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In vitro effects of triclosan and methyl-triclosan on the marine gastropod *Haliotis tuberculata*

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ABSTRACT

Triclosan (2,4,4′-trichloro-2′-hydroxy-diphenyl ether; TCS) is an antibacterial agent incorporated in a wide variety of household and personal care products. Because of its partial elimination in sewage treatment plants, TCS is commonly detected in natural waters and sediments. Moreover, due to its high hydrophobicity, TCS accumulates in fatty tissues in various aquatic organisms. TCS can be converted into methyl-triclosan (2,4,4′-trichloro-2′-methoxydiphenyl ether; MTCS) after biological methylation. In this study, the acute cytotoxicity of TCS and MTCS in short-term *in vitro* experiments was assessed on cell cultures from the European abalone *Haliotis tuberculata*. The results showed that morphology and density of hemocyte are affected from a concentration of 8 μM TCS. Using the XTT reduction assay, TCS has been demonstrated to decrease hemocyte metabolism activity in a dose- and time-dependent exposure. The IC_{50} was evaluated at 6 μM for both hemocyte and gill cells after a 24 h-incubation with TCS. A significant cytotoxicity of MTCS was also observed from 4 μM in 24 h-old hemocyte culture. Our results reveal a toxic effect of TCS and MTCS on immune (hemocytes) and/or respiratory cells (gill cells) of the abalone, species living in coastal waters areas and exposed to anthropogenic pollution.

1. Introduction

Triclosan (TCS; CAS registration number 3380-34-5), is a diphenyl ether referred to as 5-chloro-2-(2,4-dichlorophenoxy)phenol or 2,4,4′-trichloro-2′-hydroxy-diphenyl ether. TCS has been used in various consumer products since 1968 as an antiseptic, disinfectant, preservative in cosmetics and household cleaning products, and in textiles and kitchen utensils (see review, Bedoux et al., 2012). The antibacterial activity of TCS is due to the specific inhibition of fatty acid synthesis, required for building cell membranes (Schweizer, 2001). Among the different benefits of TCS, Chen et al. (2010) have demonstrated that TCS can be introduced in tooth-binding micelle formulations to inhibit biofilm formation and to treat preformed biofilm to the tooth surface. TCS is among the most commonly detected organic wastewater compounds for frequency and concentration (Fuchsman et al., 2010; Lyndall et al., 2010; Brausch and Rand, 2011). Although a part of TCS degrades during wastewater treatment (Onesios et al., 2009), TCS has been found in many environmental samples (Bedoux et al., 2012). TCS was detected in effluents (Sabaliunas et al., 2003; Gomez et al., 2007; Vieno et al., 2007; Kantiani et al., 2008; Peng et al., 2008; Fair et al., 2009; Kumar et al., 2010), and in marine sediments (Agüera et al., 2003; Fernandes et al., 2011). TCS presents moderate water solubility of 12 mg/L (Reiss et al., 2002) and high hydrophobicity. So, TCS is highly adsorbed to particulate materials (Orvos et al., 2002; Chu and Metcalfe, 2007) and bioaccumulates in fish tissue (Adolfsson-Erici et al., 2002; Orvos et al., 2002), and in the filamentous algae *Cladophora* spp. at 100–150 μg/kg fresh weight (Coogan et al., 2007). Recently, Gatidou et al. (2010) have detected TCS in 50% of the eighteen samples of the Mediterranean mussel *Mytilus galloprovincialis* which were collected from different sites along the Thermaikos Gulf and Lesvos Island (Greece) with levels ranging from <LOD to 2578 μg/kg (d.w.). Under these conditions, methyl-triclosan (MTCS or 2,4,4′-trichloro-2′-methoxydiphenyl ether; CAS registration number 4640-01-1) has been found as the main transformation product of TCS (Bedoux et al., 2012). MTCS is reported to be more persistent in the environment than TCS (Lindström et al., 2002; Balmer et al., 2004). Although MTCS is generally less prevalent in the environment than TCS, its mechanism of action is similar and can occur at measurable levels even when TCS is below the limit of detection (Lindström et al., 2002; DeLorenzo et al., 2008; Hinther et al., 2011). At common pH of surface waters (7 to 9),

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MTCS was not or very slowly degraded under natural sunlight when TCS degradation rate ranged from 0.3 to 18 day^{-1}. MTCS has also a higher potential to bioaccumulate, since it is more lipophilic (Lindström et al., 2002; Poiger et al., 2003; Balmer et al., 2004; Bester, 2005; DeLorenzo et al., 2008). *In vitro* and *in vivo* acute toxicity tests showed that both TCS and MTCS could also affect aquatic species such as algae (Wilson, 2003; Kuster et al., 2007; DeLorenzo and Fleming, 2008; DeLorenzo et al., 2008; Franz et al., 2008; Harada et al., 2008; An et al., 2009; Liu et al., 2009; Stevens et al., 2009), crustaceans and fishes (Orvos et al., 2002; Ishibashi et al., 2004; Tatarazako et al., 2004). Very few studies are available about TCS toxicity on mollusks, and especially gastropods, which are widespread along coastal areas and are particularly affected by anthropogenic pollution (Ward and Kach, 2009; Gatidou et al., 2010). *In vitro* and *in vivo* genotoxicity of TCS has been shown in the freshwater mussel *Dreissena polymorpha* for a concentration of 0.1 μM and 0.3 μM respectively (Binelli et al., 2009a,b). Cytotoxicity of TCS was also tested on marine bivalves. A concentration of 1 μM induced destabilization of hemocytes lysosomal membrane in the mussel *M. galloprovincialis* (Canesi et al., 2007). In the clam *Ruditapes philippinarum*, 0.001 μM TCS decreased total hemocyte count (THC) (Matozzo et al., 2011). Based on these studies, the ecotoxicity of TCS seems to depend on the exposure time and the species. To our knowledge, no study has been reported on MTCS ecotoxicity using marine organisms.

The European abalone *Haliotis tuberculata* is a marine benthic gastropod living in coastal areas along the eastern Atlantic to the west coast of Africa. As abalone feed on macro-algae, that can bioaccumulate TCS and MTCS (Coogan et al., 2007), it is a relevant model organism to study the toxicity of these chemicals. Furthermore, abalones have been previously shown to serve as sensitive indicator species for coastal pollutions (Gorski and Nugegoda, 2006; Zhu et al., 2011). Primary cultures derived from mollusk tissues have been successfully used for cytotoxicity tests (Domart-Coulon et al., 2000; Canesi et al., 2007; Binelli et al., 2009a; Matozzo et al., 2011). As shown in marine bivalves, short-term primary cultures from abalone target tissues would help in the assessment of marine pollutants. Since hemocytes play a key role in digestion, metabolite transport, shell repair (Mount et al., 2004) and immune system (Adema et al., 1991; Gopalakrishnan et al., 2009), they provide a suitable model to study *in vitro* toxicity of pollutants. Because gills are the first organ exposed to exogenous molecules, *in vitro* toxicity tests on gill cells could give information on the effect of toxicants on respiratory function.

The aim of this study was to assess the acute cytotoxicity of TCS and MTCS in short-term *in vitro* experiments in hemocyte and gill cell cultures of the abalone *H. tuberculata*. We used previously developed primary cultures of hemocytes to determine the effect of TCS and by-product MTCS on immunity cells (Auzoux-Bordenave et al., 2007). Explant primary cultures of gill cells were also established for *in vitro* toxicity assays of TCS on respiratory cells.

2. Materials and methods

2.1. Source and maintenance of animals

European adult abalones (*H. tuberculata*), 90 mm in shell length, were collected from the north coast of Brittany (Roscoff, France). Animals were maintained at the laboratory in an 80 L-tank supplied with seawater from the Atlantic Ocean (exchange rate 2% per hour). Natural conditions of water temperature, salinity and photoperiod were used. Abalones were fed once a week with the red algae *Palmaria palmata*. Two days before experiments, abalones were starved and seawater was UV treated to avoid contamination.

2.2. Primary cultures of hemocytes

Hemolymph was sampled with a syringe from the branchial cavity of the abalone. For each assay, 5 mL of hemolymph from a single animal were used. Hemolymph was filtered on a 40 μm-sieve and then diluted in 2 volumes of a sterile anticoagulant solution (383 mM NaCl, 115 mM glucose, 37 mM sodium dihydrogen citrate, 11 mM EDTA, 100 U/mL penicillin, and 100 μg/mL streptomycin, diluted in ultra-pure water, pH 7.5) and 1 volume of an antiseptic solution (200 U/mL penicillin, 200 μg/mL streptomycin, 250 μg/mL gentamicin, and 2 μg/mL amphotericin B, diluted in filtered sea water, pH 7.4). Cell density was evaluated using a Malassez grid and cells were plated in 96-well plates (100,000 cells/well). Two hours after seeding, as cells adhered to the bottom of the wells, solutions were replaced by 90 μL/well of modified Leibovitz L-15 medium (346 mM NaCl, 7 mM KCl, 5 mM $CaCl_2$, 4 mM $MgSO_4$, 19 mM $MgCl_2$, 4 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 200 μg/mL gentamicin, and 1 μg/mL amphotericin B) adjusted to 1100 mmol/L and pH 7.4. Plated cells were incubated at 19 ± 0.2 °C in a humidified incubator, in the dark, for 24 h before any *in vitro* experiments.

2.3. Gill explant cultures

Cells from abalone gills were obtained from 3-day-old explant primary culture. For each assay, gills from a single animal were dissected and washed in the antiseptic solution (see Section 2.2.) for two days. Gills were then minced into 2–3 mm^3 explants that were placed to adhere onto the bottom of plastic 6-well plates. After 5 min, explants were covered with modified Leibovitz L-15 medium and incubated for 3 days at 19 ± 0.2 °C in a humidified incubator, in the dark. Explants and gill cells were collected and filtered on a 40 μm mesh filter. The cell suspension was then resuspended in modified Leibovitz L-15 medium. After estimating cell density, 90 μL of gill cells were plated in 96-well plates (200,000 cells/well) and incubated at 19 ± 0.2 °C in a humidified incubator, in the dark for 24 h before any *in vitro* experiments.

2.4. Toxicants

TCS (Irgasan) and MTCS (target molecules) were purchased from Sigma-Aldrich. Considering their low solubility in water, TCS and MTCS were dissolved in DMSO. DMSO was diluted in modified Leibovitz L-15 medium at the concentrations used in the study and the mixture was incubated with hemocyte and gill cell cultures to test its potential proliferating or cytotoxic effect. For both cultures, DMSO showed no significant effect up to 2%. The solutions used in the study ranged between 0 and 0.3% of DMSO, thus, DMSO was considered as a suitable solvent for assaying TCS and MTCS toxicities in abalone cell cultures. Fresh 4 mM stock solutions in DMSO were prepared for each experiment and immediately used. Each solution was diluted in modified Leibovitz L-15 medium and 10 μL was added to experimental wells. Negative controls were performed with 10 μL of the modified Leibovitz L-15 medium. Cell cultures were incubated for 24 h at 19 ± 0.2 °C, in the dark.

2.5. Light microscopy

Plated cells were observed twice a day to control their behavior and morphology with and without target molecules. Cells were observed with an inverted phase contrast microscope (Telaval 3, Zeiss, Germany) and pictures were taken with an Olympus digital camera.

2.6. Cytotoxicity assay

Cell viability was evaluated using the XTT assay (Roche Laboratory, France), based on the reduction of a tetrazolium salt into yellow formazan salt by active mitochondria (Mosmann, 1983). As for the MTT assay, previously adapted to marine mollusk cells, the XTT assay