
# Microplastics and plastic additives as contaminants of emerging concern: a multi-biomarker approach using Rhinella arenarum tadpoles

Andrés M. Attademo , Lucila M. Curi , Ana P. Cuzziol Boccioni , Carlos E. Barrios , Paola M. Peltzer , María F. Simoniello , Rafael C. Lajmanovich , Paola M. Michlig , María R. Repetti , Juan M. Ríos

PII: S2666-7657(23)00102-3
DOI: https://doi.org/10.1016/j.envadv.2023.100444
Reference: ENVADV 100444

To appear in: *Environmental Advances*

Received date: 31 August 2023
Revised date: 12 October 2023
Accepted date: 28 October 2023

Please cite this article as: Andrés M. Attademo , Lucila M. Curi , Ana P. Cuzziol Boccioni , Carlos E. Barrios , Paola M. Peltzer , María F. Simoniello , Rafael C. Lajmanovich , Paola M. Michlig , María R. Repetti , Juan M. Ríos , Microplastics and plastic additives as contaminants of emerging concern: a multi-biomarker approach using Rhinella arenarum tadpoles, *Environmental Advances* (2023), doi: https://doi.org/10.1016/j.envadv.2023.100444

## HIGHLIGHTS

- Microplastics (PE -MPs) and tetrabromobisphenol A (TBBPA) exposure inhibited AChE in tadpoles.
- The mixture of TBBPA + PE-MPs increased CbE, GR and ALP activities, and CHOL levels in tadpoles.
- PE-MPs and TBBPA increased thyroid hormone levels in tadpoles.
- PE-MPs and TBBPA + PE-MPs generated mechanical changes in the intestinal walls of tadpoles.
- The mixture of TBBPA + PE-MPs increased melanomacrophage number in the liver of tadpoles.

# Microplastics and plastic additives as contaminants of emerging concern: a multi-biomarker approach using *Rhinella arenarum* tadpoles

Andrés M. Attademo [a,e*], Lucila M. Curi [b,e], Ana P. Cuzziol Boccioni[a,e] ; Carlos E. Barrios [c,e], Paola M. Peltzer [a,e], María F. Simoniello [d], Rafael C. Lajmanovich [a,e], Paola M. Michlig[f], María R. Repetti [f] Juan M. Ríos [g]

[a] Laboratorio de Ecotoxicología. Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral (FBCB-UNL). El Pozo S/N, Santa Fe, Argentina.

[b] Instituto de Materiales de Misiones (IMAM). Facultad de Ciencias Exactas, Químicas y Naturales (FCEQyN). Universidad Nacional de Misiones (UNAM-CONICET). Félix de Azara 1552, Posadas, Argentina.

[c] Instituto de Ictiología del Nordeste (INICNE). Facultad de Ciencias Veterinarias, Universidad Nacional del Nordeste (FCV, UNNE), Sargento Cabral 2139, PC: 3400, Corrientes, Argentina.

[d] Cátedra de Toxicología (FBCB-UNL), El Pozo S/N, Santa Fe, Argentina.

[e] CONICET-Consejo Nacional de Investigaciones Científicas y Técnicas. Buenos Aires, Argentina.

[f] Programa de Investigación y Análisis de Residuos y Contaminantes Químicos, Facultad de Ingeniería Química, Universidad Nacional del Litoral, Santa Fe, Argentina.

[g] Laboratorio de Ecotoxicología, Instituto de Medicina y Biología Experimental de Cuyo (IMBECU, CCT-CONICET), 5500, Mendoza, Argentina

*Corresponding author: Andrés Maximiliano Attademo: mattademo@hotmail.com

Tel. 005493424575215 (196)

**Abstract**

Polyethylene microplastics (PE-MPs), a whitish thermoplastic polymer with numerous applications, is one of the materials most widely used in the industrial sector, whereas tetrabromobisphenol A (TBBPA) is a brominated flame retardant. The aim of this study was to analyze the effects of PE-MPs and TBBPA on *Rhinella arenarum* tadpoles at the laboratory scale. Tadpoles were chronically exposed (30 days) to four treatments: PE-MPs (60 mg $L^{-1}$), TBBPA (10 µg $L^{-1}$), their mixture (PE-MPs +TBBPA), and dechlorinated water as negative control (CO). Biomarkers of enzymatic activity (acetylcholinesterase, AChE; carboxylesterase, CbE; glutathione reductase, GR; and glutathione-S-transferase, GST), hepatic physiological alteration (alkaline phosphatase; ALP activity, and cholesterol; CHOL level), and endocrine disruption through thyroid hormone (T4) levels were assessed. In addition, intestine and liver were histomorphologically evaluated. AChE activity in tadpoles was significantly inhibited after exposure to PE-MPs and TBBPA with respect to CO. In addition, CbE, GR, and ALP activities showed higher values in the mixture of PE-MPs + TBBPA treatment than in CO, whereas CHOL level was higher in TBBPA and PE-MPs + TBBPA treatments than in CO. GST activity did not show significant differences between treatments and CO. T4 levels increased significantly in all treatments with respect to CO. The intestinal structure of tadpoles exposed to PE-MPs and PE-MPs + TBBPA showed signs of mechanical damage. The intestinal wall of tadpoles under PE-MPs, TBBPA and PE-MPs + TBBPA treatments was thicker than that of CO individuals. The analysis of liver histology demonstrated the hepatotoxicity caused by PE-MPs + TBBPA. This study provides quantitative evidence of the harmful effects of PE-MPs, TBBPA and their mixture on enzymatic and hormonal activities, and histological evidence of intestinal wall hypertrophy and liver damage of *R. arenarum* tadpoles.

***Keywords:*** anuran tadpoles, biomarkers, polyethylene microplastics, TBBPA

## 1. Introduction

Both marine and freshwater environments receive a large amount and variety of pollutants of anthropogenic origin. The omnipresence of plastic waste at the global scale represents an environmental threat that has received increasing public attention in recent

decades (Shruti et al., 2021). Since plastics are currently considered one of the most important “technofossils”, these materials will be a long-lasting record of anthropogenic presence in this planet (Zalasiewicz et al., 2016).

Plastic pollutants are widely distributed in ecosystems in different shapes and sizes, such as megaplastics, macroplastics, mesoplastics, microplastics, and nanoplastics (Zhang et al., 2017; Thushari and Serenivathna, 2020). Since microplastics (MPs: plastic items between 0.1 and 5000 µm in size) are environmentally persistent materials, they represent a global problem mainly in freshwater environments (De Sá et al. 2018, Paul et al., 2020). Microplastic pollution has currently reached high levels and is widespread in various ecosystems (Blettler et al., 2017; Pastorino et al., 2022; Liu et al., 2022), including freshwater environments of the Earth's poles (Citterich et al., 2023). Moreover, the role of MPs as vectors of pollutants has become a topic of debate in recent years (Santos et al., 2021; Arienzo et al., 2021). Polyethylene (PE) is the most widely used polymer in plastic material production (Horton et al., 2017). Although studies of the effects of PE-MPs on aquatic organisms have increased in the last years, the literature focusing on amphibians is scarce (Araújo et al., 2020a, 2021; Balestrieri et al., 2022). It has been recently demonstrated that PE-MPs particles can bioaccumulate in amphibians (Araújo and Malafaia, 2021). PE-MPs were found to cause nuclear abnormalities in erythrocytes (Araújo et al., 2020a), hepatotoxicity effects in the liver of *Physalaemus cuvieri* tadpoles (Araújo et al. 2020b), and alterations of the normal social and antipredator behavior of that species (Araújo and Malafaia, 2020). These particles were found to modify the metabolic activities of enzymes (e.g., glutathione -*S*- transferase, carboxylesterase, and phosphatase) in other neotropical anuran species (Attademo et al., 2022; Lajmanovich et al., 2022). Because of the importance of anurans in ecosystems and their position in food

webs, the effects of PE-MPs ingestion, accumulation, and fate in the organism could reveal the environmental consequences of this type of contamination over time.

The use of additives during plastic production has increased in the last few years (Malkoske et al., 2016; Ríos et al., 2023). The ecological impact of exposure to different PE-MPs particles and their additives and the consequences of their toxicity have still not been fully explored. The virgin polymer is treated with chemical additives to improve physical and chemical characteristics, such as elasticity, UV- and fire-resistant, durability, and color (Horton et al., 2017; Sajjad et al., 2022). Hence, final plastic products contain plastic additives of concern for human health, including phthalates, bisphenols, and flame retardants (Galloway, 2015). Tetrabromobisphenol A (2,2,6,6 - tetrabromo-4,4-isopropylidenediphenol (TBBPA) is a brominated flame retardant used to increase temperature resistance of plastic material often exposed to heat sources (Zhou et al., 2020). It is considered highly contaminating to the environment and is mainly used in electronic devices such as TV sets, computers, laser printers, video displays, copying machines (Kitamura et al., 2005), manufacturing TBBPA-based products, e-waste recycling, and disposal sites. TBBPA is massively used (41,352 tons in 2016; WorldAnalytics, 2019) and has been detected in a variety of environments worldwide (Huang et al., 2014; McAvoy et al., 2016; Malkoske et al., 2016; Zhou et al., 2020). The toxic effects of TBBPA have been investigated in different organisms, such as microalgae (Zhang et al., 2022), shrimps (Ríos et al., 2023), and fish (Kiuper et al., 2007; Chan and Chan, 2012; Jarema et al., 2015; Yu et al., 2023). The effects of MPs and TBBPA on amphibian tadpoles have been poorly studied compared to those on mammals (Zhou et al., 2014; Dong et al., 2021). Since populations of amphibians are in decline, efforts to mitigate the main causes of this global phenomenon require more studies of this emergent contaminant (Green et al., 2020).

Vertebrates have antioxidant enzymes, including glutathione reductase (GR) and glutathione -*S*- transferase (GST) (Attademo et al., 2021). These enzymes are involved in the detoxification and biotransformation of many xenobiotics and in the defense against oxidative stress (Potega, 2022). Furthermore, carboxylesterase (CbE) enzymes catalyze the hydrolysis of a wide range of carboxylic esters, including different xenobiotics. In mammals, CbEs and acetylcholinesterase (AChE) are involved in important physiological processes (Lian et al., 2018) as well as in the activation of prodrugs and pesticides (Ross et al., 2006), and phthalate metabolism (Ozaki et al., 2017). Alkaline phosphatase (ALP) is a glycoprotein present in the outer layer of the cell membrane. Alkaline phosphatase is widespread in different body tissues; it has low substrate specificity and catalyzes the hydrolysis of phosphate esters. Alkaline phosphatase activity is an indicator of the general well-being of organisms and is involved in the detoxification of xenobiotics in hepatocytes (Banaee, 2020).

Total lipids include cholesterol (CHOL), phospholipids, and triglycerides. Lipids are the main energy reserve and are transported through the blood as CHOL and triglycerides. Hyperglycemic hormones accelerate the degradation of triglycerides into free fatty acids through lipolysis. These hormones also allow the passage of fatty acids into the blood and therefore increase free fatty acids. If lipolysis is inhibited, CHOL accumulates and its content in circulation increases. High CHOL concentration could be due to the transport of lipids from the site of synthesis for further use or to lipid accumulation and storage (Vaseem and Banerjee, 2013).

