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Biological effects of environmentally relevant concentrations of the pharmaceutical Triclosan in the marine mussel *Perna perna* (Linnaeus, 1758)

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ABSTRACT

Triclosan (5-Chloro-2-(2,4-dichlorophenoxy) phenol) is an antibacterial compound widely employed in pharmaceuticals and personal care products. Although this emerging compound has been detected in aquatic environments, scarce information is found on the effects of Triclosan to marine organisms. The aim of this study was to evaluate the toxicity of a concentration range of Triclosan through fertilization assay (reproductive success), embryo-larval development assay (early life stage) and physiological stress (Neutral Red Retention Time assay - NRRT) (adult stage) in the marine sentinel organism *Perna perna*. The mean inhibition concentrations for fertilization (IC_{50} = 0.490 mg L^{-1}) and embryo-larval development (IC_{50} = 0.135 mg L^{-1}) tests were above environmental relevant concentrations (ng L^{-1}) given by previous studies. Differently, significant reduction on NRRT results was found at 12 ng L^{-1}, demonstrating the current risk of the continuous introduction of Triclosan into aquatic environments, and the need of ecotoxicological studies oriented by the mechanism of action of the compound.

1. Introduction

Personal Hygiene and Care Products and Pharmaceuticals (PHCPP) comprise a diverse group of chemicals used in veterinary medicine, human health, agricultural practices and cosmetics (Daughton, 2007). After administration, a significant proportion of the PHCPPs is excreted and reach the wastewater treatment plants (WWTP) where they may not be totally eliminated by conventional treatment technologies (Fent et al., 2006; Quinn et al., 2008).

Special concern rises regarding widely used bactericides, such as those present in the formulations of PHCPP. Triclosan (5-Chloro-2-(2,4-dichlorophenoxy) phenol) (TCS) is a lipophilic organic compound, which has been used in formulations of toothpaste, facial cream, shampoo, soaps, and various types of materials such as packaging of foodstuffs, adhesives, toys, polyethylene, shoes, sealants, paints, mattresses, clothes, shower curtains, flooring, awnings and grout (USEPA, 2008). The widespread use of the TCS is due to its great effectiveness against gram negative and gram positive bacteria.

The safety of this compound has been questioned in relation to environmental and human health due to some factors, such as: (i) conversion by photodegradation into dioxins and furans; (ii) structural similarity to Bisphenol-A; (iii) biological methylation into more toxic compounds; (iv) ability to bioaccumulate and (v) toxicity to non-target organisms (Orvos et al., 2002; Lindstrom et al., 2002; Latch et al., 2003; Ishibashi et al., 2004; Sanchez-Prado et al., 2006; Aranami and Readman, 2007; Coogan et al., 2007; Binelli et al., 2009; Fair et al., 2009; Dann and Hontela, 2011). Due to these characteristics, further studies have been required to support a future decision on whether to proceed with the use of this compound (USEPA, 2008).

The presence of TCS has been frequently reported in wastewater, rivers, lakes, sediments, surface water, aquatic organisms (fish) as well as in human breast milk (McAvoy et al., 2002; Aguera et al., 2003; Dayan, 2007; Ying et al., 2007). In spite of its presence in the environment, to date there is limited data available on the toxicity of TCS for non-target aquatic organisms and most of these studies concern freshwater organisms (Yang et al., 2008; Oliveira et al., 2009; Nassef et al., 2010; Palenske et al., 2010). The lack of studies assessing toxicity of TCS to marine and estuarine organisms is of great importance since the occurrence of this compound in coastal areas has been extensively reported in the literature (Aguera et al., 2003; Nishi et al., 2008; Xie et al., 2008; Fair et al., 2009; Zhao et al., 2010).

Marine bivalves have been employed in marine pollution assessments all around the world because of their sessile habits,

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filter feeding, broad distribution, and economic importance, which make them suitable species to be employed in ecotoxicological studies (Farrington and Tripp, 1995). Different life stages of mussels can be used in toxicity tests. It has been extensively reported that the early life stages of marine invertebrates are more susceptible to environmental toxicants than are the adult forms (Kobayashi, 1984; His et al., 1999). However, mechanism of action oriented toxicity assays has been performed with adult organisms and the results are sensitive to environmentally relevant concentrations (Martín-Díaz et al., 2009).

