Toxicological effect and enzymatic disorder of non-studied emerging contaminants in *Artemia salina* model

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**A B S T R A C T**

Emerging contaminants such as sunscreens, hair dyes and flame retardants have been found at important concentrations in surface water (river, lake, ocean), but their negative impact on different aquatic species is not fully known. This study evaluated the effect of benzophenone (BZ), 2,5-diaminotoluene sulfate (PTD), p-phenylenediamine (PPD) and tetrabromobisphenol A (TBPA) on survival (LC50) and the impact of sublethal concentrations (LC25) on the activity of enzymes linked to stress oxidative process in brine shrimp under two temperature conditions (22 °C and 28 °C) for 24 h and 48 h of exposure time. LC50 values obtained for each chemical substance and the activity of GST, AChE and LDH were significantly affected by the temperature conditions and exposure time. In contrast, Gpx was only altered by the tested compound. TBBPA (LC50 from 17.05 up to 28.55 μg/L) and BZ (LC50 from 14.86 up to 24.49 mg/L) resulted in the most toxic substances for *A. salina*. The impact of dyes, such as PTD and PPD, on aquatic organisms is limited. These are the first results that show that not only dyes, but their respective by-products induce harmful effects in brine shrimp (LC50 for PTD and PPD were 23.6–396.3 and 52.0–164.9 mg/L, respectively). Although this study model was very useful to evaluate the ecotoxicity of the different ECs, additional research is needed to increase available information related to the effects of dyes and other non-studied micropollutants on aquatic systems in general.

**Keywords:**
Emerging contaminants
*Artemia salina*
Median lethal concentration (LC50)
Oxidative stress
Ecotoxicology

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1. Introduction

According to the Norman Network, emerging substances can be defined as ‘substances that have been detected in the environment, but which are currently not included in routine monitoring programs at EU level and whose fate, behavior and ecotoxicological effects are not well understood’ [1] and their concentrations in water may range from ng up to μg per liter [2]. Their presence can generally be attributed to inefficient water treatment processes, direct discharges of raw wastewater (domestic and industrial) or natural processes [2]. The Norman Network in 2016 published a list of 1036 substances catalogued as emerging contaminants [3]. The distribution and concentration of these compounds vary between locations (urban, industrial, agriculture and touristic areas), season [2], and have been bound in the tissue of several aquatic organisms such as mollusks, fish, crustacean, plankton, and benthic microalgae [2]. Antidepressants, fragrances, antimicrobials, preservatives, stimulants, hormones, UV filters, organophosphorus, antibiotics, non-steroidal anti-inflammatory drugs, diuretics are some examples of these pollutants detected in the tissues [4]. The effects of these compounds are widely diverse, including endocrine disrupting action [5], enzymatic activity alteration [AChE, superoxide dismutase (SOD), CAT, etc.], estrogenic activity change in molting time in some crustaceans, gene modification in protein and lipid metabolism, biosynthesis, energy metabolism, growth and survival reduction, body size reduction, DNA damage, etc. [4]. Although, their impact in aquatic organisms depends on the organism size, body lipid content, metabolic transformation rate, depuration rate, environment condition (temperature, pH, organic carbon in water), among others [4].

Benzophenone (BZ) is an aromatic and water-insoluble compound reported as emerging contaminant in surface water at concentrations ranging from 2 to 713 ng/L [5,7]. It is usually used as sunscreen UV filter and is incorporated in a large number of personal care products.
(perfumes, shampoos, soaps, makeup, sanitation products, etc.) [8], but also is included as an additive for plastics, coatings and adhesive formulations, insecticides, agricultural chemicals, hypnotic drugs, antihistamines, and other pharmaceuticals [9]. The inclusion levels of benzophenone in personal care products range from 0.5–10 %, but in some countries these levels go up to 25 % [10]. Phenylenediamines are an example of synthetic hair dyes, which are aniline derivatives with an inclusion level that go from 0.5 up to 10 % [11,12]. There are few studies where the concentration of hair dyes in surface water has been reported. For instance, phenylendiamine (0.058 mg/L), resorcinol (4.52 mg/L), p-aminophenol (2.1 mg/L) and p-toluenediamine (2.08 mg/L) had been found in samples collected from hairdressing salon effluent; while p-toluenediamine (0.00177 to 0.00236 mg/L) and p-aminophenol (0.0013 to 0.0019 mg/L) were detected in water inlet and outlet of drinking water treatment plant [13–15]. Hair dye substances have been associated with allergic contact dermatitis and cancer [11]. Flame retardants are classified in halogenated and organophosphorus flame retardants substances, that are included in different con
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detected in soil, surface water, groundwater and inclusively in aquatic organisms (plasma and tissues) [17]. Optimal growth, metabolic functions and survival rate in aquatic organism are also affected by the environmental temperature; consequently, any change in their optimal temperature conditions may trigger multi-stress effects and compromise their capacity to metabolize and excrete toxicant compounds [18]. As example, a reduction in GPx and glutathione reductase enzyme activities (both enzymes play an important role in the phase II detoxification system) were significantly reduced in *Mytilus galloprovincialis* kept at higher temperature (22 °C) [43]. Lowering GST activity was observed in *A. salina* exposed at 28 °C, but some other enzymes such as GPx, LDH and AChE increased due to experimental conditions [63]; consequently, the capacity to transform emerging contaminants is altered. Although many studies show the impact of emerging contaminants in different biological indicators (survival, oxidative stress, immune response, and so on), there is a lack of information about how the toxicity of these substances can be changed by the temperature and prolonged exposure period. Therefore, the current study determined the effect of exposure time and temperature over the median lethal concentration (LC50) of benzophenone (BZ), 2,5-diaminotoluene sulfate (PTD), p-phenylenediamine (PPD) and tetramobisphenol A (TBBPA) on *Artemia salina*, and how lower concentrations (LC25) may alter the activity of enzymes linked to stress oxidative process under same experimental conditions.