On the other hand, the thyroid hormones T3 (triiodothyronine) and T4 (thyroxine) play a key role in metabolism, growth, osmoregulation, acclimation, and development in many vertebrates (Brown, 1997; Heijlen et al., 2014). These hormones are affected by phthalates, bisphenols, and TBBPA (Jagnytsch et al., 2006; Veldhoen et al., 2006; Horie

et al., 2023; Ríos et al., 2023). In anurans, these hormones are key in metamorphosis and larval development.

The aim of this experiment was to analyze the effects of PE-MPs and TBBPA, and their mixture, on *Rhinella arenarum* tadpoles at the laboratory scale. This is the first study to evaluate the effect of those contaminants on this anuran species. Biomarkers of enzymatic activity (ACHE; CbE; GST, and GR), hepatic physiological alteration (ALP activity and CHOL level), and endocrine disruption through T4 levels were assessed. Histomorphological features of the intestine and liver were considered to identify the possible effects of the studied pollutants. We used scanning electron microscopy (SEM) to identify the possible MP uptake and/or accumulation and the effect on the digestive system of tadpoles. We hypothesized that the ingestion of PE-MPs and TBBPA might lead to an accumulation of particles on the intestinal walls, affecting enzyme activities and causing hepatic alterations, as well as hormonal and morphological changes.

## 2. Materials and methods

### *2.1. Study species and experimental design*

*Rhinella arenarum* is a common anuran species present in wetlands, agroecosystems, and riparian and urban areas of the neotropical countries Argentina, Brazil, Bolivia, Uruguay, and Paraguay. This species is listed as "not threatened" in the Red List of Amphibians of Argentina (Vaira et al., 2012). Surface *R. arenarum* egg strings of several oviposition events (50 cm per string) were collected from small temporary ponds situated in the natural floodplain of the Paraná River (31° 11′ 31″ S, 60° 9′ 29″ W). The collection site was considered unpolluted in previous studies (Attademo et al., 2022; Lajmanovich et al 2022). Collection was authorized by the Ministry of Environment of the Province of Santa Fe (File N° 02101-0026248-0), Argentina. Eggs were immediately transported in

dechlorinated tap water (DTW) to the laboratory, and acclimated to12-h light/dark cycle at 24 ± 2 °C (Attademo et al., 2022). Embryos were kept under these conditions in a communal aquarium and fed boiled lettuce until the start of the bioassay, when they were at premetamorphic development stages (Gosner stage 27-29, Gosner 1960).

The ecotoxicity bioassay was performed in a set of 1-L glass aquaria under controlled laboratory conditions (Lajmanovich et al., 2022). Treatment groups (N= 10 individuals per treatment, in triplicate) were: i) control tadpoles (tadpoles maintained in DTW), and tadpoles exposed to ii) PE-MPs (60 mg $L^{-1}$), iii) TBBPA (10 µg $L^{-1}$), and iv) a mixture of TBBPA + PE-MPs. Tadpoles were randomly selected from an aquarium and those with external morphological abnormalities or erratic movements were removed from the experiment. Solutions were renewed every 7 days for 30 days. Tadpoles were treated following the standardized protocols for laboratory experiments of the American Society of Ichthyologists and Herpetologists (2004). Specimens were euthanized using a solution of 0.1% tricaine methane sulfonate (MS-222), following the Institutional Animal Care and Use Committee of the FBCB-UNL (Res. CD N: 388/06) and the bioassay procedures followed ARRIVE guidelines (Kilkenny et al., 2010).

Polyethylene microplastics (PE-MPs; CAS number 9002-88-4, 40-48 µm particle size, density 0.9215 - 166 0.9255 gm $L^{-1}$; purity > 99%) and TBBPA (CAS number79-94-7 97% lot#MKCM2562 purity > 97%) were purchased from Sigma-Aldrich. Firstly, a primary solution was prepared (500 mg $L^{-1}$) and stored in the freezer at - 20 °C until the moment of preparing the aquarium water. The primary solution was used to spike the aquarium water (10 µg $L^{-1}$) every 7 days. To verify the correct dosage, TBBPA concentration in the aquarium weekly were measured by direct UHPLC-MS/MS analysis. (Table 1 supplementary data).

The PE-MPs concentration (60 mg $L^{-1}$) used for the study was previously tested in amphibian tadpoles (Attademo et al. 2022; Lajmanovich et al. 2022) and was suggested as a realistic pollution scenario (Anbumani and Kakkar, 2018). This selected particle size (40 - 48 µm) is within the range of sizes of items naturally consumed by wild amphibian species (Lajmanovich, 1994). The TBBPA concentration used was 10 µg $L^{-1}$, according to findings of Liu et al. (2018), who reported that the most serious case of TBBPA pollution in China was found in samples of lake water containing TBBPA at concentrations from 5 to 10 µg $L^{-1}$.

*2.2. Biomarkers*

*2.2.1. Samples homogenization*

To determine the activity of enzymes (AChE, CbE, GR, GST, and ALP) as well as CHOL levels after 30 days of control and xenobiotic exposure, 10 tadpoles per treatment were individually weighed (g) and homogenized (1:10, w/v) in ice-cold 25 mM sucrose, 20 mM Tris-HCl buffer (pH = 7.4) with 1 mM EDTA, using a polytron tissue grinder. Another pool of 10 individuals was homogenized after 30 days to determine T4 levels, following the same protocol. The homogenates were then centrifuged at 10,000 rpm at 4 ± 1°C for 15 min, and stored at - 80°C until the analysis of biomarkers.

*2.2.2. B-esterases and stress oxidative enzymes*

Total protein (TP) concentration was determined using the Biuret method (Kingbley, 1942). Activity of AChE was determined calorimetrically following the procedure of Ellman et al. (1961). The activity of AChE was expressed as nmol $min^{-1}$ $mg^{-1}$ TP (total protein).

Activity of CbE was measured using 1-naphthyl acetate (1-NA) as substrate. Briefly, the hydrolysis of 1-NA was determined according to Gomori (1953) and adapted by Bunyan and Jennings (1968). The activity of CbE was expressed as nmol $min^{-1}$ $mg^{-1}$ TP. Activity of GST was determined by method described by Habig et al. (1974) and adapted for mammal serum GST activity by Habdous et al. (2002).

The activity of GST was expressed as nmol $min^{-1}$ $mg^{-1}$ TP. GR activity was assessed following the method of Ramos-Martínez et al. (1983), and was expressed as nmol $min^{-1}$ $mg^{-1}$ TP.

*2.2.3 Physiological endpoints*

*2.2.3.1. ALP activity, CHOL and T4 levels*

The levels of ALP activity were determined using commercially available kits (Wiener Lab®), according to the producer's instructions and standardized procedures (Attademo et al., 2022). The enzymatic activity was expressed in Units $mg^{-1}$ of TP.

CHOL level was determined using a similar procedure to that used for ALP activity, and was expressed in milligrams (Units $mg^{-1}$ of TP).

Total T4 level was determined using enzyme-linked electrochemical luminescent immunoassay (ECLIA) kits (COBAS®, Roche Diagnostics, IN, USA), according to the manufacturer's instructions. The detection limit for T4 was 0.42 ng $g^{-1}$ (Attademo et al., 2021).

*2.3. Histological analysis of intestinal tract and liver, and Scanning Electron Microscopy (SEM) method*

At the end of the assay, the whole bodies of two tadpoles per treatment, fixed in 10% formalin solution, were processed according to the conventional procedures for

histological analysis of the liver and intestinal tract. Samples were dehydrated in an ethanol series, impregnated in a butyl alcohol-paraffin mixture, embedded in paraffin, serially sectioned at 3-4 μm, and mounted on slides pretreated with silane (3-amino-propyltriethoxysilane; Sigma Chemical, St Louis, MO, USA). For final observation, samples were routinely stained with hematoxylin and eosin (H & E), according to De Souza Santos et al. (2014). The sections were observed under a Leica DM500 optical microscope equipped with a Leica ICC50HD digital camera. Four micrographs (x40 magnification) of three intestinal tract sections were analyzed per treated and control tadpole. In each micrograph, the thickness of the intestinal wall was measured using ImageJ software according to Romero Arauco et al. (2007), with several modifications. The histological appearance of the intestine was evaluated and compared between control and exposed individuals.

In addition, the melanomacrophages (MMs) were counted in whole hepatic tissue from two non-consecutive histological sections per individual using the Image J software. The sections were also observed under the microscope to identify other histological alterations (Cakici, 2015; Sayed and Younes, 2016).

Thus, intestinal fragments of three treated and three control tadpoles were fixed in 10% formalin solution to scan for microplastics. The tissues were prepared for SEM analysis following the standardized method, which consisted of dehydration in increasing concentration of acetone solution (12.5, 25, 50, 75, 100%), drying at a critical point, and gold plating (Goldstein, 1992). Observations were performed under a JEOL JSM-5800 LV scanning electron microscope.

*2.4 Data analysis*

Data normality and homogeneity of variance were evaluated using Kolmogorov-Smirnov and Levene's tests, respectively. The activities of enzymes (AChE, CbE, GR,

GST, and ALP) as well as CHOL and T4 levels were compared between control and treated (PE-MPs; TBBPA or mixture of TBBPA + PE-MPs) tadpoles using Kruskal-Wallis (KW) and Dunn post-hoc tests (Zar, 1999). For the analysis of intestinal wall thickness and MM number contrast among treatments, ANOVA and Tukey's multiple comparison *a posteriori* test was performed. Values are expressed as mean ± standard error. All statistical analyses were performed using Infostat (Universidad Nacional de Córdoba, Argentina). The level of significance used was $p < 0.05$.

## 3. Results

### *3.1. B-esterase and stress oxidative enzymes*

AChE activity was significantly inhibited (KW = 9.79, $p = 0.02$; Fig. 1) in tadpoles exposed to PE-MPs (15.09 ± 1.91) and TBBPA (16.54 ± 0.88) with respect to control tadpoles (21.88 ± 1.51). In contrast, tadpoles exposed to TBBPA + PE-MPs showed increased activity of CbE (26.41 ± 0.91, KW = 14.42, $p = 0.002$, Fig. 2) and GR (0.32 ± 0.03, KW = 9.12, p = 0.02, Fig. 3) with respect to control tadpoles (CbE= 18.44 ± 0.95, GR= 0.24 ± 0.01). GST did not show significant differences among treatments (KW = 5.91, $p = 0.11$, Fig. 4).

### *3.2. ALP activity, and CHOL and T4 levels*

The activity of ALP significantly increased (KW = 14.42, $p = 0.002$; Fig 5) in the tadpoles exposed to TBBPA + PE-MPs (1.92 ± 0.10) with respect to control (1.32 ± 0.15). Tadpoles in the TBBPA treatment (17.04 ± 0.55) exhibited the highest CHOL levels (KW = 14.22, $p = 0.002$; Fig. 6), followed by tadpoles treated with TBBPA + PE-MPs (16.73 ± 0.57) and control tadpoles (11.68 ± 0.72).

T4 levels were significantly higher in *R. arenarum* tadpoles exposed to MP, TBBPA, and TBBPA + PE-MPs (KW = 11.34, $p$ = 0.01, Fig. 7) than in control tadpoles (7.30 ± 0.11).

