b**) and TX100 (**c**) on AChE activity (mean ± SE) of *Gambusia holbrooki*. There are no significant differences between experimental groups (one-way ANOVA, $p > 0.05$)

No specific gross morphological alterations were observed in the organs of fish in any of the treatments. Almost all gills presented a normal architecture (Fig. 2a), but a few presented intraepithelial oedema, epithelial lifting, and partial fusion of the secondary lamellae (respectively, 27, 16, and 11 % of the overall individuals) (Fig. 2b). Livers showed a normal architecture (Fig. 2c), but some of them exhibited some degree of cytoplasmic vacuolization of hepatocytes (11 % of the overall individuals) (Fig. 2d). These histological changes were, however, observed for all individuals, exposed and unexposed, without no evidence of any dose-effect relationship (SDS: $X^2 = 12.267$, $n = 15$, $p = 0.659$; BZC: $X^2 = 8.715$, $n = 15$, $p = 0.892$; TX100: $X^2 = 5.218$, $n = 15$, $p = 0.990$).

Discussion

Due to the fact that detergents are common anthropogenic compounds that are released into the wild, it is with great concern that their effects are assessed by ecotoxicologists. A large number of studies point to the involvement of detergents in the impairment of key physiological functions in several test organisms. Several studies report effects such as mortality of crustaceans (Warne and Schifko 1999; Chukwu and Odunzeh 2006; Sibila et al. 2008), growth inhibition of algal cultures (Sibila et al. 2008), genotoxicity (Liwarska-Bizukojc et al. 2005), and activity inhibition in key enzymes (Guilhermino et al. 2000; Nunes et al. 2006; Li 2008; Nunes, 2011). The fact that a large number of distinct detergents are simultaneously released into the aquatic compartment can turn the analysis of their combined effects even more complicated, since the consequent toxic activity may be enhanced due to combination effects (Warne and Schifko 1999). Thus, it is licit to conclude that a biochemical/enzymatic/histological marker for aquatic contamination and effects by detergents is most needed. According to this trend, some studies pointed acetylcholinesterase inhibition as a putative marker for detergent contamination. It has been suggested that TX100 could promote a direct interaction with this enzymatic form that could culminate in its inhibition (Millar et al. 1979). However, the general interaction of tensioactive compounds with living cells was mediated via a previously solubilization of membranar portions, to which AChE may be adherent, and the inclusion of these membranar portions into micellar structures (Foster et al. 1976). A more recent study showed that the inhibition of AChE of *G. holbrooki* by an anionic detergent (such as sodium dodecylsulfate) was reversible and was partially abolished through alteration of the dieletric constant of an aqueous medium (Nunes et al. 2005a, b). This finding suggests that the inhibition of AChE was mediated through

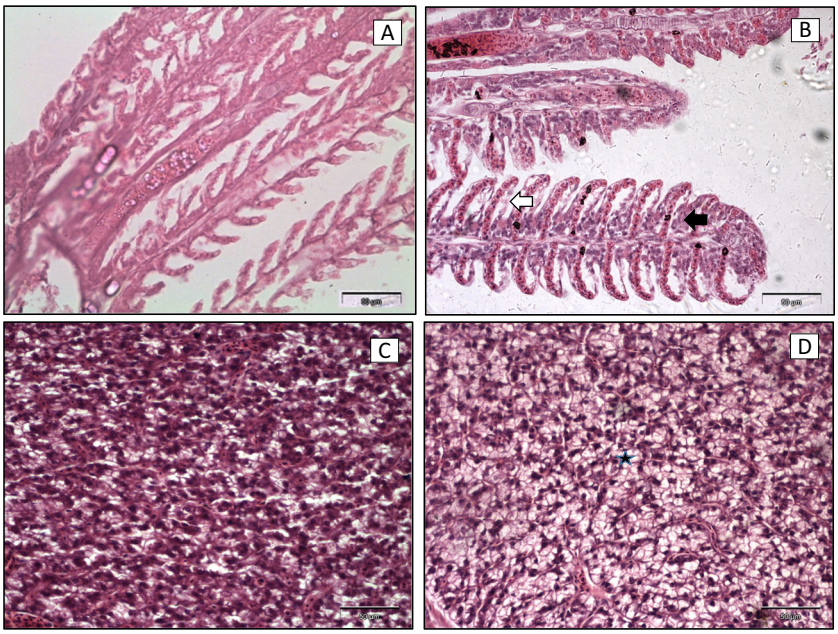

Fig. 2 **a** Gills of *Gambusia holbrooki* showing a normal architecture (control group). **b** Gills from an individual exposed to SDS (0.08 mg L^{-1}) showing epithelial lifting (*white arrow*) and interlamellar hyperplasia (*black arrow*). **c** Liver of *Gambusia holbrooki* showing a normal parenchyma (control group). **d** Liver from and individual exposed to TX100 (0.002 mg L^{-1}) showing cytoplasmatic vacuolization (*asterisk*). H&E ×40

the same mechanism described earlier, consequent to the solubilization of membrane-anchored AChE residues. Due to the fact that AChE is inside micelles, it is not possible to establish a hydrolytic interaction with the substrates used to quantify this enzyme's activity. However, it is noteworthy that this apparent inhibition caused by SDS only occurred at high doses. In fact, several previous studies reported that the deleterious interaction of SDS with AChE of aquatic organisms was possible, with inhibition of these enzymatic forms, but only for doses much higher than those employed in our study. For instance, Feng et al. (2008) reported that SDS was capable of interfering with the hydrolytic capacity of *Tilapia nilotica* AChE, but only following an in vitro exposure to extremely high levels (e.g., 0.5 and/or 1 g L^{-1}). Similarly, the work conducted by Wang et al. (2014) showed that exposure levels ranging from 0.8 to 4 mg L^{-1} of SDS were also causative of a significant inhibition of AChE of the crustacean *Moina macrocopa*. These levels are well above those we selected to develop our study, since we aimed to increase the ecological relevance of our data by setting exposure levels to amounts already reported to occur in the wild. Thus, it is possible to assume that the pathway of AChE inhibition may not occur at low (and environmentally relevant) concentrations, as shown by the here-obtained results.

None of the three types of tensioactive compounds (anionic, cationic, and non-ionic detergents) showed to have AChE inhibition properties, at concentrations similar to the ones found in the aquatic compartment and under the lethal levels documented for several fish species. In fact, the presence of several detergents may even enhance the hydrolytic activity of AChE of selected species. The work by Rosenfeld et al. (2001) showed that the presence of Triton X-100 in mammalian tissue homogenates increased the overall hydrolytic activity of the cholinesterasic forms present. The study by Martín-Valmaseda et al. (1995) showed that another form of cholinesterase, from sheep platelet, was not altered after extraction with Triton X-100. In fact, extraction of AChE from tissue homogenates is a common practice, in order to obtain purified extracts for subsequent testing (Vidal et al. 1987; Cabezas-Herrera et al. 1997 Perrier et al. 2002). This type of interaction is physical (i.e., it is established between amphiphilic tetrameric and/or globular forms of anchored AChE and the micelles of the detergent, usually Triton X-100) but may not constitute a true inhibition of the hydrolytic activity of the enzyme. This fact is of the uttermost importance for environmental monitoring, since it is not predictable, likely, or even possible, to attain in the wild the high levels of detergents necessary to elicit measurable effects on AChE activity, thus leading to a re-evaluation of the role of this enzyme as an environmental assessment tool.

The existent works concerning the effect of detergents in fish gills and liver are scarce. However, for linear alkylbenzene sulfonates (LAS), which is the most widely utilized class of synthetic anionic surfactants for cleaning purposes, changes in the gill architecture of fish have been observed after acute and chronic exposures. These include epithelial lifting, fusion of gill lamellae, stagnation of gill vessels, oedema, and aneurisms, especially after acute exposure (Alvarez-Munoz et al. 2009; Naeemi et al. 2013). Chronic exposures resulted in hypertrophy, hyperplasia, fusion of adjacent lamella, and telengeastases were observed (Hampel et al. 2008; Rejeki et al. 2008). Moreover, in the liver tissue, hepatocyte degeneration, congestion, and dilation of sinusoid and vacuolar degeneration were observed for a short-term

exposure (Naeemi et al. 2013). Concerning the hereby tested compounds, a single work exists which reported histopathological gill damage in *Oncorhynchus tshawytscha* after an acute exposure (1 h) to SDS, in levels above 3.0 mg L^{-1} (Hoskins and Dalziel 1984). However, no specific gross morphological alterations were observed in the present study. Only occasional, slight, and reversible alterations that do not alter the function of the tissues (stage I alterations: Poleksic and Mitrovic-Tutundzic 1994; Simonato et al. 2008), were observed in the gills and liver. Furthermore, since these alterations were also recorded in some fish of the control group, they cannot be associated with the presence of the tested xenobiotics and could be considered as a natural occurrence (Costa et al. 2009). Moreover, the progressive gills histological alterations recorded hereby (epithelial lifting, hyperplasia, and lamellar fusion) can be also considered responses to an unspecific stressor agent, such as water temperature or pH, and not necessarily to a chemical xenobiotic (Mallatt 1985). Additionally, cytoplasmic vacuolation of hepatocytes is frequently considered a common non-pathological liver histological change which may reflect any nutritional problem resulting from the adoption of a different feeding regime of fish in captivity (Caballero et al. 2004).

In summary, the results obtained in this work clearly showed the absence of toxic effects of the three selected detergents towards *G. holbrooki* individuals, even when fish were chronically exposed to environmentally realistic levels (especially for the detergents SDS and BZC). Since no effects were registered concerning the impairment of the cholinesterasic activity, it is possible to suggest that the cholinergic impairment pathway is not likely to occur for realistic levels of exposure to detergents. Furthermore, no tissue damage or indication of initial noxious alterations was also reported. Absence of liver damage reinforces the absence of uptake of all three detergents. On the contrary, and considering that the gills are directly exposed to the external media, the absence of toxic modifications in this tissue demonstrates the lack of toxic potential of all tested detergents, under the adopted experimental conditions. Despite the non-occurrence of toxicity, it is possible to suggest that other physiological pathways may be involved in the response to exposure to detergents, such as increased detoxification capacity or enhanced metabolism of exposed organisms. In addition, it is also possible to hypothesize that *G. holbrooki* might be refractory to detergents, under the here-proposed experimental conditions. Even only considering the here-presented lethality data (please see "Exposure to the detergents" subsection), it is possible to conclude about large differences among distinct taxa—evidencing that species-specific mechanisms of toxicity may explain this large diversity in terms of toxicity data. This assumption is in agreement with biochemical data previously obtained by Nunes et al. (2008) after exposing *G. holbrooki* to a series of xenobiotics, including the detergent SDS. In this study, authors found that the antioxidant, biochemical, behavioral, oxidative stress defense, and metabolic response of this fish species towards SDS was null or negligible, evidencing its refractory behavior when exposed to detergents. These possibilities require further research to devise the nature of toxicological interactions between aquatic organisms from distinct taxa and levels of organization and detergents.

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Compliance with ethical standards

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

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