Marine Environmental Research 74 (2012) 40–46

Contents lists available at SciVerse ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

# A multi-biomarker approach to assess effects of Triclosan in the clam *Ruditapes philippinarum*

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ARTICLE INFO

*Article history:*
Received 5 November 2011
Received in revised form
7 December 2011
Accepted 8 December 2011

*Keywords:*
Triclosan
Clams
Biomarker
Vitellogenin
Lipid peroxidation
Antioxidant enzymes
Acetylcholinesterase
Ecotoxicology
Sublethal effects

ABSTRACT

The effects of 7 days' exposure to differing Triclosan (TCS) concentrations (300, 600, and 900 ng/L) were investigated in the clam *Ruditapes philippinarum*. Vitellogenin (Vg)-like protein levels in haemolymph and digestive gland from males and females, gill acetylcholinesterase (AChE) activity, superoxide dismutase (SOD) and catalase (CAT) activities in gills and digestive gland, and gill lipid peroxidation (LPO) were measured. The highest TCS concentrations decreased significantly Vg levels in male haemolymph and digestive gland, whereas no significant variations were found in females. The highest TCS concentrations increased significantly SOD activity in gills, but decreased it in digestive gland. No changes in CAT activity were observed. In gills, TCS reduced significantly AChE activity, but it did not induce significant variations in LPO. Our study demonstrates that TCS alters biochemical parameters in *R. philippinarum*, even at environmentally realistic concentrations, and suggests differing modes of action of the contaminant, in clams at least.



## 1. Introduction

Among emerging environmental contaminants, pharmaceuticals and personal care products (PPCPs) are a large group of substances used either by human for personal health and cosmetic reasons or by agribusiness to enhance growth or health of livestock. PPCPs are produced in large quantities – thousands of tons per year – and comprise numerous chemicals, including prescriptible drugs, veterinary drugs, diagnostic agents, fragrances, lotions, and cosmetics (EPA, http://www.epa.gov/ppcp/basic2.html). As a consequence, main sources of PPCPs in the environment are human activities (e.g., swimming), residues from both pharmaceutical manufacturing and hospitals, illicit drug use, veterinary drug use (antibiotics, steroids, in particular) and agribusiness.

Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether – TCS) is an ionisable chlorinated biphenyl ether widely used as an antimicrobial and antifungal agent in soaps, shampoos, deodorants, toothpastes, cosmetics, skin creams, and plastics (Daughton and Ternes, 1999; Jones et al., 2000). TCS has a moderate water solubility (12 mg/L), a pKa of 8.1 at 20 °C, and is lipophilic (log $K_{ow}$ of 4.8) (Reiss et al., 2002). The wide use of TCS has contributed to its presence in aquatic ecosystems. For example, TCS has been detected in 57.6% of the water bodies analysed (139 streams) across 30 states of the USA, with a maximum level of 2.3 μg/mL and a median level of 0.14 μg/mL (Kolpin et al., 2002). TCS has been detected at ng/l levels in freshwater, mainly near urban areas (Kolpin et al., 2002; Flaherty and Dodson, 2005). TCS concentrations ranging from 0.8 to 6870 pg/L and from <1 to 95 pg/L have been detected in the dissolved phase and suspended particulate matters, respectively, of seawater samples from the German Bight, especially in the estuaries of the Elbe and the Weser rivers (Xie et al., 2008). TCS has also been found at concentrations ranging from 15 to 110 ng/L in seawater from Tai Po and Victoria Harbours in Hong Kong (Wu et al., 2007).

In aquatic ecosystems, TCS can bioaccumulate and affect non-target organisms (Orvos et al., 2002). For example, the average concentration of TCS in bivalves (*Modiolus barbatus* L., *Mytilus galloprovincialis*, *Venus gallina*) sampled in Greece was 461 ng/g (dry weight) (Gatidou et al., 2010). In the same study, exposure for 28 days of *M. galloprovincialis* to 300 ng/l TCS increased the contaminant concentration in animals, demonstrating that Mediterranean mussels can accumulate TCS (Gatidou et al., 2010). In fish, TCS has been shown to be androgenic (Foran et al., 2000) or estrogenic (Ishibashi et al., 2004). Sublethal effects, such as loss of

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doi:10.1016/j.marenvres.2011.12.002

equilibrium, fish jaw locked open, erratic swimming, spinal curvature, and quiescence were observed after exposure of the rainbow trout, *Oncorhynchus mykiss*, at the highest TCS concentration tested (80 μg/L) (Orvos et al., 2002). In bivalve molluscs, cytotoxic and genotoxic effects of TCS have recently been demonstrated in the zebra mussel, *Dreissena polymorpha*, after in vitro and in vivo experiments (Binelli et al., 2009a,b). In *M. galloprovincialis*, TCS affected immune functions both in vitro and in vivo, stimulated the activity of glutathione transferase (GST) in digestive gland from injected mussels, but it did not induce changes in total glutathione levels (Canesi et al., 2007).