*3.3. SEM and histological analysis of intestine*

PE-MPs were present in the intestine of the tadpoles exposed to PE-MPs and TBBPA + PE-MPs treatments (Fig. 8 B-D), and absent in tadpoles under control and TBBPA treatments, as observed in SEM images (Fig. 8 A). The histological structure of the intestine was normal in the tadpoles of the control treatment (Fig. 9 A). The intestinal wall was composed of a homogeneous mucosa epithelium of normal cell shape (Fig. 9 A). The intestinal structure of tadpoles exposed to PE-MPs and TBBPA + PE-MPs treatments showed highly irregular arrangement and shape of cells of the mucosa epithelial layer (Fig. 9 B, D). The epithelial layer was partially damaged by PE-MPs (Fig. 9 B, D). However, in tadpoles exposed to TBBPA treatment, mucosal cells of the intestinal wall structure showed moderately irregular arrangement and shape (Fig. 9 D, E). Melanocytes were also observed in the mucosa epithelial cells of TBBPA and TBBPA + PE-MPs treated tadpoles (Fig. 9 F). The intestinal wall of individuals under PE-MPs, TBBPA, and TBBPA + PE-MPs was thicker than that of control individuals (F=113.37, $p$=0.0001). However, no significant differences were observed among the individuals exposed to PE-MP, TBBPA, and TBBPA + PE-MPs treatments (Tukey's Test $p \geq 0.05$).

*3.4. Histological analysis of liver*

The number of liver MMs increased with respect to control only in tadpoles exposed to TBBPA + PE-MPs treated tadpoles (H=8.58, $p$=0.03, Fig. 10 A). The liver of tadpoles exposed to TBBPA and TBBPA + PE-MPs showed a high level of hepatocyte

vacuolization and congestion (Fig. 10 B, C), whereas a low level of vacuolization was observed in MP-treated and control tadpoles (Fig. 10 B).

## 4. Discussion

The present study provides evidence of the effects of exposure to PE-MP particles, alone or in combination with TBBPA, on a neotropical tadpole, *Rhinella arenarum*, using biochemical, physiological and histological biomarkers. The findings of this study highlight the importance of using different biomarker screening methods to characterize the ecotoxicity of these pollutants (Rochman and Hoellein, 2020). The metabolic analysis of the studied enzymes (Attademo et al., 2022) and of important metabolic parameters such as ALP and CHOL, and the histological analysis, suggest alterations of physiological condition of individuals.

Significant inhibition of AChE was observed in treated tadpoles (under both PE-MPs and TBBPA) with respect to control tadpoles, whereas in the mixture treatment (TBBPA + PE-MPs) the activity of this enzyme was lower than in control, but without significant differences. Inhibition of AChE results in an accumulation of acetylcholine, leading to continuous and excessive stimulation of the nerve/muscle fibers (Minier et al., 2008). Treatment with a glufosinate-based herbicide in a mixture with MP inhibited AChE activity in *Scinax squalirostris* tadpoles (Lajmanovich et al., 2022). The authors suggested spontaneous interactions between the herbicide and polyethylene MPs (Lajmanovich et al., 2022).

Likewise, exposure to PE-MPs and TBBPA inhibited AChE activity in a decapod crustacean (Ríos et al., 2023). On the other hand, CbE acts as a hydrolase, catalyzing the hydrolysis of a wide variety of compounds, including plastic additives such as phthalates and TBBPA (Solé et al., 2022). Similarly, CbE hydrolyzes the surface chains of

polystyrene and polyethylene (Kawai et al., 2019; Lajmanovich et al., 2022). The increased CbE activity detected in the tadpoles exposed to TBBPA + PE-MPs might be related to the enzyme hydrolytic capacity to degrade PE-MPs and TBBPA. It has been reported that several plastic additives (e.g., TBBPA) and other plasticizers are chemically bound to the polymer (Hermabessiere et al., 2019). In addition, MPs act as "Trojan horses", carrying other environmental contaminants (Schell et al. 2022) and be released -probably driven by the activity of the CbE- into the organism's gut. However, further research is needed.

Glutathione S-transferase (GST) plays a role in phase II of biotransformation, catalyzing the conjugation of electrophilic substrates and protecting the cells against the oxidative stress caused by xenobiotics (van der Oost et al., 2003). Therefore, the increase or decrease of GST activity is an indicator of a defense mechanism of tadpoles against free radicals, particularly in the natural environment (e.g., Attademo et al., 2014). In this study, at 30 days of exposure to PE-MPs and TBBPA, no change in this enzyme was observed; one of the causes of non response in chronic exposure could be habituation to the long-term conditions (Rich and Romero, 2005). Probably, the reaction occurred earlier and by the end of the assay, it was no longer noticeable. In this context, Lajmanovich et al. (2022) found that 48-h exposure to PE-MPs in amphibian larvae (*Scinax squalirostris*) produced significant changes in GST. Likewise, Ríos et al. (2023) showed that the mixture of both xenobiotics (PE-MPs and TBBPA) at environmental relevant concentration inhibits GST activity in the freshwater shrimp *Palaemonetes argentinus* at 96 h of exposure. The antioxidant system of PE-MPs -exposed organisms has shown diverse responses, which varied from induction through reduction to non-significant changes, depending on MP size, type, and concentration, as well as on the tissues and species studied (D'Costa, 2022).

Glutathione reductase (GR) increased in *R. arenarum* during 30 days of exposure to the mixture of PE-MPs and TBBPA. GR is an essential enzyme that recycles oxidized glutathione back to the reduced form, and plays a fundamental role in the cellular control of reactive oxygen species (Couto et al., 2016). The measured levels of GR indicate that PE-MPs and TBBPA affected the enzyme performance in terms of antioxidant response; therefore, this enzyme seems to be a suitable biomarker to evaluate the emerging plastic contamination in aquatic ecosystems.

Reactive oxygen species can attack polyunsaturated fatty acids through oxidation processes and lead to lipid peroxidation. High-density lipoprotein (HDL) is also known to help clear CHOL from extra hepatic tissues. Hence, a decrease in HDL caused by TBBPA could be due to lipid peroxidation, with the consequent increase in circulating CHOL levels (Javed et al., 2017). Zhu et al. (2022) suggest that bisphenol A (BPA) caused a rise in Apolipoprotein A1 (ApoA1: is the major component of HDL), and consequently a fall in plasmatic CHOL levels of the fish *Goby cyprisrarus* exposed to BPA. In contrast to these findings, we found that TBBPA activates oxidative stress through the enzyme GR (Fig. 3) and would oxidize HDL, causing an increase in CHOL levels in exposed tadpoles. Although both animal models, fish and tadpoles, have gill respiration, tadpoles also breathe through their permeable skin, which means they have more uptake routes for xenobiotics, making them more sensitive to xenobiotics in the water.

However, CHOL showed significant increases in mice exposed to BPA (Moghaddam et al., 2015). The discrepancy of these findings with previous findings would be due to the different mixtures tested in the present investigation; therefore, further studies incorporating HDL and other markers of oxidative stress should be conducted.

In this study, the significant increase in ALP activity caused by the mixture (MP+TBBPA treatment) compared to the values from the control suggests

hepatotoxicity. Similarly, higher ALP levels were observed in the fish *Nile tilapia* exposed to BPA than in the control group (Hamed and Abdel-Tawwab, 2017). TBBPA is a brominated form of bisphenol A (BPA), a compound known to have numerous endocrine-disrupting effects in wildlife and lab organisms. The high similarities of these brominated chemicals to other well-known toxic environmental contaminants such as DDTs and PCBs are a great concern for environmental and human/animal health (Kodavanti and Loganathan, 2019). Attademo et al. (2022) observed a decrease of ALP activity in PE-MPs-treated tadpoles and suggested damage of the immune system. An increase or decrease in ALP could be related to the presence of environmental stressors (Revel et al., 2020). The ingestion of TBBPA + PE-MPs may lead to change in the physiological responses.

The levels of T4 hormone were higher in all the treatments evaluated than in the control. Different studies demonstrated the toxic effects of PE-MPs and TBBPA in zebrafish *Danio rerio* (Kiuper et al., 2007; Godfrey et al., 2017; Liu et al., 2018, Yu et al., 2023), in other vertebrates (Siracusa et al. 2018; Yu et al. 2019) and in invertebrates (Ríos et al., 2023). The effect of TBBPA on the thyroid hormone (TH) system has been previously investigated (Yu et al., 2019). TH action at the cellular level is highly conserved across vertebrate species (Heimeier and Shi, 2010). Overall, TBBPA affects the hypothalamic-pituitary-thyroid axis, influencing the function of the thyroid gland (Zhou et al., 2020). TH is the most important hormone in amphibian metamorphosis (Denver et al., 2002). Many chemical, physical and biological stressors may affect the endocrine system in amphibian larvae. An increase in T4 levels was observed in crustaceans only after 96-h acute exposure to a mixture of PE-MPs and TBBPA (Ríos et al., 2023). Veldhoen et al. (2006) found that low levels of TBBPA may accelerate anuran metamorphosis through an agonistic action of TH and enhance TH-mediated gene

expression. Jagnytsch et al. (2006) also found that a high TBBPA concentration inhibited larval development in *Xenopus leavis*, suggesting endocrine disruption. Future studies should assess the effects of exposure to theses xenobiotics on anuran development.

Intestinal damage after PE-MPs ingestion has been reported in different vertebrates (Pedá et al., 2016; Jovanovic, 2017; Stock et al., 2019; Ahrendt et al., 2020; Mbugani et al., 2022; Lajmanovich et al., 2022), and in invertebrates (see Law, 2017; Wang et al., 2019). Lei et al. (2018) suggested that the effect of MP on *D. rerio* and *C. elegans* would depend on MP size. The ingestion of MP also exerts a functional and oxidative effect on organisms (Lei et al., 2018; Yu et al., 2020). Most studies available in the literature focus on fish species (see Hirt and Body-Malapel, 2020). Exposure of *X. tropicalis* tadpoles to polystyrene microspheres (1-10 µm) resulted in uptake and accumulation in gills and digestive tract after 1 h of exposure, and then elimination via feces after 6 h of exposure (Hu et al., 2016); however, the authors did not explain the consequences of the ingestion. Attademo et al. (2022) detected PE-MPs in the gut of *Scinax squalirostris* tadpoles after exposure to 60 mg $L^{-1}$ of PE-MPs and warned about the potential risk of this contaminant to aquatic organisms. The physical damage (tissue abrasion) observed after histological evaluation as well as the increase in thickness of the intestinal wall in exposed individuals highlight the potential harmful effects of the ingestion of PE-MPs alone or mixed with TBBPA. Balestrieri et al. (2022) also tested two amphibian species under realistic contamination conditions and showed that the adverse effects of MPs depended on the species and that MP intake varied with MPs characteristics such as density, size, shape and color. Several effects of plastic ingestion have been described in different organisms, such as blue mussel *Mytilus edulis* L, in which MPs are taken up into cells and cause significant effects at the tissue and cellular levels (Von Moos et al., 2012). In addition, in juveniles of the interstitial fish *Girella laevifrons*, feeding with polystyrene incorporated

in the diet produced severe mechanical lesions in intestine as well as leukocyte infiltration and hyperemia (Ahrendt et al. 2020). We hypothesize that this damage could affect the physiological process in the digestive system of anurans such as secretory, digestive, and absorptive functions. MP ingestion may also pose a risk due to chemicals related to plastics incorporated during manufacture or accumulated from contaminated environments (Law, 2017).