The aim of this study was to evaluate sub-lethal effects of TCS in different life stages of the marine mussel *Perna perna* (Bivalvia: Mytilidae) in order to support future ecological risk assessments and regulation of TCS use and discharges in aquatic ecosystems.

2. Materials and methods

The effects of TCS in different life stages of *Perna perna* were assessed through the following assays: (i) the fertilization assay (USEPA, 1991 with modifications of Zaroni et al., 2005), which measures the ability of sperm cells, after the exposure to TCS, to promote fertilization; (ii) the embryo-larval development assay (ASTM, 1992 with modifications of Zaroni et al., 2005), which assesses the early life development of zygotes exposed to TCS; and (iii) Neutral Red Retention Time Assay (NRRT) (Lowe et al., 1995), which evaluates the integrity of lysosomal membrane of haemocytes of adults after exposure to TCS.

2.1. Dilution water

Dilution water was collected at the Cocanha beach, Caraguatatuba, São Paulo, an environmental protection area in Southeastern Brazilian coast. Following ASTM (1992) with minor adaptations proposed by Zaroni et al. (2005) regarding salinity (slightly elevated), natural seawater was previously filtered through a cellulose membrane of 0.22 μm for the acute and chronic assays. For the NRRT (Lowe et al., 1995), natural seawater was filtered (150 μm) and maintained under aeration during the assays. Physical-chemical parameters of the dilution water and treatments were measured at the beginning and at the end of the assays (fertilization and embryo-larval assays), or at every replacement of test solutions (NRRT assays). Temperature ranged from 22 °C to 25 °C, pH ranged from 7.87 to 8.03, salinity ranged from 34 to 36 ppt, and dissolved oxygen ranged from 5.9 to 6.6 mg L^{-1}.

2.2. Test organisms

Adult mussels *Perna perna* were acquired from a farming zone located at the Cocanha beach. The organisms were transported to the laboratory where they were kept for 24 h prior to the assays under temperature (25 °C) and salinity (35 ppt) controlled in a 300 L aquarium with seawater.

2.3. Test solutions

Due to its low solubility in water, Triclosan (Merck®) was firstly dissolved in dimethyl sulfoxide (Merck®) 1 g L^{-1}, before being diluted in seawater to prepare a stock solution with a final concentration of 5 mg L^{-1}. From this stock solution all test solutions was prepared. In order to assess possible effects of the solvent on the test organisms, the highest DMSO concentration used in each experiment was simultaneously tested as the solvent control.

2.4. Fertilization assay

This assay followed the USEPA protocol (USEPA, 1991) developed for assessment of acute and chronic toxicity of effluents and receiving waters for different marine and estuarine organisms. In the present study, this method was adapted to the mussel *Perna perna*, according to Zaroni et al. (2005).

Sixty adult individuals were induced to spawn by thermal stimulation (ASTM, 1992). Firstly, *Perna perna* adults were placed in a tray and exposed to dilution water at 15 °C for 30 min. The individuals were then transferred to another tray with dilution water at 25 °C for the same period. As soon as the organisms started releasing the gametes, they were removed from the tray to prevent fertilization. The gametes from males and females were collected separately and transferred to glass beakers.

The sperm was exposed to different concentrations of Triclosan for 60 min. After this period, a suspension containing approximately 2000 ovules were added to the test recipients. Fourty minutes after adding the eggs, the test was finished by adding 0.5 mL of formaldehyde in each replicate. The first 100 eggs from each replicate were analyzed and fertilization was identified by observation of the occurrence of the membrane of fertilization or first cellular divisions. The assay was repeated ($n = 4$) to obtain mean values of concentration of TCS that cause fertilization inhibition to 50% of the exposed cells (IC_{50}; 1 h). The assays were validated with 70% or more eggs in the control.