2. Material and methods

2.1. Reagents

Benzophenone (BZ), 2,5-diaminotoluene sulfate (PTD), p-phenylenediamine (PPD), p-tetramobisphenol (TBBPA), potassium dichromate (K₂Cr₂O₇) were supplied in analytical grade by Sigma-Aldrich® (St. Louis, MO, USA). Hair dyes substances (2,5-diaminotoluene sulfate and p-phenylenediamine) and K₂Cr₂O₇ were dissolved in milli Q water before use. PTD and PPD were prepared just before use to reduce the autoxidation. Benzophenone and tetramobisphenol A were dissolved in methanol. Chemical information and toxicity levels reported for these substances in other aquatic organisms are presented in Supplementary Table A. A preliminary trial was run to evaluate the exposure to the methanol present in the concentrated dilutions, confirming that its maximum concentration (5.5 %), which is lower than the LC50 (6.4 %) described for *Artemia* [19], did not cause mortality at the experimental evaluated conditions (data not shown). Therefore, only a seawater blank (control group) was included in all the experimental conditions tested as a negative control.

2.2. Hatching of brine shrimp

*Artemia salina* was purchased as canned cysts (Biogrow, Proaqua®, Mazatlan Sinaloa, Mexico). A solution 1:1 commercial chlorine (bleach) and distilled water containing 150 mg of sodium hydroxide was used as a pretreatment for decapsulation of the artemia cysts. For the LC50 experiment, one gram of cysts was mixed in decapsulation solution (25 mL) and stirred continuously for 7–10 min, washed with tap-water, filtrated and placed in a 500 mL glass brine shrimp egg hatchery containing marine water (Instant Ocean® 29.9 mg/L distilled water). It was then aerated for 24 h in a controlled water bath shaker (28 °C). For enzymatic assays, five gram of cysts were mixed in decapsulation solution (75 mL) following the protocol described above. Decapsulated eggs were placed in a 1 L clear plastic bottle and incubated for 24 h at 28 °C. At least 20 g of eggs were decapsulated to obtain enough biomass for each enzymatic test. Starved 24 h-old artemia nauplii were used to carry out all the experimental trials.

2.3. Lethal concentration 50 (LC50) tests

Preliminary trials were done to establish the best range of concentration to evaluate each substance for every single experimental condition (data not shown). Test compounds were prepared at different graded concentrations using seawater to maintain an equal salinity concentration among treatments (Supplementary Table B). Contaminant concentrations tested in the bioassays correspond to nominal concentrations. Each test substance was evaluated at two temperatures (22 °C and 28 °C) and two exposure times (24 h and 48 h). The temperature of 22 °C represented room temperature. Individuals were kept fasting during the total length of the bioassays. Trials were performed in 96-microwell cell culture plates: 10 nauplii in 20 μl of marine water were placed in each plate well. 230 μl of each contaminant dilution was added to each well resulting in 250 μl total working volume. Negative (seawater) and positive (K₂Cr₂O₇) controls were included in all the experiments. Each compound was evaluated by triplicate for each experimental condition, and after 24- or 48-h exposure, the survival rate was calculated for each treatment. Lethal concentration for half the population (LC50) was determined by linear regression for each bioassay and then compared to calculate an average. These regressions were obtained using Microsoft Excel® software.