Signs of hepatotoxicity –the presence of hepatocyte vacuolization and congestion of blood vessels– have been observed in liver of individuals after exposure to TBBPA and the mixture of TBBPA + PE-MPs. Similarly, Araújo et al. (2020b) detected blood vessel dilation, infiltration, congestion, hydropic degeneration, and other histological alterations in the liver of *P. cuvieri* tadpoles after 7 days of exposure to MPs (60 $mgL^{-1}$). They demonstrated that the pollutant bioaccumulation in liver was correlated to different histopathological changes.

The number of MMs increased in the liver of individuals exposed to the mixture of TBBPA + PE-MPs. This result reinforces the function of MMs in detoxification and indicates the detrimental effect of the co-exposure of contaminants, as occurs in the aquatic environment. Finally, their function in detoxification of pollutants and their role as contamination biomarkers in several tissues (Aguis and Robert, 2003; De Souza Santos et al., 2014) have been confirmed in this study.

## 5. Conclusion

In recent years, the concern about plastic pollution has increased, since these products are incorporated in biota and their effects are not fully understood. Ecotoxicity of PE-MPs and TBBPA on *R. arenarum* was found to be high in terms of biochemical and physiological impairments, such as inhibition of AChE; induction of CbE, GR, and

ALP activities; and variation in CHOL and T4 levels, respectively. Those enzymes are related to immunological and physiological processes, and overall tadpole fitness could be threatened in MP-polluted environments. The histological analysis reveals the capacity of PE-MPs to damage the intestine structure, producing hypertrophy. Hepatotoxicity was also demonstrated by the alterations of the cell arrangement and the increase in MM number, mainly in the TBBPA + PE-MPs treated tadpoles. The findings of this study suggest that the exposure to PE-MPs, TBBPA, and mainly of both pollutants together by anuran tadpoles potentially affects their survival, with consequences on population viability in the mid and long term. This phenomenon might lead to ecological death and local extinction of anuran populations in Pampean wetlands where the worst pollution scenarios can occur with high levels of contaminants concentrated in lentic aquatic systems as breeding ponds of amphibians.

**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.

**Acknowledgements**

This study was supported in part by the National Agency for Promotion of Science and Technology (ANPCyT FONCyT PICT, 2018 No. 3293; and 1069), and the Course of Action for Research and Science Promotion (CAI + D-UNL, PIC No. 50620190100036LI), Argentina. We also thank Brasca J. for English style editing and comments.

**Authors Contributions**

Andres M. Attademo: Conception: Design, Execution, Interpretation and writing.

Lucila M. Curi: Execution, interpretation and writing.

Carlos E. Barrios: Execution, interpretation and writing

Rafael C. Lajmanovich: Conception, Design, Execution and Interpretation.

Paola Peltzer: Design and Interpretation.

Ana P. Cuzziol Boccioni: Execution and Interpretation.

Fernanda Simonielo: Execution and Interpretation

Paola M. Michlig: Chemical Analyses

María R. Repetti: Chemical Analyses

Juan M. Ríos: Interpretation.

**References**

Agius, C., Roberts, R.J., 2003. Review: melano-macrophage centres and their role in fish patology. J. Fish. Biol. 26(9), 499-509

Ahrendt, C., Perez-Venegas, D.J., Urbina, M., Gonzalez, C., Echeveste, P., Aldana, M., Pulgar, J., Galbán-Malagón, C., 2020. Microplastic ingestion cause intestinal lesions in the intertidal fish *Girella laevifrons.* Mar. Pollut. Bull. 151, 110795. https://doi.org/10.1016/j.marpolbul.2019.110795

Anbumani, S., Kakkar, P. 2018. Ecotoxicological effects of microplastics on biota: a review. Environ. Sci. Pollut. Res. 25, 14373-14396. https://doi.org/10.1007/s11356-018-1999-x

Araújo, A.P.C, Malafaia, G. 2020. Can short exposure to polyethylene microplastics change tadpoles' behavior? A study conducted with neotropical tadpole species belonging to order Anura (*Physalaemus cuvier*i). J. Hazard. Mater. 391, 122214.

Araújo, A.P.C., Malafaia, G., 2021. Microplastic ingestion induces behavioral disorders in mice: A preliminary study on the trophic transfer effects via tadpoles and fish. J. Hazard. Mater. 401, 123263.

Araújo, A.P.C., Silva de Melo, N.F., De Oliveira, J.A.G., Rodrigues, F.P., Fernandes, T., Andare, V.J.E., Lopes Rocha, T., Malafaia, G., 2020a. How much are microplasticsharmful to the health of amphibians? A study with pristine polyethylene microplastics and *Physalaemus cuvieri*. J. Hazard. Mater. 382, 121066.

Araújo, A.P.C., Gomes, A.R., Malafaia, G., 2020b. Hepatotoxicity of pristine polyethylene microplastic in neotropical *Physalaemus cuvieri* tadpoles (Fitzinger, 1826). J. Hazard. Mater. 386, 121992.

Araújo, A.P.C., Rocha, T.L., De Silva, D.D.M., Malafaia, G., 2021. Micro(nano)plastics as an emerging risk factor to the health of amphibians: a scientometric and systematic review. Chemosphere, 131090.

Arienzo, M., Ferrara, L., Triffuogi, M., 2021. The Dual Role of Microplastics in Marine Environment: Sink and Vectors of Pollutants. J. Mar. Sci. Eng. 9(6), 642. https://doi.org/10.3390/jmse9060642

Attademo, M.A., Peltzer, P.M., Lajmanovich, R.C., Cabagna-Zenklusen, M.C., Junges, M.C., Bassó, A., 2014. Biological endpoints, enzyme activities, and blood cell parameters in two anuran tadpole species in rice agroecosystems of mid-eastern Argentina. Environ. Monit. Assess. 186, 635–649. https://doi.org/10.1007/s10661-013-3404-z

Attademo, A.M., Lajmanovich, R.C., Peltzer, P., Cuzziol Boccioni, A.P., Martinuzzi, C., Simonielo, F., Repetti, M.R., 2021. Effects of the emulsifiable herbicide Dicamba on amphibian tadpoles: an underestimated toxicity risk? Environ. Sci. Pollut. Res. 28, 31962-31974. https://doi.org/10.1007/s11356-021-13000-x.

Attademo, M.A., Cuzziol Boccioni, A.P., Peltzer, P.M., Franco, V.G., Simoniello, M.F., Passeggi, M.C.G., Lajmanovich, R.C., 2022. Effect of microplastics on the activity of carboxylesterase and phosphatase enzymes in *Scinax squalirostris* tadpoles. Environ. Monit. Assess. 194, 718.

Balestrieri, A., Winkler, A., Scribano, G., Gazzola, A., Lastrico, G., Grioni, A., Pellitteri-Rosa, D., Tremolada, P., 2022. Differential effects of microplastic exposure on anuran tadpoles: A still underrated threat to amphibian conservation? Environ. Pollut. 303, 119137. https://doi.org/10.1016/j.envpol.2022.119137

Banaee, M., Akhlaghi, M., Soltanian, S. et al., 2020. Combined effects of exposure to sub-lethal concentration of the insecticide chlorpyrifos and the herbicide glyphosate on the biochemical changes in the freshwater crayfish *Pontastacus leptodactylus*. Ecotoxicology 29, 1500–1515. https://doi.org/10.1007/s10646-020-02233-0

Blettler, M.C.M., Ulla, M.A., Rabuffetti, A.P., Garello, N., 2017. Plastic pollution in freshwater ecosystems: macro-, meso-, and microplastic debris in a floodplain lake. Environ. Monit. Assess. 189, 581. https://doi.org/10.1007/s10661-017-6305-8

Brown, D.D., 1997. The role of thyroid hormone in zebrafish and axolotl development. Proc. Natl. Acad. Sci. U.S.A. 94, 13011–13016. https://doi.org/10.1073/pnas.94.24.13011

Bunyan, P.J., Jennings, D.M., 1968. Organophosphorus poisoning; some properties ofavian esterase. J. Agric. Food Chem. 16, 326–331

Cakici, O., 2015. Histopathologic changes in liver and kidney tissues induced by carbaryl in *Bufo tesvariabilis* (Anura: Bufonidae). Exp. Toxicol. Pathol. 67, 237-243. https://doi.org/doi:10.1016/j.etp.2014.12.003

Chan, W.K., Chan, K.M., 2012. Disruption of the hypothalamic-pituitary-thyroid axis in zebrafish embryo-larvae following waterbone exposure to BDE-47, TBBPA and BPA. Aquat. Toxicol. 108, 106-111.

Citterich, F., Lo Giudice, A., Azzaro, M., 2023. A plastic world: A review of microplastic pollution in the freshwaters of the Earth's poles. Sci. Total Environ. 869, 161847. https://doi.org/10.1016/j.scitotenv.2023.161847

Couto, N., Wood, J., Barber, J., 2016. The role of glutathione reductase and related enzymes on cellular redox homoeostasis network. Free Radic. Biol. Med. 95, 27-42.

D'Costa, A. H., 2022. Microplastics in decapod crustaceans: Accumulation, toxicity and impacts, a review. Sci. Total Environ. 832, 154963.

De Sá, L.C., Oliveira, M., Ribeiro, F., Lopes Rocha, T. Futter, M.N., 2018. Studies of the effects of microplastics on aquatic organisms: What do we know and where should we focus our efforts in the future? Sci. Total Environ. 645, 1029-1039. https://doi.org/10.1016/j.scitotenv.2018.07.207

De Souza Santos, L.R., Franco-Belussi, L., Zieri, R., Borges, R.E., de Oliveira, C., 2014. Effects of thermal stress on hepatic melanomacrophages of *Eupemphix nattereri* (Anura). Anat. Rec. 29, 864–75. https://doi.org/10.1002/ar.22884

Denver, R.J., Glennemeier, K.A., Boorse, G.C., 2002. Endocrinology of complex life cycles: amphibians. Horm. Brain Behav. 2, 469–513.

Dong, M., Li, Y. and Zhu, M., Qin, Z., 2021.Tetrabromobisphenol A: a neurotoxicant or not? Environ. Sci. Pollut. Res. 28, 54466-54476.

Ellman, G.L., Courtney, K.D., Andreas, V. Jr., Featherstone. R.M., 1961. A new and rapid colorimetric determination of cholinesterase activity. Biochem. Pharmacol. 7, 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Galloway, T.S., 2015. Micro-and nano-plastic and human health, in: Bergmann, M., Gutow, L., Klages, M. (Eds), Marine anthropogenic litter. Springer, Cham, pp. 343-66.