2.5. Embryo-larval development assay

In order to assess the zygote viability to develop in normal larvae when exposed to TCS, experiments were performed according to the protocol recommended by ASTM (1992) for mussels, with minor adaptations proposed by Zaroni et al. (2005) regarding salinity, which was slightly elevated to 35 ± 1 ppt. Sixty adult individuals were induced to spawn by thermal stimulation (refer to Section 2.4 for the detailed characterization of this procedure). The gametes from males and females were collected separately and transferred to glass beakers. The fertilization was attained by adding 2 mL of sperm solution to the 200 mL of ovules solution.

With the aid of a Sedgwick-Rafter chamber, the density of fertilized eggs was estimated, an about 500 embryos were transferred to glass tubes containing different nominal concentrations of TCS ranging from 0.01 to 0.32 mg L^{-1}, for a period of 48 h at a temperature of 25 °C and salinity of 35 ppt. Four replicates were used for each group, including seawater control and solvent control. After the exposure period, the assay was finished and the first 100 larvae from each replicate were analyzed. Larvae developed to D-phase were considered normal, whereas those presenting delay and/or morphological anomalies in their development were considered abnormal. A mean percentage of normal development was obtained for each tested concentration. As it was done for the fertilization assay, the embryo-larval development assay was also repeated ($n = 4$) to obtain mean values of (i) concentration of TCS that causes embryo-larval development inhibition to 50% of the exposed organisms (IC50; 48 h); (ii) the highest tested concentration of TCS showing no embryo-larval development inhibition (No Observable Effect Concentration, from now on referred to as "NOEC"); and (iii) the lowest tested concentration of TCS showing significant embryo-larval development inhibition (Lowest Observable Effect Concentration, from now on referred to as "LOEC").

2.6. Neutral Red Retention Time Assay (NRRT)

NRRT assay is based on the principle that only lysosomes in healthy cells take up and retain the vital dye neutral red. Lysosomal membrane damage caused by the impact of xenobiotics can decrease the NRRT times by inducing the leaking of lysosomal components (Dailianis et al., 2003). NRRT assay was carried out following the method described by Lowe et al. (1995).

Specimens of adult mussels ($n = 15$) were placed in 30 L aquaria with different concentrations of TCS. A first assay tested the concentrations 120 ng L^{-1}; 1200 ng L^{-1} and 12,000 ng L^{-1}. A second assay employed the concentrations 1.2 ng L^{-1}; 12 ng L^{-1} and 120 ng L^{-1}. A seawater control and a solvent control were set in parallel with the TCS bioassay. The test solutions were changed daily in all tanks. For each 24, 48 and 72 h of exposure, the mussels were removed from the aquaria for the extraction of haemolymph. After this procedure the organism were replaced in the tanks to continue the experiment.

This non-destructive method employed haemolymph withdrawn from the posterior adductor muscle of living mussels. The haemolymph was mixed to physiological saline solution (pH 7.3 containing HEPES 4.77 g L^{-1}, NaCl 25.48 g L^{-1}, $MgSO_4$ 13.06 g L^{-1}, KCl 0.75 g L^{-1}, $CaCl_2$ 1.47 g L^{-1}), spread on slides and transferred to a lightproof chamber for 15 min to allow cells attachment. Excess liquid was removed and 40 μL of the Neutral Red (NR) dye were added to the cell monolayer. A cover slip was added. After a 15-min incubation period, slides were examined every 15 min by optical microscopy (400×) for both structural abnormalities and NR dye loss from the lysosomes to the cytosol. The test was terminated when at least 50% of the examined cells exhibited these characteristics and an NRRT mean value was calculated for each group. The same analyst carried out the assessment for all slides during the study.

2.7. Statistics

2.7.1. Linear interpolation

The linear interpolation method (Norberg-King, 1988) was used to calculate the set of IC50 (1 h) for the fertilization assays and the set of IC50 (48 h) for the embryo-larval development assays.

2.7.2. Analysis of Variance

Our hypothesis considered that different life stages of *P. perna* were significantly affected by exposure to environmentally relevant concentrations of TCS; which was statistically tested by Analysis of Variance (ANOVA).