2.4. Enzymatic activity

Sublethal concentration (LC25) was selected to determine the impact of BZ, PTD, PPD and TBBPA on the enzymatic activity of glutathione S-transferase (GST), glutathione peroxidase (GPx), lactate dehydrogenase (LDH) and acetylcholinesterase (AChE) at the different temperature conditions evaluated for LC50 determination. One gram of artemia was exposed to each emerging contaminant for 24 h or 48 h. After that, Artemia nauplii were collected by filtration in Whatman paper and washed three times with milli Q water and then stored at ~80 °C until enzymatic analysis. Three replicates were run for each experimental condition. Enzymatic extraction was done as followed: 100 mg of frozen artemia was resuspended into 1 mL extraction buffer and sonicated (Bransonic® 5510R-DTH ultrasonic cleaner, Danbury, CT, USA) for 10 min, then centrifuged (Labnet Prism R, Edison, NJ, USA) at 3500 g for 10 min at 4 °C, and afterwards the supernatant was transferred to a clean Eppendorf tube and stored at ~80 °C until enzymatic analyses. Two different extraction buffers were employed: 50 mM phosphate buffer pH 7 for LDH and AChE enzymes and 50 mM phosphate buffer pH 7.2 with 5 mM EDTA for GPx and GST.

The quantification of GPx and LDH in 100 μL supernatant samples were performed using kits and protocols provided by Cayman chemical® (MI, USA) and reactions were measured quantifying the absorbance at the wavelengths of 340 and 490 nm, respectively. GST activity was determined according to the procedure described by [20], using
1-chloro-2, 4-dinitrobenzene as substrate and measuring the absorbance at 340 nm. AChE activity was measured using the colorimetric Assay Kit ab138871 from ABCAM® (Cambridge, UK) and a wavelength of 410 nm. All enzymatic activities were standardized against protein content measured by Bradford method: 50 μL of the supernatant of each assay extract was mixed with 200 μL of Bradford reagent (B6916–500ML Sigma-Aldrich®, St. Louis, MO, USA) and incubated for 5 min before reading the absorbance at 595 nm wavelength. Each condition was analyzed per triplicate.

2.5. Statistics analysis

Three-way ANOVA and Tukey’s test at the significant level of $P \leq 0.05$ were used to evaluate the significant differences of LC50 and enzymatic activity data regarding the different experimental conditions (emerging contaminant, exposure time, and temperature conditions). If significant differences were observed, a one-way analysis of variance and Tukey’s test (for separation of means) were carried out to find a significant difference between data observed for the respective contaminant evaluated under condition tested. All statistical analyses were executed using the software SPSS® version 22 (Chicago, IL, USA). The results are presented as mean ± standard deviation (SD).

3. Results and discussion

3.1. Median lethal concentration (LC50) test

Brine shrimp species can grow and survive in a wide range of temperature (15–55 °C) and salinity (3–300 g/L). Nevertheless, the optimal conditions for lethality experiments ranged between 20–28 °C and 25–35 g/L for temperature and salinity respectively [21]. In this study, the survival rate in the non-contaminated group ranged from 93 to 98 % in all the experimental conditions tested (data not shown), indicating that the mortality rate observed was due to the test substances and experimental conditions. The factorial analysis showed a significant effect ($P < 0.001$) among test contaminants, exposure time and temperature condition on LC50 values, as well as a significant interaction ($P < 0.001$) between test variables (Table 1). Since there was a significant difference among experimental variables, a two-way statistical analysis was performed for each test product to evaluate the impact of temperature and exposure time.

Potassium dichromate ($K_2Cr_2O_7$) was used as a positive control and the results showed that exposure time, temperature and the interaction of these factors increased significantly ($P < 0.001$) its toxicity around 4 times on brine shrimp (Fig. 1). LC50 values at 22 °C condition after 24 h and 48 h of exposure were 39.3 ± 3.0 μg/mL and 20.1 ± 2.1 μg/mL respectively, while at 28 °C, LC50 was reduced to 9.6 ± 0.7 μg/mL and 5.0 ± 0.4 μg/mL after 24 h and 48 h of exposure respectively. The toxicity of $K_2Cr_2O_7$ observed in the present study agree to previous studies in brine shrimp, where the LC50 values reported varied from 9 up to 78 μg/mL after a 24–72 h period at 28 °C and 30 °C [22,23]. The influence of exposure time and incubation conditions over potassium dichromate toxicity had been observed in some other crustacean species (A. salina, Brachionus plicatilis, Daphnia magna and Streptocephaulus proboscides) when exposed to this substance, but at higher temperature conditions [24].