Godfrey, A., Abdel-Moneim, A., Sepulveda, M.S., 2017. Acute mixture toxicity of halogenated chemicals and their next generation counterparts on zebrafish embryos. Chemosphere 181, 710-712

Goldstein, J.I., Newbury, D.E, Echlin, P., Joy, D.C., Romig, A.D., Lyman, C.E., Fiori, C., Lifshin, E., 1992. Scanning Electron Microscopy and X-Ray Microanalysis. Springer, New York.

Gomori, G., 1953. Human esterases. J. Lab. Clin. Med. 42, 445–453.

Gosner, K.L., 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16(3), 183-190.

Green, D.M., Lannoo, M.J., Lesbarreres, D., Muths, E., 2020. Amphibian population declines: 30 years of progress in confronting a complex problem. Herpetologica 76(2), 97–100.

Habdous, M., Visvikis, S., Visvikis, S., 2002. Rapid spectrophotometric method for serum glutathione S-transferases activity. Clin. Chem. Acta 326, 131–142.

Habig, W. H., Pabst, M. J., Jakoby, W., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. Int. J. Biol. Chem. 249(22), 7130–7139.

Hamed, H. S., Abdel-Tawwab, M., 2017. Ameliorative effect of propolis supplementation on alleviating bisphenol-A toxicity: Growth performance, biochemical variables, and oxidative stress biomarkers of Nile tilapia, *Oreochromis niloticus* (L.) fingerlings. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 202, 63-69.

Heijlen, M., Houbrechts, A. M., Bagci, E., Van Herck, S.L., Kersseboom, S., Esguerra, C.V., Blust, R., Visser, T.J., Knapen, D., Darras, V.M., 2014. Knockdown of type 3 iodothyroninedeiodinase severely perturbs both embryonic and early larval development in zebrafish. Endocrinol. 155, 1547–1559. https://doi.org/10.1210/en.2013-1660

Heimeier, R.A., Shi, Y., 2010. Amphibian metamorphosis as a model for studying endocrine disruption on vertebrate development: Effect of bisphenol A on thyroid hormone action. Gen. Comp. Endocrinol. 168, 181-189.

Hermabessiere, L., Paul-Pont, I., Cassone, A. L., Himber, C., Receveur, J., Jezequel, R., Soudant, P., 2019. Microplastic contamination and pollutant levels in mussels and cockles collected along the channel coasts. Environ. Pollut. 250, 807-819. https://doi.org/10.1016/j.envpol.2019.04.051

Hirt, N., Body-Malapel, M., 2020. Inmunotoxicity and intestinal effects of nano-and microplastics: a review of the literature. Part. Fibre Toxicol. 17, 57.

Horie, Y., Yamagishi, T., Yamamoto, J., et al., 2023. Adverse effects of thyroid-hormone-disrupting chemicals 6-propyl-2-thiouracil and tetrabromobisphenol A on Japanese medaka (*Oryzias latipes*). Comp. Biochem. Physiol. Part C Pharmacol. Toxicol. 263, 109502.

Horton, A.A., Walton, A., Spurgeon, D.J., Lahive, E., Svendsen, C., 2017. Microplastics in freshwater and terrestrial environments: evaluating the current understanding to identify the knowledge gaps and future research priorities. Sci. Total Environ. 586, 127–141.

Hu, L., Su, L., Xue, Y., Mu, J., Zhu, J., Xu, J., Shi, H., 2016. Uptake, accumulation and elimination of polystyrene microspheres in tadpoles of *Xenopus tropicalis*. Chemosphere 164, 611-617. https://doi.org/10.1016/j.chemosphere.2016.09.002

Huang, D.Y., Zhao, H.Q., Liu, C.P., Sun, C.X., 2014. Characteristics, sources, and transport of Tetrabromobisphenol A and bisphenol A in soils from a typical e-waste recycling area in South China. Environ. Sci. Pollut. Res. 21, 5818–5826

Jagnytsch, O., Opitz, R., Lutz, I., Kloas, W., 2006. Effects of tetrabromobisphenolA on larval development and thyroid hormone-regulated biomarkers of the amphibian *Xenopus leavis*. Environ. Res. 101, 340-348.

Jarema, K.A., Hunter, D.L., Shaffer, R.M., Behl, M., Padilla, S., 2015. Acute and developmental behavioral effects of flame retardants and related chemicals in zebrafish. Neurotoxicol. Teratol. 52, 194–209. https://doi.org/10.1016/j.ntt.2015.08.010.

Javed, M., Ahmad, M. I., Usmani, N., Ahmad, M., 2017. Multiple biomarker responses (serum biochemistry, oxidative stress, genotoxicity and histopathology) in *Channa punctatus* exposed to heavy metal loaded waste water. Sci. Rep. 7(1), 1675.

Jovanovic, B., 2017. Ingestion of microplastics by fish and its potential consequences from a physical perspective. Integr. Environ. Asses. 3, 510–515.

Kawai, F., Kawabata, T., Oda, M., 2019.Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. Appl. Microbiol. Biotechnol. 103 (11), 4253–4268. https://doi.org/10.1007/s00253-019-09717-y

Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G., 2010. Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br. J. Pharmacol. 160(7), 1577.

Kingbley, G.R., 1942. The direct biuret method for the determination of serum proteins as applied to photoelectric and visual calorimetry. J. Lab. Clin. Med. 27, 840–845.

Kitamura, S., Kato, T., Lida, M., Jinno, N., Suzuki, T., Ohta, S., Fujimoto, N., Hanada, H., Kashiwagi, K., Kashiwagi, A., 2005. Anti-thyroid hormonal activity of tetrabromobisphenol A, a flame retardant, and related compounds: Affinity to the mammalian thyroid hormone receptor, and effect on tadpole metamorphosis. Live Sci. 76 (14), 1589-1601.

Kiuper, R.V., van den Brandhof, E.J., Leonards, P.E.G., van der Ven, P.W., Vos, J.G., 2007.Toxicity of tetrabromobisphenol A (TBBPA) in zebrafish (*Danio rerio*) in a partial life-cycle test. Arch. Toxicol. 81, 1-9.

Kodavanti, P. R. S., & Loganathan, B. G. (2019). Polychlorinated biphenyls, polybrominated biphenyls, and brominated flame retardants. In *Biomarkers in toxicology* (pp. 501-518). Academic Press

Lajmanovich, R.C., 1994. Contribución al conocimiento de la alimentación de larvas de la rana criolla *Leptodactylus ocellatus* (Amphibia, Leptodactylidae) en el Paraná medio, Argentina. Stud. Neotrop. Fauna Environ. 29 (1), 55-61.

Lajmanovich, R.C., Attademo, A.M., Lener, G., Cuzzion Boccioni, A.P., Peltzer, P.M., Martinuzzi, C.S., Demonte, L.D., Repetti, M.R., 2022. Glyphosate and glufosinate-

ammonium, herbicides commonly used on genetically modified crops, and their interaction with microplastics: Ecotoxicity in anuran tadpoles. Sci. Total Environ. 804, 150177.

Law, K., 2017. Plastics in the marine environment. Annu. Rev. Mar. Sci. 2017(9), 205–229. https://doi.org/10.1146/annurev-marine-010816-060409

Lei, L., Wu, S., Lu, S., et al., 2018. Microplastic particles cause intestinal damage and other adverse addicts in zebrafish *Danio rerio* an nematode *Caenorhabditis elegans*. Sci. Total Environ. 619-620, 1-8.

Lian, J., Nelson, R., Lehner, R., 2018. Carboxylesterases in lipid metabolism: from mouse to human. Protein Cell 9, 178-195.

Liu, H., Ma, Z., Zhang, T.,Yu, N., Su, G., Giesy, J.P., Yu, H., 2018. Pharmacokinetics and effects of tetrabromobisphenol a (TBBPA) to early life stages of zebrafish (*Danio rerio*) Chemosphere 190, 243-252.

Liu, Y.,You, J., Tang, Y., He, Y., Breider, F., Tao, S., 2022. Quantified effects of multiple parameters on inputs and potential sources of microplastics from a typical river flowing into the sea. ACS EST Water 4, 556–564.

Malkoske, T., Tang, Y., Xu, W., Yu, S., Wang, H., 2016. A review of the environmental distribution, fate, and control of tetrabromobisphenol A released from sources. *Sci. Total Environ.569*, 1608-1617. https://doi.org/10.1016/j.scitotenv.2016.06.062.

Mbugani, J.J., Machiwa, J.F., Shilla, D.A., Kimaro, W., Joseph, D., Khan, F.R., 2022. Histomorphological Damage in the Small Intestine of Wami Tilapia (*Oreochromis urolepis*) (Norman, 1922) Exposed to Microplastics Remain Long after Depuration. Microplastics 2020 (1), 240-253. https://doi.org/10.3390/microplastics1020017

McAvoy, D.C., Pittinger, C.A., Willis, A.M., 2016. Biotransformation of tetrabromobisphenol A (TBBPA) in anaerobic digester sludge, soils, and freshwatersediments. Ecotoxicol. Environ. Saf. 131, 143–150.

Minier, C., Forget-Letay, J., Bjørnstad, A., Camus, L., 2008. Multixenobiotic resistance, acetyl-choline esterase activity and total oxyradical scavenging capacity of the Arctic spider crab, *Hyas araneus*, following exposure to bisphenol A, tetra bromodiphenyl ether and diallyl phthalate. Mar. Pollut. Bull. 56, 1410-1415.

Moghaddam, H. S., Samarghandian, S., Farkhondeh, T., 2015. Effect of bisphenol A on blood glucose, lipid profile and oxidative stress indices in adult male mice. Toxicol. Mech. Methods 25(7), 507-513.

Ozaki, H., Sugihara, K., Watanabe, Y., Moriguchi, K., Uramaru, N., Sone, T., Ohta, S., Kitamura, S., 2017. Comparative study of hydrolytic metabolism of dimethyl phthalate, dibutyl phthalate and di(2-ethylhexyl) phthalate by microsomes of various rat tissues. Food Chem. Toxicol. 100, 217-224.

Pastorino, P., Prearo, M., Pizzul, E., Concetta, E., Renzi, M., Ginebreda, A., Barceló, D., 2022. High-mountain lakes as indicators of microplastic pollution: current and future perspectives. Water Emerg. Contam. Nanoplastics. 1, 3. https://dx.doi.org/10.20517/wecn.2022.01

Paul, M.B., Stock, V., Cara-Carmona, J., Lisicki, E., Shopova, S., Fessard, V., Braeuning, A., Sieg, H., Bohmert, L., 2020. Micro-and nano plastic-current state of knowledge with the focus on oral uptake and toxicity. Nanoscale Adv. 2, 4350.

Pedá, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T., Maricchiolo, G., 2016. Intestinal alterations in European sea bass *Dicentrarchus labrax (*Linnaeus, 1758) exposed to microplastics: preliminary results. Environ. Pollut. 212, 251–256.

Potega, A., 2022. Glutathione Mediated Conjugation of Anticancer Drugs: An Overview of Reaction Mechanisms and Biological Significance for Drug Detoxification and Bioactivation. Molecules 27, 5252.https://doi.org/10.3390/

Ramos-Martínez, J.I., Bartolomé, T.R., Pernas, R.V., 1983. Purification and properties of glutathione reductase from hepatopancreas of *Mytilus edulis* L. Comp. Biochem. Physiol. B 75(4), 689-692.