The data were firstly analyzed for normality using Chi-square (χ^2) goodness of fit test, and subsequently analyzed for homoscedasticity by the Bartlett's test. Student's *t*-test was employed to identify significant differences between control and the highest concentration of the solvent DMSO in each assay. ANOVA followed by the Dunnett's test were used to identify the concentrations significantly different from the control (NOEC and LOEC) for each embryo-larval development assay and NRRT assay. For all assays, significant differences were determined when $p < 0.05$. Statistical analysis was performed employing TOXSTAT 3.5.

3. Results

The data passed normality and homoscedasticity tests (alpha = 0.01) for every assay, and therefore no transformations were done in the datasets.

3.1. Fertilization assay

The concentrations of TCS that inhibited the fertilization of *P. perna* (IC_{50}; 1 h) ranged from 0.410 mg L^{-1} to 0.540 mg L^{-1}, with a mean value of 0.49 ± 0.048 mg L^{-1}, as presented in Table 1. There was no statistically significant difference (Student's *t*-test, $p = 0.851$) between control and the highest concentration of the solvent DMSO.

3.2. Embryo-larval development assay

The concentrations of TCS that inhibited the normal embryo-larval development of *P. perna* (IC_{50}; 48 h) ranged from 0.101 mg L^{-1} to 0.170 mg L^{-1}, with a mean value of 0.135 ± 0.028 mg L^{-1}, as presented in the Table 2. No significant difference was found between the control and the highest concentration of the solvent DMSO (Student's *t*-test, $p = 0.903$).

The NOEC of TCS ranged from 0.03 mg L^{-1} to 0.08 mg L^{-1}, with a mean value of 0.056 ± 0.021 mg L^{-1}, whereas LOEC ranged from 0.06 mg L^{-1} to 0.10 mg L^{-1}, with a mean value of 0.076 ± 0.018 mg L^{-1}. The results are presented in the Table 2.

3.3. Neutral red retention time assay

The results of the first assay of cytotoxicity (NRRT) are shown in Fig. 1. In the first 24 h, the retention time decreased significantly (Student's *t*-test $p < 0.05$) at concentrations of 1200 ng L^{-1} and 12,000 ng L^{-1} when compared with control. After 48 h, the organisms exposed to all concentrations (120 ng L^{-1}; 1200 ng L^{-1} and 12,000 ng L^{-1}) showed a significant decrease in the retention time of the dye. There was no statistically significant difference (Student's *t*-test, $p < 0.05$) between control and the highest concentration of the solvent DMSO.

The results of the second assay of cytotoxicity (NRRT) are shown in Fig. 2. In the first 48 h, retention time of neutral red dye in the lysosomes decreased significantly (Student's *t*-test $p < 0.05$) in concentration of 120 ng L^{-1}. After 72 h of exposure, significant differences were observed in concentrations of 12 ng L^{-1} and 120 ng L^{-1}.

4. Discussion

Our study assessed the biological effects of TCS in different life stages of the marine mussel *Perna perna* through fertilization, embryo-larval development and cytotoxicological assays. Considering the mean results of the fertilization assay ($IC_{50} = 0.49$ mg L^{-1}) and embryo-larval development assay ($IC_{50} = 0.13$ mg L^{-1}), it could be suggested that such effects are unlikely to occur in the environment, since current environmental concentrations of TCS in surface waters have been reported within the range from pg L^{-1} to ng L^{-1} (Nishi et al., 2008; Xie et al., 2008; Fair et al., 2009; Zhao et al., 2010).

Table 1
Results of the fertilization assays ($n = 4$) of Triclosan (IC_{50} and confidence limits) to *Perna perna*.

Assay	IC_{50} mg L^{-1}	Confidence limits–95% (lower–upper)
1	0.500	(0.498–0.517)
2	0.410	(0.384–0.462)
3	0.540	(0.528–0.579)
4	0.510	(0.498–0.514)
Mean	0.490	–
SD	0.048	–

Table 2
Results of the embryo-larval assays ($n = 4$) of Triclosan (NOEC, LOEC, IC_{50} and confidence limits) to *Perna perna*.