There was a significant difference for TBBPA LC50 values (μg/L) in brine shrimp exposed at different temperature conditions ($P < 0.001$), but not for the exposure time ($P > 0.05$) (Fig. 2). LC50 for TBBPA at 24 h and 48 h exposure was estimated at 17.0–27.6 and 19.4–28.5 μg/L respectively. TBBPA particularly displayed higher toxicity at 28 °C (17–19.4 μg/L) than the 22 °C condition (27.6–28.5 μg/L) either after 24 or 28 h exposure. These results showed that A. salina is more susceptible to TBBPA toxicity than other aquatics organism such as D. magna (LC50 0.4–960 μg/L) [25–28], copepods (Oithona similis, Acartia pacifica and Pseudodiaptomus inopinus) (LC50 40.0 μg/L-566 mg/L) [29], fish (Danio rerio, Carassius auratus, Pseudorasbora parva, Channa punctatus) (LC50 0.8–1.0 mg/L) [30–33] and microalgae (Chlorella pyrenoidosa and

![Fig. 1. Lethal concentration 50 (LC50) in A. salina after exposure to potassium dichromate (K$_2$Cr$_2$O$_7$) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same capital letter (A or B) are not significantly different (α = 0.05) between temperature conditions; means with the same lowercase letter (a or b) are not significantly different (α = 0.05) with respect to exposure time. Values are mean ± SEM of all treatments for each condition (n = 20).](image1)

![Fig. 2. Lethal concentration 50 (LC50) in A. salina after exposure to tetrabromobisphenol A (TBBPA) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same capital letter (A or B) are not significantly different (α = 0.05) between temperature conditions; means with the same lowercase letter (a or b) are not significantly different (α = 0.05) with respect to exposure time. Values are mean ± SEM of all treatments for each condition (n = 20).](image2)

| Parameters                  | LC50  | GST  | GPx  | AChE | LDH  |
|-----------------------------|-------|------|------|------|------|
| Contaminant                  | 0.000 | 0.000| 0.000| 0.000| 0.002|
| Temperature (°C)             | 0.001 | 0.000| 0.211| 0.000| 0.000|
| Exposure time (h)            | 0.000 | 0.000| 0.145| 0.001| 0.000|
| contaminant*temperature      | 0.000 | 0.000| 0.000| 0.002| 0.000|
| contaminant*exposure time    | 0.000 | 0.000| 0.000| 0.017| 0.000|
| temperature*exposure time    | 0.000 | 0.577| 0.000| 0.000| 0.035|
| contaminant*temperature*exposure time | 0.000 | 0.000| 0.000| 0.000| 0.000|

GST, glutathione S-transferase; GPx, glutathione peroxidase; LDH, lactate dehydrogenase; AChE, acetylcholinesterase.
Scenedesmu quadricauda (LC50 1.2–3.1 mg/L) [30,34]. The lack of TBBPA toxicity by temperature condition likely was because it tends to sediment due to its low to moderate water solubility (0.1–2.3 mg/L at 25 °C) [35].

Interestingly, PPD was 2.17 times more toxic than PTD (Fig. 3). For both substances, a significant effect for temperature (P < 0.001) and exposure time (P < 0.001) with a significant interaction (P < 0.001) was observed. The exposure to PPD at 28 °C showed higher toxicity in brine shrimp than 22 °C conditions (52.0–52.3 mg/L vs 81.8–164.9 mg/L). The same trend was observed for PTD. LC50 values were 2.7 and 8.4 times higher as the temperature increased, and 1.9 and 6.1 times higher as exposure time was extended. There is an absence of information about the toxicity of hair dyes on A. salina. For other aquatic species, there is little information about the LC50 values for PPD and PTD. The LC50 values for PPD in different fish species (D. rerio, Oreias latipes, Cyprinus carpio, Oncorhynchus mykiss and Pimephales promelas) ranged from 60 μg/mL up to 140.7 mg/L after 48 h or 96 h exposure (Supplementary Table A); whereas in the crustacean D. magna and the microalgae Pseudokirchneriella subcapitata, the LC50 was about 0.28 mg/L for both aquatic species (Supplementary Table A). In the case of PTD, limited data is available (Supplementary Table A). In the fish D. rerio, the crustacean D. magna and microalgae (unspecified specie), the LC50 value for PTD ranged from 0.3–12 mg/L. In the present study, the LC50 values obtained for PPD in brine shrimp were closed to the aforementioned, while for PTD the LC50 values were much higher than those reported in another aquatic organism. The difference among aquatic species could be related to the Artemia resistance to hair dye toxicity among other aquatic species. Hair dyes, such as PPD and PTD, easily can oxidize [13] and some by-products are generated as monomeric, dimeric and trimeric products [36], which can be detected from 30 min to 8 h of being exposed to air saturated aqueous medium [37]. Some of these compounds are highly toxic than the original compounds, for example, PPD (3%–5%) transforms into Bandrowski first step of the elimination process [46]. GST activity in control groups showed an important decrement in enzyme activity (47.7 and 27.3 %) after being exposed to PPD, PTD, BZ and TBBPA and untreated control groups after 24 h exposure of the aquatic midge Chironomus riparius to benzophenone-3 at elevated temperature conditions (25 °C) induced the expression of genes related to stress (Hsp22, Hsp27, Hsp70), immune (phenol oxidase activity), and nervous system (AChE activity) causing a lower survival rate [44].