Revel, M., Yakovenko, N., Caley, T., Guillet, C., Châtel, A., Mouneyrac, C., 2020. Accumulation and immunotoxicity of microplastics in the estuarine worm Hediste diversicolor in environmentally relevant conditions of exposure. Environ. Sci. Pollut. Res. 27, 3574-3583.

Rich, E. L., & Romero, L. M., 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. Am. J. Physiol.-Regul., Integr. Compar. Physiol. *288*(6), R1628-R1636.

Ríos, J.M., Attademo, A.M., Horie, Y., Ginevro, P.M., Lajmanovich, R.C., 2023. Sublethal Biochemical Effects of Polyethylene Microplastics and TBBPA in experimentally exposed Freshwater Shrimp *Palaemonetes argentinus*. Biology 12, 391.

Rochman, C.M., Hoellein, T., 2020. The global odyssey of plastic pollution. Science 368 (6496), 1184–1185

Romero Arauco, L. R., De Stéfani, M. V., Okada Nakaghi, L. S., Lobato de Oliveira-Bahia, V. R., 2007. Histologia do rim, fígado e intestino de girinos de rã-touro (*Rana catesbeiana*) alimentados com dietas contendoprópolis. Ciênc Rural, 37, 1436-1441. [Histology of the kidney, liver and intestine of bullfrog tadpoles (*Rana catesbeiana*) fed diets containing propolis. Rural Sci. 37, 1436-1441.

Ross, M.K., Borazjani, A., Edwards, C.C., Potter, P.M., 2006. Hydrolytic metabolism of pyrethroids by human and other mammalian carboxylesterases. Biochem. Pharmacol. 71, 657-669.

Sajjad, M., Huang, Q., Khan, S., Khan, M.A., Liu, Y., Wang, J., Lian, F., Wang, Q., Guo, G., 2022. Microplastic in the soil environment: A critical review. Environ.Technol. Innov. 27, 102408.

Santos, L.H., Rodríguez-Mozaz, S., Barceló, D., 2021. Microplastics as vectors of pharmaceuticals in aquatic organisms – An overview of their environmental

implications. Case Stud. Chem. Environ. Eng. 3, 100079. https://doi.org/10.1016/j.cscee.2021.100079

Sayed, A.H., Younes, H.A.M., 2016. Melanomacrophage centers in *Clarias gariepinus* as an immunological biomarker for toxicity of silver nanoparticles. J. Microsc. Ultrastruct. 5, 97–104. https://doi.org/10.1016/j.jmau.2016.07.003

Schell, T., Rico, A., Cherta, L., Nozal, L., Dafouz, R., Giacchini, R., & Vighi, M. (2022). Influence of microplastics on the bioconcentration of organic contaminants in fish: Is the "Trojan horse" effect a matter of concern?. Environ. Pollut. 306, 119473.

Shruti, V.C., Pérez-Guevara, F., Kutralam-Muniasamy, G., 2021. The current state of microplastic pollution in the world's largest gulf and its future directions. Environ. Pollut. 291, 118142 .

Siracusa, J.S., Yin, L., Measel, E., Liang, S., Yu, X., 2018. Effects of bisphenol A and its analogs on reproductive health: a mini review. Reprod. Toxicol. 79, 96–123. https://doi.org/10.1016/j.reprotox.2018.06.005

Solé, M., Bassols, A., Labrada-Martagón, V., 2022. Plasmatic B-esterases as potential biomarkers of exposure to marine plastics in loggerhead turtles. Environ. Res. 213, 113639. https://doi.org/10.1016/j.envres.2022.113639

Stock, V., Böhmert, L., Lisicki, E., Block, R., Cara-Carmona, J., Selb, R., Pack, L.K., Lichtenstein, D., Voss, L., Henderson, C.J., Zabinsky, E., Sieg, H., Braeuning, H., Lampen, A., 2019. Uptake and effects of orally ingested polystyrene microplastic particles in vitro and in vivo. Arch. Toxicol. 93, 1817–1833 https://doi.org/10.1007/s00204-019-02478-7

Thushari, J.D.M., Senevirathna, D.M., 2020. Plastic pollution in the marine environment. Helyon 6(8), e04709.

Vaira, M., Akmentis, M., Attademo, A.M., et al. 2012. Categorización del estado de conservación de los Anfibios de la República Argentina. Cuadernos de Herpetología. 26 (1), 131-159.

Van der Oost, R., Beyer, J., Vermeulen, N.P., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13(2), 57-149. https://doi.org/10.1016/S1382-6689(02)00126-6

Vaseem, H., Banerjee, T.K., 2013.Contamination of the River Ganga and its toxic implication in the blood parameters of the major carp *Labeorohita* (Ham). Environ. Sci. Pollut. Res. 20, 5673–5681. https://doi.org/10.1007/s11356-013-1570-8

Veldhoen, N., Bodds, A., Walzak, K., Helbing, C.C., 2006. Exposure to tetrabromobisphenol-A alters TH-associated gene expression and tadpole metamorphosis in the Pacific tree frog *Pseudacris regilla*. Aquat. Toxicol. 78, 292-302.

Von Moos, N., Burkhardt-Holm, P., Kohler, A., 2012. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. Environ. Sci. Technol. l46(20), 11327–11335. https://doi.org/10.1021/es302332w

Wang, Y., Mao, Z., Zhang, M., et al., 2019. The uptake and elimination of polystyrene microplastics by the brine shrimp, *Artemia parthenogenetica*, and its impact on its feeding behavior and intestinal histology. Chemosphere 234, 123–31.

World Analytics, 2019. Global Tetrabromobisphenol-A (TBBPA) (CAS 79-94-7) Market Competitive Analysis, Market Size, Share, Consumption, Production Forecast 2019-2023. https://worldanalytics24.com/global-tetrabromobisphenol-a-TBBPA-cas-79-94-7-market/236248/ (accessed November 12, 2019)

Yu, Y., Yu, Z., Chen, H., Han, Y., Xiang, M., Chen, X., Ma, R., Wang, Z., 2019. Tetrabromobisphenol A: disposition, kinetics and toxicity in animals and humans. Environ. Pollut. 253, 909-917.

Yu, Y., Chen, H., Hua, X., Dang, Y., Han, Y., Yu, Z., Chen, X., Ding, P., Li, H., 2020. Polystyrene microplastics (PS-MPs) toxicity induced oxidative stress and intestinal injury in nematode *Caenorhabditis elegans*. Sci. Total. Environ. 726, 138679. https://doi.org/10.1016/j.scitotenv.2020.138679

Yu, Y., Zheng, T., Li, H., Hou, Y., Dong, C., Chen, H., Wang, C., Xiang, M., Hu, G., Dang, Y., 2023. Growth inhibition of offspring larvae caused by the maternal transfer effects of tetrabromobisphenol A in zebrafish. Environ. Pollut. 322, 121143. https://doi.org/10.1016/j.envpol.2023.121143

Zalasiewicz, J., Waters, C. N., Ivar do Sul, J., Corcoran, P. L., Barnosky, A. D., Cearreta, A., Edgeworth, M., Gałuszka, A., Jeandel, C., Leinfelder, R., McNeill, J. R., Steffen, W., Summerhayes, C., Wagreich, M., Williams, M., Wolfe, A. P.,Yonan, Y., 2016. The geological cycle of plastics and their use as a stratigraphic indicator of the Anthropocene. Anthropocene 13, 4–17.

Zar, J.H., 1999. Biostatistical analysis, fourth ed. Prentice Hall, New Jersey.

Zhang, W., Zhang, S., Wang, J., et al., 2017. Microplastic pollution in the surface waters of the Bohai Sea, China. Environ. Pollut. 231, 541- 548. https://doi.org/10.1016/j.envpol.2017.08.058

Zhang, W., Sun, S., Du, S., Han, Y., Tang, Y., Zhou, W., Shi, W., Liu, G., 2022. Toxic impacts of microplastics and tetrabromobisphenol A on the motility of marine microalgae and potential mechanisms of action. Gondwana Res. 108, 158–170.

Zhou, X., Guo, J., Zhang, W., Zhou, P., Deng, J., Lin, K., 2014. Tetrabromobisphenol A contamination and emission in printed circuit board production and implications for human exposure. J. Hazard. Mater. 273, 27-35.

Zhou, H., Yinm N., Faiola, F., 2020. Tetrabromobisphenol A (TBBPA): A controversial environmental pollutant. J. Environ. Sci. 97, 54-66.

Zhu, Z., Ju, J., Zhang, M., Yang, H., Wei, W., Zhang, Y., 2022. Bisphenol A disturbs hepatic apolipoprotein A1 expression and cholesterol metabolism in rare minnow *Gobio cyprisrarus*. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 252, 109239.

**Figure captions**

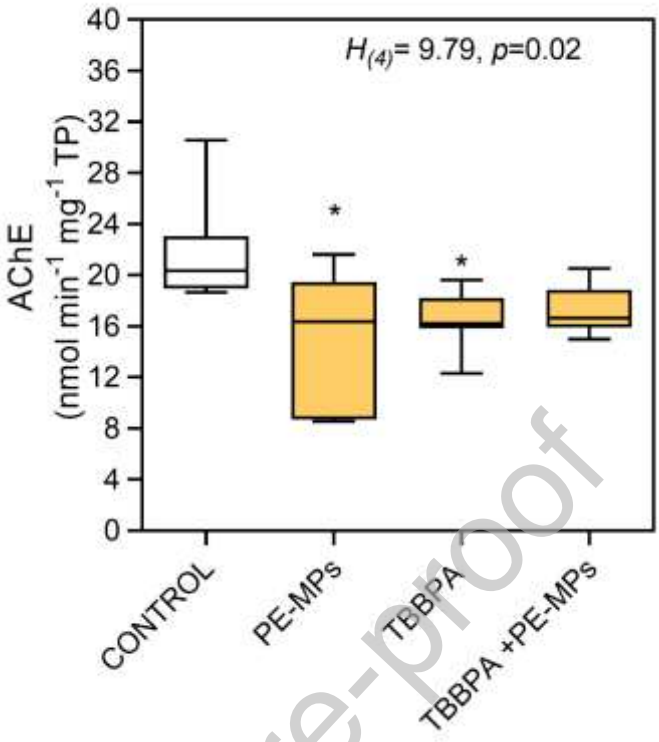



**Fig. 1** Acetilcholinesterase activity (AChE, nmol $min^{-1}$ $mg^{-1}$ TP) in *Rhinella arenarum* control tadpoles (CO), tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10 µg $L^{-1}$ of tetrabromobisphenol A (TBBPA) and their mixture (TBBPA+ PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * $p < 0.05$ compared with the control