We have recently demonstrated that a 7-day exposure of the clam *Ruditapes philippinarum* to sublethal TCS concentrations can induce significant alterations in immune parameters, suggesting that the contaminant is immunotoxic, in clams at least (Matozzo et al., in press). Conversely, information about the potential neurotoxicity and estrogenicity of TCS, as well as its role in promoting oxidative stress in *R. philippinarum*, lack in the literature. As a consequence, in the present study, effects of TCS on various biochemical parameters of *R. philippinarum* were evaluated for the first time. In particular, vitellogenin (Vg)-like protein levels were measured by the alkali-labile phosphate assay (ALP) in haemolymph and digestive glands from both males and females. Activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were measured in gills and digestive gland, whereas acetylcholinesterase (AChE) activity and lipid peroxidation (LPO) were evaluated in gills. Gills and digestive gland were selected to assess TCS toxicity in clams as they are target tissues for many contaminants, the former tissue being in immediate contact with waterborne pollutants, the latter being mainly involved in accumulation and metabolism of organic contaminants. The multibiomarker approach was chosen in order:

i. to obtain further information about toxic effects of TCS in bivalve molluscs;
ii. to compare results obtained in *R. philippinarum* with those available in the literature for both freshwater and marine bivalves.

## 2. Materials and methods

### 2.1. Animals

Specimens of *R. philippinarum* (3.0–3.5 cm shell length) were collected from a reference site in the southern basin of the Lagoon of Venice (Italy) and acclimatised in the laboratory for 5 days before exposure to TCS. They were kept in large aquaria with sandy bottoms and aerated sea water (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C) and fed with microalgae (*Isochrysis galbana*).

### 2.2. TCS solutions, clam exposure and tissue collection

TCS was purchased from Sigma–Aldrich (Milano, Italy). A stock solution of the contaminant was prepared in ethanol and stored at room temperature for the duration of the experiments. Working solutions were prepared daily by diluting the stock solution in sea water. Two series of experiments were carried out.

The first experiment was performed to assess the potential estrogenicity of TCS measuring Vg-like protein levels in male and female clams. To this aim, clams were exposed for 7 days during the pre-spawning phase, when it was possible to distinguish sex by microscopic observation of gonadal smears. Bivalves were exposed to 0, 0 + ethanol, 300, 600, and 900 ng/L. The nominal concentrations were chosen on the basis of the reported data on TCS bioaccumulation and toxicity in bivalves (Canesi et al., 2007; Binelli et al., 2009a; Gatidou et al., 2010; Matozzo et al., in press). In ethanol controls, solvent was added at the highest concentration used in TCS treatments (16 μl/L). During exposure, clams were maintained in glass aquaria (without sediment) containing aerated sea water (1 L/animal), in the same thermo-haline conditions used in the acclimatisation period. Every day water was changed, and TCS and microalgae added (initial concentration of about 100,000 cells/L). At the end of exposure, prior to sexing, haemolymph was individually collected from the anterior adductor muscle of both control and TCS-exposed clams with a 1-mL plastic syringe and placed in Eppendorf tubes in ice. Haemolymph from males and females was pooled to obtain 5 replicates (2 animals each) per sex from each experimental conditions. Pooling was necessary to obtain enough haemolymph protein material for ALP analysis. Haemolymph was centrifuged at 780 *g* for 10 min to remove the haemocytes. The shells were then opened and sexing (identification of oocytes and spermatozoa) was performed by microscopic observation (400×) of smears of gonadal tissue. Digestive glands were then excised from 10 individual males and females. Both cell-free haemolymph and digestive glands were frozen and stored at −80 °C until processing.

The second experiment was performed to evaluate TCS effects on antioxidant enzyme and AChE activities, and LPO. Clams (10 animals per concentration) were exposed for 7 days to the same TCS concentrations indicated above. SOD and CAT activities were individually measured in gills and digestive gland, whereas AChE activities and LPO were individually measured in gills only. After exposure to TCS, tissues were excised, frozen in liquid nitrogen, and stored at −80 °C until processing.

### 2.3. Tissue preparation

Gills and digestive glands were homogenised individually at 4 °C with an Ultra-Turrax homogeniser (model T8 basic, IKA) in four volumes of 10 mM Tris–HCl buffer, pH 7.5, containing 0.15 M KCl, 0.5 M sucrose, 1 mM EDTA and 40 μg/ml aprotinin (Sigma), sonicated for 1 min at 0 °C with a Braun Labsonic U sonifier at 50% duty cycles, and centrifuged at 12,000 *g* for 45 min at 4 °C. Supernatants (SN) were collected for analyses.

### 2.4. Determination of Vg-like proteins: ALP assay

ALP levels were measured in both cell-free haemolymph and homogenised digestive glands from clams exposed for 7 days to TCS. We selected the 7 days exposure because this is an adequate period to induce variations in Vg levels in bivalves (Matozzo and Marin, 2005; Marin et al., 2008; Ricciardi et al., 2008). ALP levels were measured following the method of Blaise et al. (1999). This method, based on the determination of labile phosphates released by Vg after hydrolysis with alkalis, was shown to be significantly correlated with the other direct assays (Kramer et al., 1998; Blaise et al., 1999). Five hundred μL of cell-free haemolymph or tissue SN were mixed with 500 μL of *t*-butyl methyl ether (Sigma) for 30 min at room temperature. These emulsions were mixed by a Vortex agitator at least 3 times during the extraction period. A 400-μL sample of the ether phase was then mixed with 100 μL of 2 M NaOH for 60 min at 50 °C, to allow hydrolysis of bound phosphates. The levels of free phosphates were determined in the aqueous phase according to the phosphomolybdenum method proposed by Stanton (1968). A standard curve of known concentrations of inorganic phosphate was drawn. Results were expressed as μg ALP/mg proteins. Protein concentrations in both cell-free haemolymph and homogenised digestive glands were quantified