Assay	NOEC mg L^{-1}	LOEC mg L^{-1}	IC_{50} mg L^{-1}	Confidence limits–95% (lower–upper)
1	0.030	0.060	0.131	(0.127–0.132)
2	0.065	0.080	0.137	(0.130–0. 142)
3	0.080	0.100	0.170	(0.167–0.174)
4	0.050	0.065	0.101	(0.097–0.105)
Mean	0.056	0.076	0.135	–
SD	0.021	0.018	0.028	–

However, it cannot be entirely discarded the possibility of acute and chronic toxicity caused by TCS in the vicinities of effluent discharges from sewage outfalls or wastewater treatment plants. Farré et al. (2008) found synergism between TCS and LAS (linear alkylbenzene sulfonate) with acute effects in *Vibrio fischeri* and the authors concluded that TCS and methyl-triclosan as two of the main organic pollutants of domestic sewage that contribute to acute and chronic toxicity measured by standardized toxicity tests. In addition, it must also be considered that in highly populated areas, the environmental concentrations of TCS in the matrix sediment can reach levels with potential to cause chronic effects. In the study performed by Aguera et al. (2003), TCS has been detected in marine sediments with concentrations above 130.7 μg kg^{-1}.

It is noteworthy that sediment can be considered as a secondary source of TCS and other pollutants, because of the possibility of natural or anthropogenic processes resuspending chemicals to the water column. Halden and Paull (2005) estimated the half life of TCS at 540 days in estuarine sediments, although information about its fate in the long term is limited.

The NRRT results demonstrated the occurrence of significant adverse effects on concentrations of Triclosan in the order of magnitude of ng L^{-1} (LOEC = 12 ng L^{-1}). The lowest tested concentration (1.2 ng L^{-1}) did not produce a significant alteration in the lysosomal membrane integrity, and this was considered as NOEC. Our results corroborate the study of Binelli et al. (2009), which found adverse effects of this compound in the freshwater bivalve *Dreissena polymorpha* exposed to 289.5 ng L^{-1} of TCS for 48 h.

These results pointed out the possibility of TCS environmentally relevant concentrations cause adverse effects in non-target aquatic organisms. Previous studies have reported the occurrence of TCS in surface water in concentrations ranging from 90.2 ng L^{-1} to 478 ng L^{-1} in Chinese rivers (Zhao et al., 2010), from 55 ng L^{-1} to 134 ng L^{-1} in Channel Tone - Japan (Nishi et al., 2008), or ranging from 6.87 ng L^{-1} to 21 ng L^{-1} in Bay Bight – Germany (Xie et al., 2008).

It is worth noting that the results of the cytotoxicity assay, in which adult organisms were exposed to TCS, showed a greater sensitivity to the substance when compared to the early life stages assays. The sensitivity of the NRRT in detecting TCS toxicity may be related to the mechanism of action of this compound. Heath et al. (2001) and Newton et al. (2005) demonstrated the ability of TCS to inhibit the synthesis of fatty acids and impair mitochondrial function. Villalaín et al. (2001) observed that antibacterial effects of TCS are mediated at least in part through its membranotropic effects, which results in destabilized structures, thus affecting the functional integrity of cell membranes.

The disturbances in the lysosomal membranes have been widely used as early indicators of adverse effects to several factors,

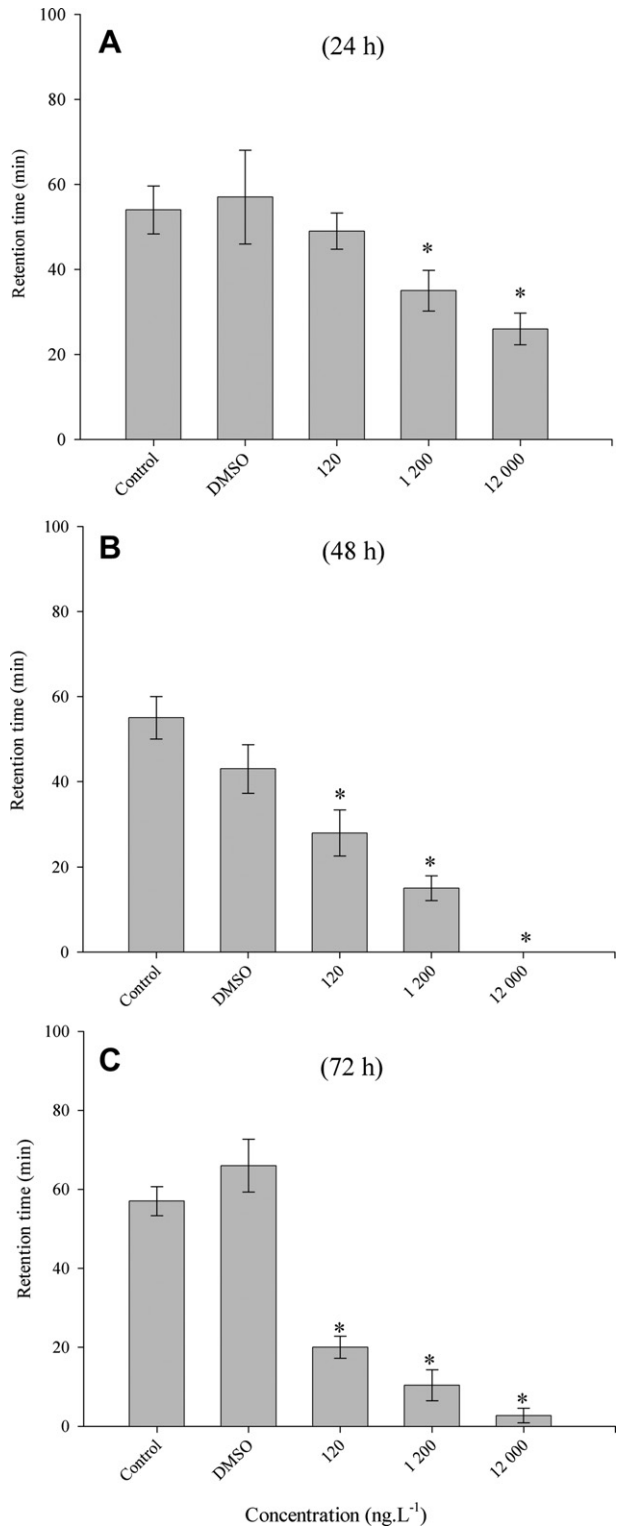

Fig. 1. Results of the first Neutral Red Retention Time Assay (ng L^{-1}; mean ± SD). A = exposure time of 24 h; B = exposure time of 48 h; C = exposure time of 72 h. An asterisk indicates a significant difference from the control (ANOVA—Dunnett's test, $p < 0.05$).

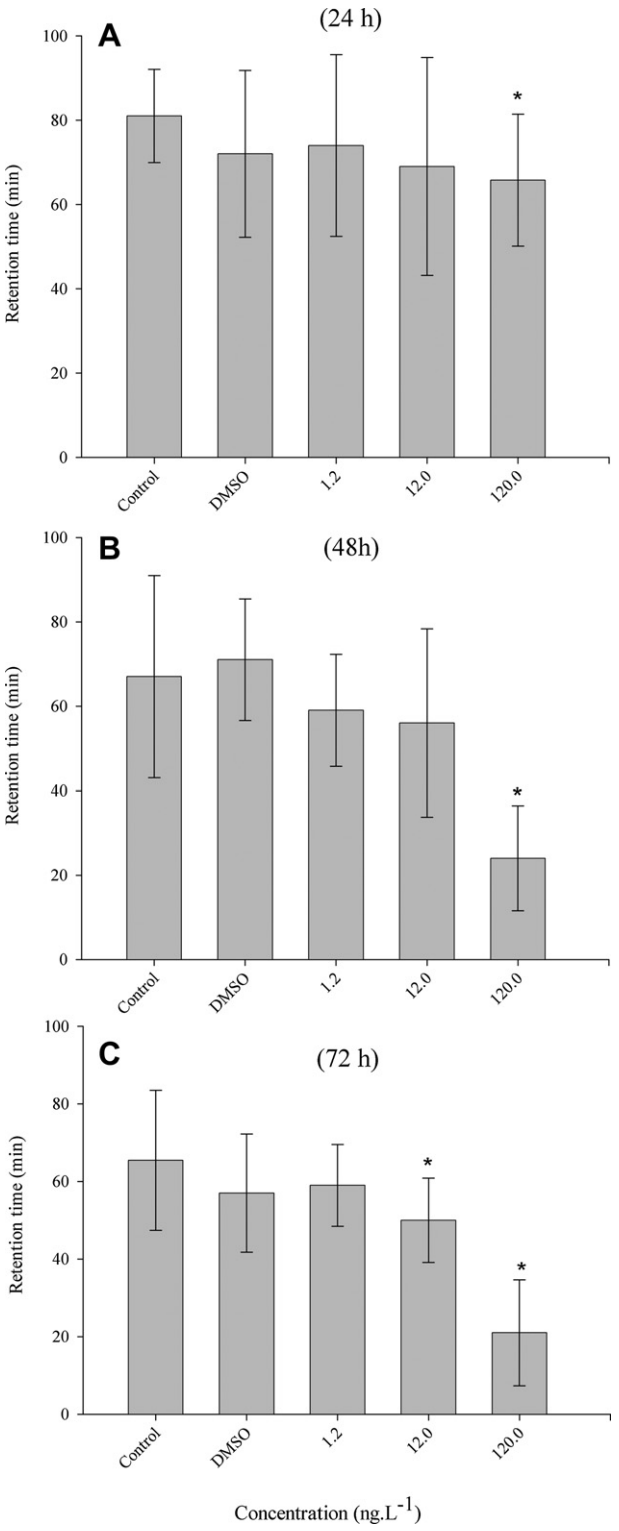

Fig. 2. Results of the second Neutral Red Retention Time Assay (ng L^{-1}; mean ± SD). A = exposure time of 24 h; B = exposure time of 48 h; C = exposure time of 72 h. An asterisk indicates a significant difference from the control (ANOVA—Dunnett's test, $p < 0.05$).

including exposure to chemicals such as drugs (Binelli et al., 2009). The integrity or stability of this membrane is considered an indicator of "well being" of the cell, being an important cellular and nonspecific biomarker of stress (Moore et al., 2007). Previous studies showed that reduced lysosomal membrane stability was associated with effects in different levels of biological organization, such as: genetic damage observed as incidence of micronuclei or DNA strand breaks (Dailianis et al., 2003; Pereira et al., 2011); biochemical alterations concerning antioxidant enzymes; cellular injury observed as lysosomal swelling, lipidosis and lipofuscinosis;

besides hepatopancreatic degeneration in mollusks (Moore et al., 2006a; Pereira et al., 2007, 2012).

Furthermore, disturbances in the integrity of lysosomal membrane can be associated to ecological impact at population and community level. It has been reported that altered lysosomal integrity can reduce gametes viability of oysters (Ringwood et al., 2004), or lead to the atrophy of the reproductive tissue of mussels by autophagy, reducing the reproductive potential (Moore and Viarengo, 1987; Allen and Moore, 2004). Moreover, lysosomal stability was directly correlated with diversity of macrobenthic organisms in a Norwegian fjord (Moore et al., 2006b). This background reinforces that the lysosomal stability is suitable to be used prognostically to evaluate the environmental risk of Triclosan in aquatic environments.

5. Conclusion

Triclosan caused acute and chronic toxicity to gametes and embryos of *Perna perna* in concentrations not yet reported in marine surface waters. However, biological adverse effects were observed at environmentally relevant concentrations when the mechanism of action of the pharmaceutical was considered by the cytotoxicological assay. Such approach provided an early warning of ecological risks for aquatic ecosystems, which should be considered in future legislation and management decisions of this compound.

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