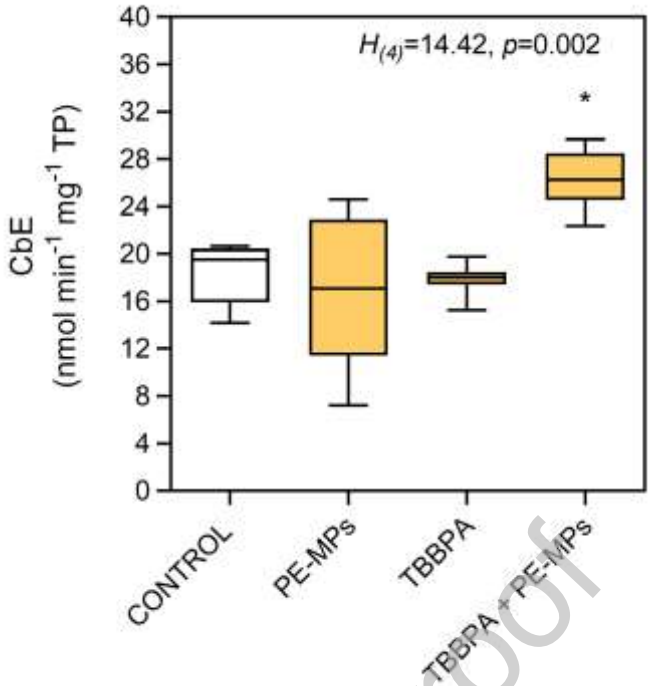



**Fig. 2** Carboxylesterase activity with 1-naphthyl acetate substrate (CbE nmol $min^{-1}$ $mg^{-1}$ TP) in *R. arenarum* control tadpoles (CO), tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10 µg $L^{-1}$ of tetrabromobisphenol A (TBBPA), and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * $p < 0.05$ compared with the control

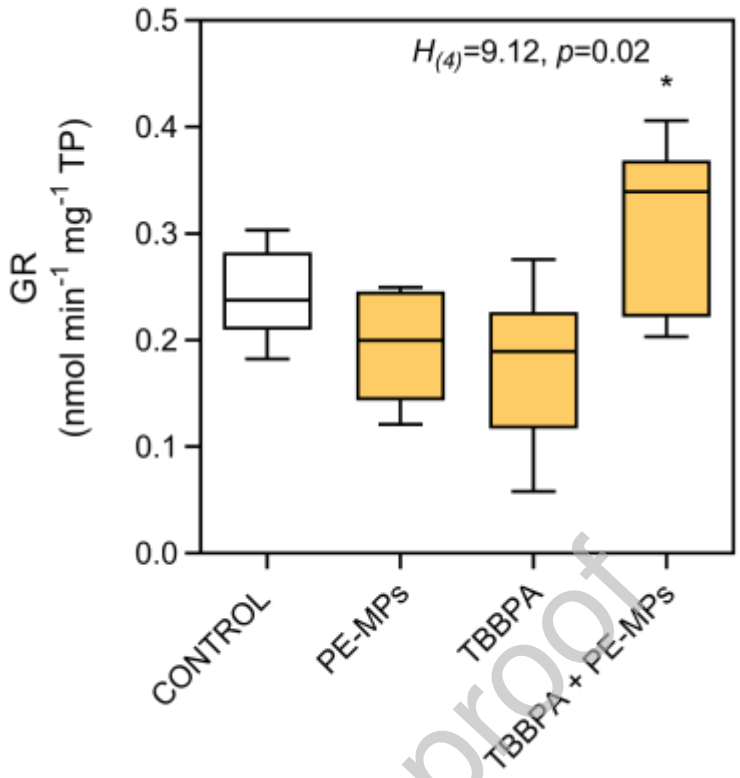



**Fig. 3** Glutathione reductase activity (GR nmol $min^{-1}$ $mg^{-1}$ TP) in *R. arenarum* control tadpoles, tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10µg $L^{-1}$ of tetrabromobisphenol A (TBBPA) and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * $p < 0.05$ compared with the control

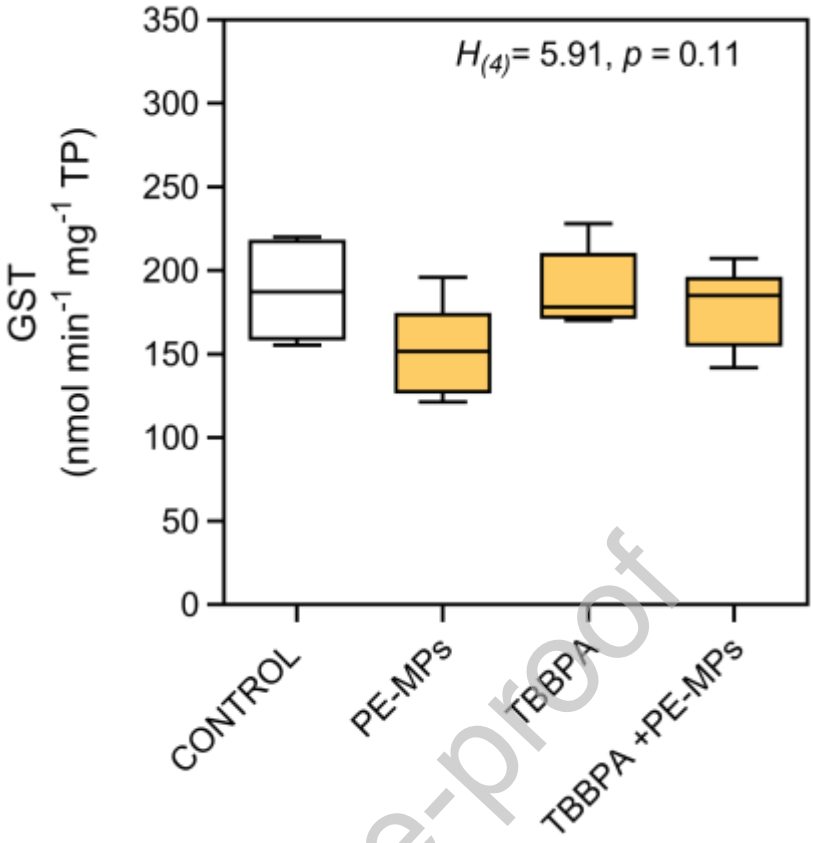



**Fig. 4** Glutathione-S-transferase activity (GST nmol $min^{-1}$ $mg^{-1}$ TP) in *R. arenarum* control tadpoles, tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10 µg $L^{-1}$ of tetrabromobisphenol A (TBBPA) and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. *p< 0.05 compared with the control

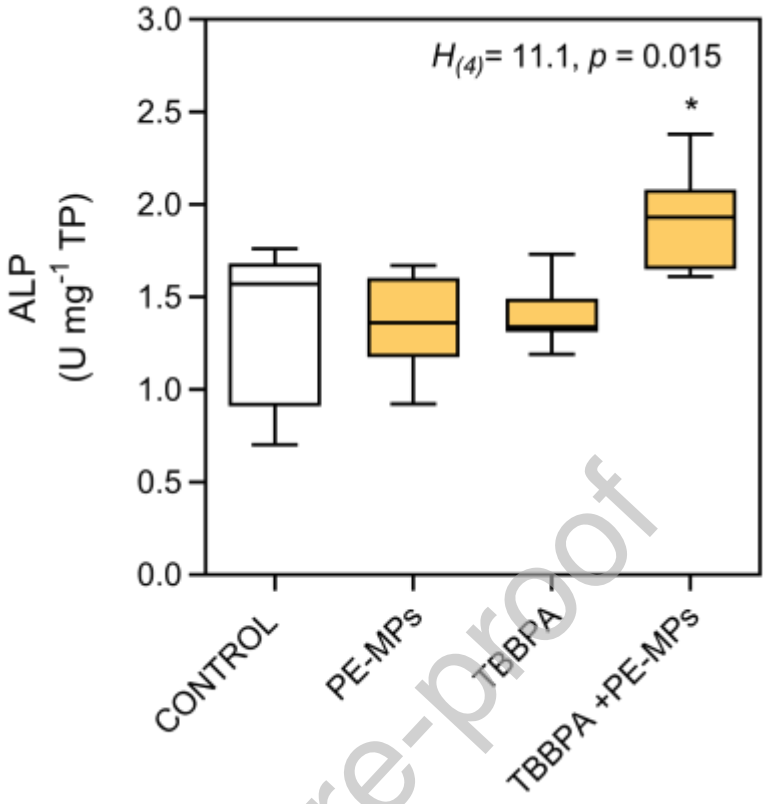



**Fig. 5** Alkaline phosphatase activity (ALP U $mg^{-1}$ TP) in *R. arenarum* control tadpoles, tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10 µg $L^{-1}$ of tetrabromobisphenol A (TBBPA) and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * p< 0.05 compared with the control

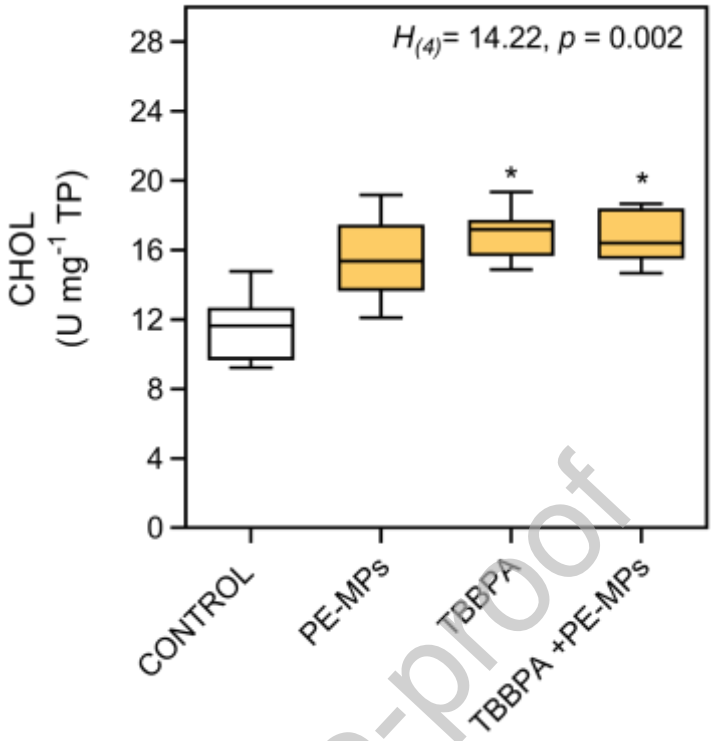



**Fig. 6** Cholesterol level (CHOL U mg $^{-1}$ PT) in *R. arenarum* control tadpoles, tadpoles treated with 60 mg L$^{-1}$ of microplastics (PE-MPs), 10 µg L$^{-1}$ of tetrabromobisphenol A (TBBPA) and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * $p< 0.05$ compared with the control