3.2. Biochemical analysis

The impact on enzymes involved in the cellular antioxidant system (GST and Gpx), neurotoxicity marker (AChE), and energy metabolism (LDH) at sublethal concentrations for PPD, PTD, BZ and TBBPA were evaluated in A. salina nauplii following the same experimental conditions for toxicity tests. GST, AChE and LDH enzymatic activities were significantly (P < 0.05) affected by the contaminant and experimental conditions. In contrast, Gpx activity was significantly affected (P < 0.001) by the test contaminant, but not for the temperature and exposure time (P > 0.05). A significant interaction (P < 0.05) for contaminant and temperature, contaminant and exposure time and contaminant, temperature and exposure time were observed for all the enzyme activities evaluated (Table 1). Particularly for GST activity, a significant interaction (P < 0.01) was observed for most of the variables, except for temperature and exposure time (P > 0.05).

3.2.1. Antioxidant enzyme activities

The antioxidant enzymes are wide distributing in different organisms, including aquatic species [44]. They play an important role in antioxidative mechanisms against xenobiotic substances, whose main purpose is suppressing or preventing the formation of free radicals or reactive species in cells. SOD, CAT, GSH and Gpx enzymes represent the first line of defense responsible for metabolizing reactive oxygen species (hydrogen peroxides, hydroperoxides) into less toxic substances, and preventing cell from an oxidation stress, and further cell damage [45].

GST enzyme plays an important role in Phase II detoxification system by binding xenobiotic compounds and several products from oxidative metabolism, lowering reactive oxygen species and intervening in the first step of the elimination process [46]. GST activity in A. salina exposed to PPD, PTD, BZ and TBBPA and untreated control groups after 24 h or 48 h exposure time at 22 °C or 28 °C is shown in Fig. 4. GST activity varied from 26.7–171.8 mL/mg/L among treatments. Contaminant, temperature, and exposure time conditions affected the activity of this enzyme. The GST activity in control groups showed an important reduction as exposure time increased to 48 h (27.3 and 61.6 %) or temperature from 22 °C to 28 °C (39.1–67.9 %). Exposure to PPD and TBBPA resulted in a significant decrement in enzyme activity (47.7 and 63.3 %) after 48 h incubation at 22 °C, but there was no difference at 28 °C in the treatment with PPD. Brine shrimp exposed to PTD showed the highest GST activity at 22 °C and 48 h of exposure (83.5 % in

Fig. 3. LC50 in A. salina after exposure to p-phenylenediamine (PPD), 2,5-diaminotoluene sulfate (PTD) and benzophenone (BZ) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same capital letter (A or B) are not significantly different (α = 0.05) between temperature conditions; means with the same lowercase letter (a or b) are not significantly different (α = 0.05) with respect to exposure time. Values are mean ± SEM of all treatments for each condition (n = 3).
comparison to control group); and the lowest GST activity (47.6 mU/mL) at 28 °C and 24 h of exposure. In the presence of BZ, the maximum GST activity (117 mU/mL) was observed at 28 °C and 48 h of exposure, followed by the condition at 22 °C at both exposure times (63.5–82.4 mU/mL). The exposure at 28 °C for 24 h resulted with the lower GST activity (38 mU/mL). GST activity was lower (23–45%) in all the BZ treatment groups when they were compared to their respective control groups, with the exception for the condition of 28 °C and 48 h exposure where GST activity was increased by 337% in comparison to the control group.

GPx enzyme acts as an antioxidant enzyme to eliminate the excess reactive oxygen species during normal and stress conditions [47]. The variation on GPx activity observed in the present study for the control group showed the same alteration for A. salina exposed to test contaminants. GPx activity ranged from 12.7–36.2 mU/mL among treatments (Fig. 5). The activity of GPx was significantly (P < 0.001) altered by the addition of the test contaminant, but not for temperature and exposure time (P > 0.05). The exposure of the brine shrimp to PPD increased in general the GPx activity (from 36.8 up to 113.7%), whereas TBBPA induced a lower activity (from 2.8 up to 8%) in comparison to the respective control groups. BZ treatment showed GPx activity similar to the control groups (from 17.95 up to 29.18 mU/mL vs. 15.8 up to 36.0 mU/mL). Meanwhile, GPx activity in artemia exposed to PTD was lower to the control group at 22 °C (from 36.3 up to 44.2%) and increased when temperature raised to 28 °C (from 12.7 up to 75.5%).

Negative effects have been observed in aquatic organisms exposed to hair dyes [48,49], UV filters [50–52] and TBBP [33,34,53,54]. The exposure of the zebrafish to PPD (25–50 mg/L) resulted in diverse abnormalities (reduction trend in body axes, cardiac looping, less pigmentation, mortalities, DNA damage, etc.) [49,55], including a state of redox imbalance as consequence of ROS production [49]. This latter triggered important changes in other biochemical mechanisms; for example, lipid peroxidation reduced the antioxidant system [56] and impaired immune system [57]. Kaur and Kaur [48] observed that low concentrations (LC10) of the azo dye acid black altered the activity of some antioxidant/detoxification enzymes (GST, GR, GPx, CAT and SOD) in different tissues (liver, kidney, gill, muscle and brain) of the fish Labeo rohita, even after 90 days of period recovery. In the present study, higher GST activity (41–127%) was observed in brine shrimp treated with PTD at different temperature for 24 h and 48 h. In contrast, this contaminant tended to reduce GPx levels (36–44%) at 22 °C, whereas the exposure to the other hair dye (PPD) increased this activity up to 113.7%. The GPx concentration depended on GSH levels, which is oxidized to GSSG during the process. Additionally, GSH is needed for the GST enzymatic reaction [48]. The increase in GST activity observed in the present study suggested that GSH was consumed to produce this enzyme and remove the pollutants in Artemia. Consequently, GPx activity resulted in minimal variations among test compounds, and the lack of significant effects by temperature and exposure time probably due to the low availability of GSH. Similar results had been found in Labeo rohita exposed to the azo dye acid black [48].

In addition to reduced growth, mortalities and estrogenic effects [50, 51] by UV filters, Nataraj et al. [51] observed an increase in lipid peroxidation and impairing SOD, CAT, GPx and GST activities after zebrafish larva were exposed to octyl methoxycinnamate and its photoproducts (2-ethylhexanol and 4-methoxybenzaldehyde). Huang et al. [52] found subsequently that benzophenone-4, 4-aminobenzoic acid, and 2-phenylbenzimidazole-5-sulfonic acid significantly modified the enzymatic activity of SOD, GST and GSH with hepatic lipid peroxidation. The latter being the ones that caused the most critical oxidative stress in the liver of zebrafish. Here, GST and GPx enzymatic activities in brine shrimp exposed to BZ were lower than the control group, except for 28 °C conditions; this was probably caused by enzymatic inhibition due to increasing cellular H2O2 levels and GSH depletion because the increasing ROS production by tissue BZ accumulation [58].

Exposure to the flame retardants such as TBBPA [33] produced oxidative stress and triggered some antioxidant/detoxification enzymes (GPx, GST, CAT) in different fish species (Channa punctatus and Gobio cyprius rarus). Here, the exposure of brine shrimp to TBBPA modified their GPx and GST activities. GST tended to be reduced (22–63%), but as temperature increased to 28 °C, GST also increased by 146–375%. In contrast, GPx activity was inhibited in all the experimental conditions up to 52% compared to the control group. Enzymes such as SOD and GSH were affected in yellow perch Perca flavescens reared at 9 °C in presence of heavy metals, but a higher temperature (28 °C) enhanced both enzyme activities [39]. Temperature stress conditions tended to increase reactive oxygen species (ROS), oxygen-free radicals and modified the balance of reactive oxygen metabolism resulting in cellular damage [60]. It may also affect the GSH production, and therefore GPx and GST enzymes. In the present study, the remarkable GST activity under higher temperatures suggested that this enzyme is binding xenobiotic compounds and lowering toxic effects [46]. While the intact activity of GPx enzyme indicated that it is possible that GSH was used to produce GST or it was not enough to produce GPx [46].

Overall, the GPx and GST levels observed in Artemia further prove related studies correct. GST enzymatic activity tends to be reduced by the exposure time and the temperature. On the contrary, GPx was not significantly affected by these two factors when artemia was exposed to graphene oxide, Fe3O4 nanoparticles, furosemide or hydrochlorothiazide [61–63]. The reduction in GST activity by temperature and

Fig. 4. Glutathione S-transferases (GST) activity in A. salina nauplii exposed to p-phenylenediamine (PPD), 2,5-diaminotoluene sulfate (PTD), benzophenone (BZ), tetrabromobisphenol A (TBBPA) and uncontaminated group (control) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same letter (a, b, c, x, y or z) are not significantly different (α = 0.05) among tested compounds at 22 °C or 28 °C. Significant differences (P < 0.05) among tested compounds at = 0.05) among tested compounds at

Fig. 5. Glutathione peroxidase (GPx) activity in A. salina nauplii exposed to p-phenylenediamine (PPD), 2,5-diaminotoluene sulfate (PTD), benzophenone (BZ), tetrabromobisphenol A (TBBPA) and uncontaminated group (control) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same letter (a, b, c, x, y or z) are not significantly different (α = 0.05) among tested compounds at 22 °C or 28 °C. Values are expressed as mean ± SEM (n = 3). NS: no significant.
The lack of the effect on GPx after 48 h exposure corrob
inactive state after activation [68]. In aquatic organisms, AChE can be
sodium dodecyl sulfate [67], diazepam [67], furosemide and hydro-
exposed to increasing temperature conditions and methylparaben
during 24 h and 48 h at 22 °C; significant differences (P<0.001) by the test substance, exposure time, and temperature, with a
significant interaction among test variables (Table 1). In general, the exposure to PPD, BZ and TBBPA for 24 h at 28 °C reduced AChE activity from 19.8–36.9 in comparison to the control groups, but not with PTD that showed similar activity. Particularly, TBBPA showed the highest inhibition rate (36.9 %) among test compounds. While at 22 °C and 24 h exposure, the AChE activity was lowered by 17.2–59.5 % when brine shrimp were exposed to PTD, BZ and TBBPA, except in the treatment with PPD which increased enzymatic activity by 35.8 % in comparison to its control group. A longer exposure time (48 h) at this temperature resulted in a higher AChE activity (61.9–127.7 %), PPD and PTD reached the highest AChE activity among test compounds (Fig. 6).

AChE activity observed in treatment groups was between the range reported for brine shrimp exposed to other microcontaminants [63]. Although, there is not information about the impact of PPD or PTD on AChE activity in A. salina, there are reports for other dyes and species. Küçükkilinç and Özür [71] observed a significant AChE enzymatic inhibitory effect on electric eel exposed to cationic triarylmethane (TAM) dyes (pararosaniline, malachite green, and methyl green) by binding at peripheral sites of this enzyme instead of the active site. This later is a common mechanism observed in the AChE inhibition by other contaminants such as pesticides, heavy metals, detergents, among others [72]. Zanoni et al. [73] observed that after one hour, 100 mg/mL of PPD increased the levels of intracellular ROS in HaCaT cells, but when it was mixed with hydrogen peroxide (10.5 mg/mL) the ROS production was reduced or increased.

3.2.2. Neurotoxicity marker

AChE belongs to the cholinesterase family enzymes and its role is
hydrolyzing acetylcholine (neurotransmitter) into choline and acetic acid, a reaction necessary for a cholinergic neuron to return to its
condition of 22 °C and 24 h; significant differences (P<0.01) among these factors (Table 1). LDH activity in the brain of zebrafish, although this enzyme was inhibited in the larvae from the adults exposed to this compound. Usenko et al. [31] reported that the flame retardants PH4 (10 mg/L), HBB (10 mg/L), DBNG (10 mg/L), TBPH (2.5 mg/L), TB (10 mg/L) enhanced AChE activity (1.8-folds), while the exposure to HBCD (2.5 mg/L), TBBPA (1.25 mg/L), and the mixture TBBPA–OHEE (1.25 mg/L) did not modify AChE enzyme activity. Previously, Key et al. [77] observed that the exposure to BDE-47 (12.5 μg/L) for 4 days also increased AChE activity up to 20 % in the killifish Fundulus heteroclitus; whereas the exposure of the Atlantic crab Hyas araneus to the same contaminant (50 μg/L, BDE-47) resulted in a substantial decline in AChE enzyme activity (32 %) [78]. In A. salina (present study), AChE activity was reduced by TBBPA up to 37 % compared to the control group; only the condition 22 °C and 48 h increased this by 90 %. It was clear that temperature and time exposure impacted AChE activity, but it is difficult to conclude a general tendency for PTD, PTD and BZ on this enzyme since for some conditions it was reduced or increased.

3.2.3. Energy metabolism (LDH)

LDH enzyme is present in terrestrial and aquatic organisms [79]. It is
a terminal enzyme in the glycolytic route during anaerobiosis, but also is responsible for converting lactate into pyruvate during the gluconeogenesis pathway [80]. Test contaminant, exposure time and temperature conditions modified significantly the LDH activity (P<0.001) with a notable interaction (P<0.01) among these factors (Table 1). LDH activity in control groups at 22 °C and 28 °C were 1.27 and 1.53mU/mL, and 1.38 and 1.19mU/mL at 24 h and 48 h of exposure (Fig. 7) and are in accord with previous results [63]. The exposure to PPD reduced LDH activity of Artemia from 23 up to 36 % in comparison to the control group at 22 °C and 28 °C after 48 h and 24 h respectively. In contrast, the condition of 22 °C and 28 °C at 24 h and 48 h increased LDH enzymatic activity from 32 up to 71 %. A different finding was observed in the PTD treatment, while the condition of 22 °C and 24 h at 24 h and 48 h of exposure reduced LDH activity by 22–37 % in comparison to their corresponding control groups, the enzyme activity increased 19.6 and 126 % at 28 °C and 22 °C after 24 h and 48 h respectively. BZ reduced LDH activity from 34.4–43.9 % at 24 h exposure (both temperatures), but it increased 15.6 and 180.4 % in comparison to the control group after 48 h of exposure at 22 °C and 28 °C respectively. TBBPA also increased LDH activity (8.3–63.2 %), except in the condition of 22 °C and 48 h where LDH activity was reduced by 2.2 % though this difference was not statistically significant in comparison to the control group.

Contrary to the increase observed in brine shrimp by the synthetic dyes (Fig. 6), Kaur and Kaur [48] detected an LDH reduction in different

![Fig. 6](image-url). Acetylcholinesterase (AChE) activity in A. salina nauplii exposed to p-phenylenediamine (PPD), 2,5-diamotoluene sulfate (PTD), benzophenone (BZ), tetrabromobisphenol A (TBBPA) and uncontaminated group (control) during 24 h and 48 h at 22 °C and 28 °C. Columns with the same letter (A, B, C, X, Y and Z) are not significantly different (α = 0.05) among tested compounds at 22 °C or 28 °C; significant differences (P<0.05) with respect to the exposure time are marked with an asterisk (*). Values are expressed as mean ± SEM (n = 3). NS no significant.
tissues of the fish *Labeo rohita* after treatment with the azo dye acid black (6–10 ml/L) even after 90 days. PTD further showed greater impact than PPD, suggesting the oxidized products generated from PDI may be more toxic than PDD by-products; more research is needed to explain in detail how these compounds affect *A. salina*.

Barely information related to the impact of BZ on LDH activity was able to be found. Li et al. [81] observed that zebrabfish embryos exposed to a mixture of UV filters (3,2-ethyl-hexyl-4-trimethoxycinnamate, benzophenone and octocrylene) presented an increment in LDH (from 1.2 up to 2.80 folds). An increase in LDH as the temperature and exposure time rise was also detected when catfish were grown in tannery wastewater [82]. Wang et al. [83] reported a neurotoxic effect (LDH release increment, stimulated ROS generation, caspase-3 activation) in rat primary astrocytes when exposed to zinc oxide nanoparticle (ingestion also used in sunscreen products). In the present work, *A. salina* exposure to BZ resulted in the highest LDH activity inhibition in comparison to the test compounds. Meanwhile the higher LDH activity on parallel to the test compounds. Meanwhile the higher LDH activity on the BZ treatment exposed at 28 °C for 48 h was in line to previous studies in yellow perch exposed to a medium-high temperature (20–28 °C) or hypoxia conditions [84]. The changes observed in LDH activity in brine shrimp exposed to BZ could also be attributed to the high mortality caused by this emerging contaminant.

Brine shrimp exposed to a low concentration of TBBPA increased DLH activity, especially at high temperatures (up to 63.2 %). Same trend had been reported in zebra fish (*D. rerio*) treated with different bisphenols (BPA, BPS or BPF) at two temperature conditions (20 °C and 28 °C) for 24 h [80]. Particularly, bisphenol exposure caused some endocrine disruptions and interfering in some processes such as locomotor performance and energy metabolism [85]. Therefore, the exposure to TBBPA may have altered carbohydrate catabolism and other physiological processes in artemia.

4. Conclusions

Our results revealed that the exposure to PTD, PPD, BZ and TBBPA dramatically affects survival and enzyme activity involved in antioxidant, neurotoxic and energy processes in brine shrimp. Temperature and exposure time conditions also influenced the effects observed in artemia, in addition to the nature of the contaminant. The impact of TBBPA on aquatic organisms definitely needs further research, since temperature conditions played an important role in the results obtained here. The LC50 values obtained for each chemical substance were between the range reported for other aquatic species, although for some ECs (BZ and TBBPA) this work represent the first report about their toxicity in *A. salina*. Also for some contaminants, such as BZ, the concentration observed to induce an enzymatic disruption is within the concentrations range reported in surface water samples. The results obtained in the exposure to PTD and PPD clearly indicate that these substances and/or their respective by-products may affect aquatic organisms, and more research is needed to increase available information related to the impact of dyes on aquatic systems in general.

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CRediT authorship contribution statement

Mireya Tapia-Salazar: Conceptualization, formal analysis, data curation, investigation, methodology, review, writing the original draft, and editing. Veronica R. Díaz-Sosa: Formal analysis, data curation, investigation, methodology, trials, validation, visualization, writing original draft, review, and editing. Diana L. Cardenas-Chavez: Conceptualization, formal analysis, methodology, original draft review, editing, funding acquisition, project administration and supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2022.01.007.

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