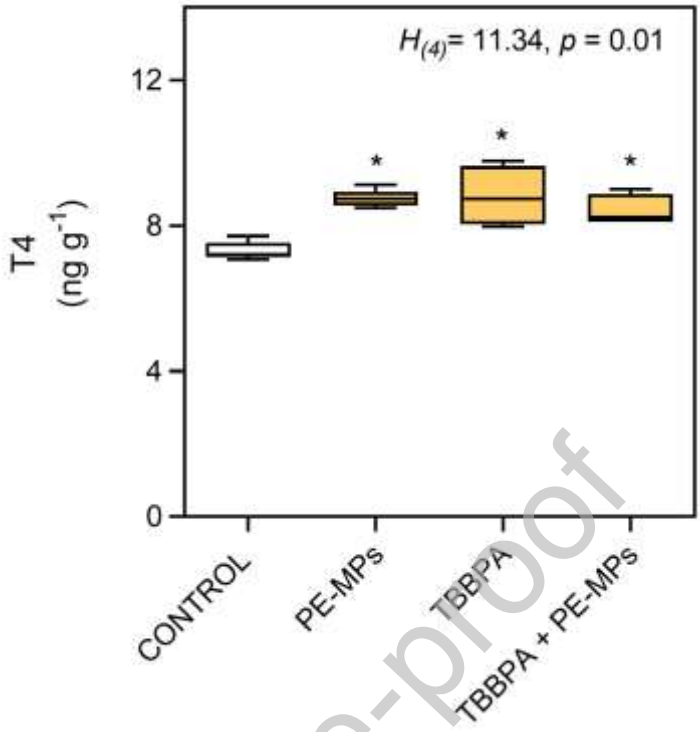



**Fig. 7** Thyroid hormone (T4 U ng $g^{-1}$) in *R. arenarum* control tadpoles, tadpoles treated with 60 mg $L^{-1}$ of microplastics (PE-MPs), 10µg $L^{-1}$ of tetrabromobisphenolA (TBBPA) and their mixture (TBBPA + PE-MPs) at 30 days of exposure. Boxes indicate the median, the 25th and 75th percentiles (box edges), and whiskers indicate the range. * $p < 0.05$ compared with the control

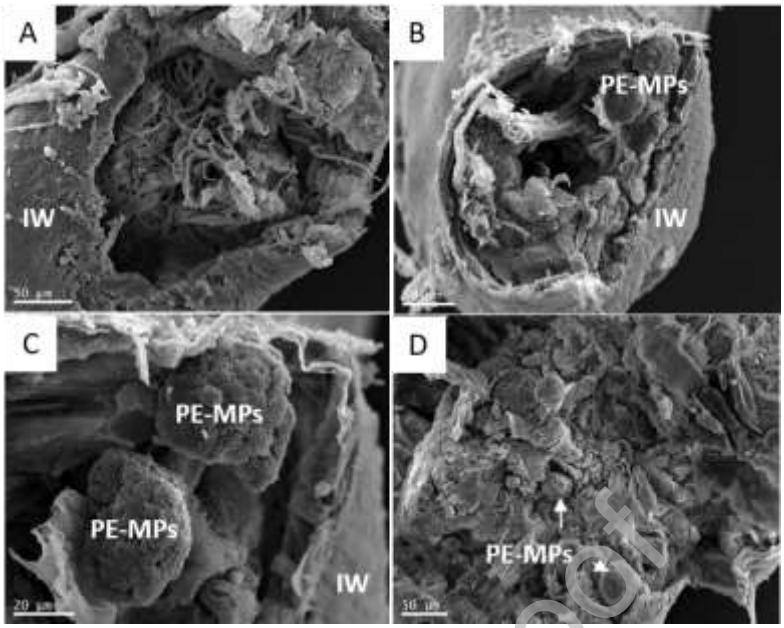



**Fig. 8** Transverse scanning electron microscope images of the intestine of tadpoles. Detail of intestine of control individual (A); and detail of intestine of tadpoles treated with PE-MPs at low (B) and high magnification (C); detail of intestine of tadpoles treated with TBBPA + PE-MPs showing the presence of MPs (D). References: Polyethylene microplastic (MP), intestinal wall (IW).

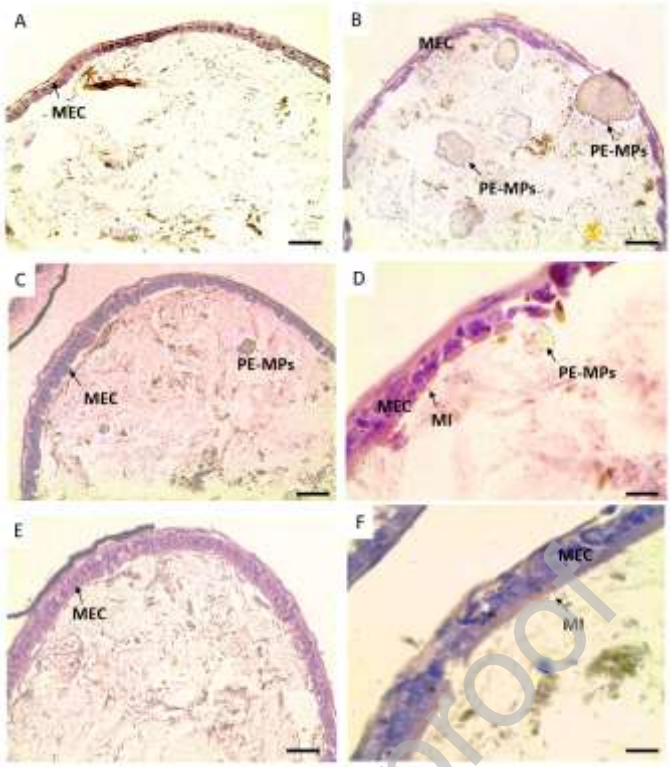



**Fig. 9** Histological images of intestine of *R. arenarum* tadpoles in different treatments. Intestine of control tadpole (A), intestine of tadpoles treated with PE-MPs (B), TBBPA + PE-MPs (C and D) and TBBPA (E, F). References: microplastic (PE-MPs), mucosal epithelial cells (MEC), intestinal wall (IW), melanocytes (asterisk).

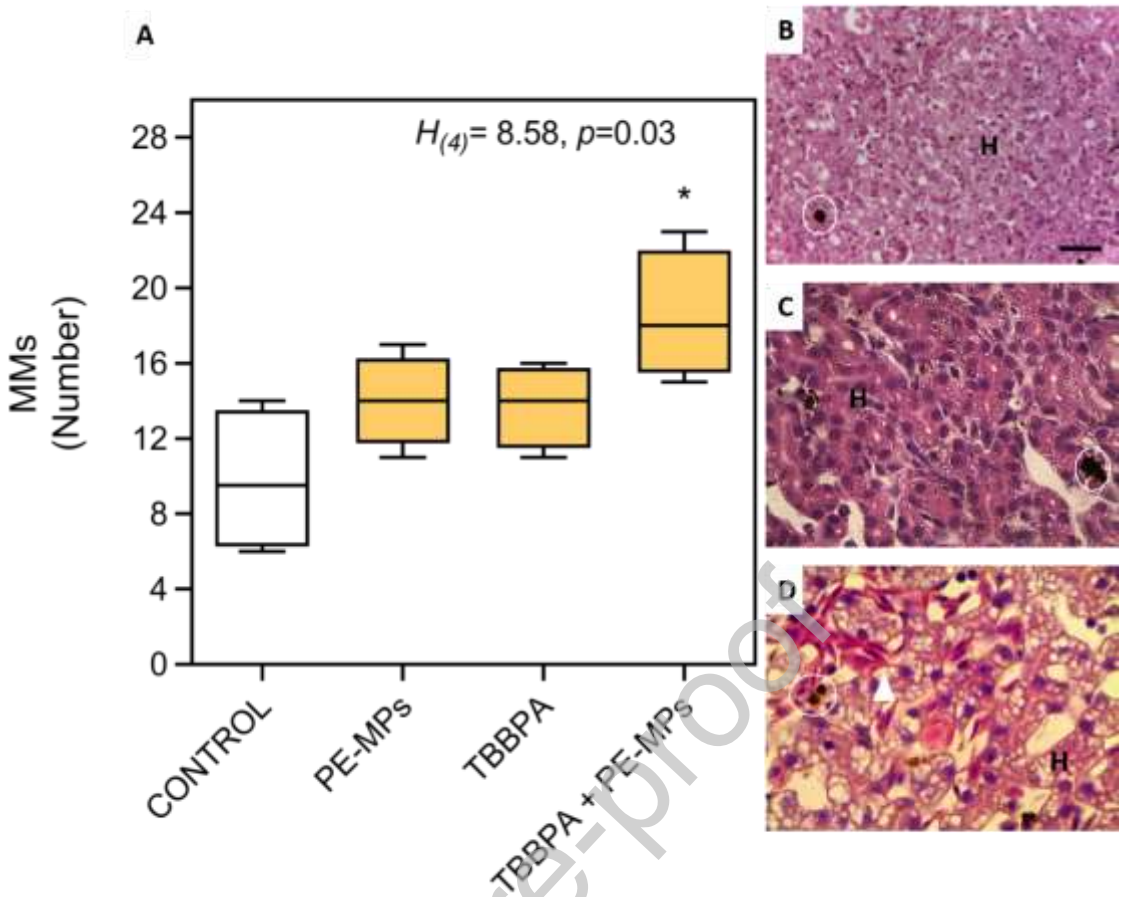
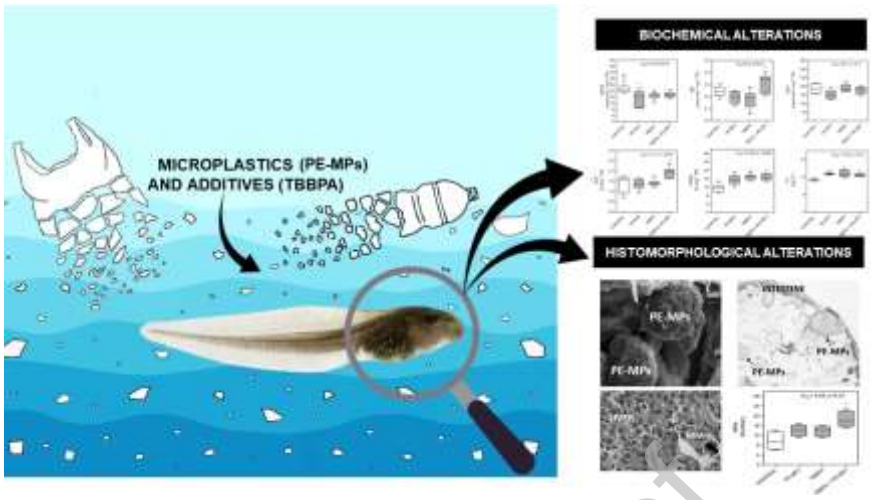



**Fig. 10** Analysis of the liver of *R. arenarum* tadpoles. Melanomacrophage number in liver expressed as mean±standar deviation in different treatments. The different letters above the bars indicate significant differences between groups ($p<0.05$) (A); histological microphotographs of liver (H&E) from control, PE-MPs and TBBPA + PE-MPs (B, C, and D respectively) treatments. References: hepatocyte (H), melanomacrophage (black circle), hepatocyte vacuolization (head arrow). B: 40x, scale bar: 20 µm; C,D: 100x, scale bar: 10 µm.

Graphical Abstract




**Authors Contributions**

Andres M. Attademo: Conception: Design, Execution, Interpretation and writing.

Lucila M. Curi: Execution, interpretation and writing.

Carlos E. Barrios: Execution, interpretation and writing

Rafael C. Lajmanovich: Conception, Design, Execution and Interpretation.

Paola Peltzer: Design and Interpretation.

Ana P. Cuzziol Boccioni: Execution and Interpretation.

Fernanda Simonielo: Execution and Interpretation

Paola M. Michlig: Chemical Analyses

María R. Repetti: Chemical Analyses

Juan M. Ríos: Interpretation.

**Declaration of interests**

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

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: