

Toxic Assessment of Triclosan and Triclocarban on *Artemia salina*

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Abstract In this study, we investigated the possible acute toxic and genotoxic effects of triclosan (TCS) and triclocarban (TCC) on *Artemia salina*. Genotoxicity was evaluated using single-cell gel electrophoresis and apoptotic frequency assays (Annexin V-FITC/PI assay). Acute toxicity test results showed that TCC ($LC_{50-24\text{ h}} = 17.8\ \mu\text{g/L}$) was more toxic than TCS ($LC_{50-24\text{ h}} = 171.1\ \mu\text{g/L}$). Significant increases in both genotoxic biomarkers were observed at 24 h after initial exposure, indicating that these two chemicals are potentially dangerous for this aquatic biological model. Although further studies are required, a comparison of data both in vitro and in vivo allowed us to suggest possible mechanisms of action for TCS and TCC in this sentinel organism.

Keywords *Artemia salina* · Triclosan · Triclocarban · Acutetoxicity · Genotoxicity

The antimicrobial agents triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether; TCS) and triclocarban (3,4,4'-trichlorocarbanilide; TCC) are high-production-volume

chemicals that are widely used in a variety of personal care products (PCPs) (Richardson 2010). Currently, TCS and TCC are among the top ten most commonly detected organic wastewater compounds for frequency and concentration in U.S. (Halden and Paull 2005).

Although these two compounds are broadly classified as halogenated aromatic hydrocarbons, TCS has functional moieties representing phenols, diphenyl ethers, and polychlorinated biphenyls (PCBs). Both TCS and TCC have been detected in wastewater effluent in quantities up to micromolar concentrations ($\mu\text{g/L}$) in Asia, Europe, and North America, indicating widespread environmental contamination. Although the detection of the instrument can quantify TCS and TCC concentrations, it cannot measure directly the influences on test organisms. In a laboratory-based study, TCS has been shown to inhibit enoyl-acyl carrier-protein reductase, an enzyme that catalyzes an essential lipid biosynthesis step in the membranes of many bacteria and fungi (Heath et al. 2000). TCC acts predominantly against Gram-positive bacteria (Richardson 2010). Due to the persistence and toxicity of TCS and TCC, their aquatic ecotoxicity has attracted wide attention (Brausch and Rand 2011). Several studies provided evidence that TCC and TCS are toxic to aquatic organisms, demonstrating that these substances interfere with biological receptors in vitro, including a stably transfected aryl hydrocarbon receptor (AhR)-, androgen receptor (AR)-, or estrogen receptor (ER)-responsive firefly luciferase reporter gene that responds to chemicals that can bind to and/or activate the respective receptor (Ahn et al. 2008). Moreover, TCC could also be a significant concern when it coexists with TCS, whose hazard quotient was reported to be over ten by Brausch and Rand (2011).

Artemia salina is routinely employed as a test organism for ecotoxicological studies. Normally, the molecular,

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cellular, and physiological levels of *A. salina* change dramatically when they are under contamination stress (Margómez et al. 2004). The aim of this study was to evaluate the possible acute and genotoxic effects of TCS and TCC on *A. salina*. Acute toxicity tests were conducted to estimate the median lethal concentration (LC_{50}). Genotoxicity was evaluated using the SCGE and Annexin V-FITC/PI assays. The study presents result for toxicity and genotoxicity of TCS and TCC in *A. salina* at the larval stage, a vulnerable developmental stage.

Materials and Methods

TCS and TCC were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) with minimum purity of 97 %. Commercial TCS and TCC compounds were further purified to approximately 100 % after recrystallization three times from petroleum ether and ethanol. Unless otherwise noted, all of the other chemicals and solvents (obtained from Sangon Biotech Chemical Co., Shanghai, China) were of analytical grade, with secondary sub-boiling distilled being used.

Concentrations of TCS and TCC were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Instrumental conditions and quality assurance and quality control (QA/QC) were followed by the method of Chen et al. (2010). An Agilent 1200 rapid resolution liquid chromatograph (Agilent Technologies Inc., Santa Rosa, CA, USA) coupled to Agilent G6460A triple quadrupole mass spectrometer. The chromatographic separation was performed on an Agilent SB-C18 column (3.0×100 mm, $1.8 \mu\text{m}$) with a RRLC in-line filter kit (4.6 mm, $0.2 \mu\text{m}$ filter) (Zoz Group, Wenden, Germany). The column temperature was maintained at 40°C . The chromatographic mobile phases were run at a flow rate of 0.3 mL/min. The mobile phase for analysis of TCS and TCC consisted of (A) Milli-Q water and (B) acetonitrile, with the gradient programmed as follows: 0 min, 40 %B; 15 min, 70 %B; and 20 min, 95 %B. The initial mobile phase was equilibrated for 8 min after each run. The mass spectrometer was operated at a flow in electrospray ionization mode (ESI) with optimized parameters as follows: drying gas temperature 350°C and flow rate 8 mL/min, capillary voltage 3500 V, nebulizing gas pressure 50 psi, sheath gas temperature 350°C and flow rate 12 mL/min. The injection volume was $10 \mu\text{L}$. The parameters for the analyzed chemicals are shown in Table 1. The recovery test with four replicates ($n = 4$) was performed. For water samples, the recoveries of TCC and TCS were $103 \pm 5 \%$ and $97 \pm 1 \%$, respectively. Limit of detection (LOD) and quantitation (LOQ) for TCC and TCS were also

calculated. The minimum detection limits were both lower than 1 ng/L.

Approximately 1 – 2 mL of *A. salina* eggs were incubated in approximately 10 mL of artificial salt water (25 g NaCl/L oxygen-saturated distilled water, pH 8.0) in a dish at a temperature of 25°C , with exposure to constant illumination of 1000 – 2000 lx. After 24 h from the start of the incubation, all larvae had molted to the 2nd–3rd instar stages, at which point they can swim freely. These instars have been shown in several papers (Sánchez-Fortún et al. 1996, 1997) to be the most sensitive stages and were used for our bioassay.

A major factor to consider when conducting experiments in a laboratory setting is the concentrations used in order to accurately reflect what occurs in the environment. Since no data existed on acute toxicity and cytotoxicity of TCS and TCC for *A. salina* prior to, chemical concentrations were selected from a preliminary experiment. TCS and TCC were prepared in at least 12 different concentrations (TCS was in the range of 100 – $500 \mu\text{g/L}$ and TCC was in the range of 1 – $100 \mu\text{g/L}$), followed by solubilization in an artificial marine solution with 1% (v/v) dimethyl sulfoxide (DMSO) as a solubilization agent. The tested concentrations also reflect TCS and TCC levels that have been measured in moderate to highly polluted ecosystems (Binelli et al. 2009). Five replicates for each treatment were performed, and a solvent blank was also included for TCS and TCC. During the test, ten nauplii of *A. salina* (incubated as previously described) were added to each beaker (10 mL) and maintained in direct contact with the solutions. All exposures were conducted in an artificial climate incubator at 25°C under continuous illumination. The number of dead individuals was counted at 24 h and the LC_{50} value was determined on the basis of the geometrical means from the dose–response curves (DRC) of the measured test chemical concentrations.

Ten nauplii of *A. salina* with similar body lengths and activity (movements per minute) were also selected and treated with TCS and TCC at the $LC_{50-24 \text{ h}}$ concentration (obtained from the acute toxicity test above) for 0 , 6 , 12 , and 24 h. Ten replicates were used for each group, and the controls were exposed to the artificial marine solution with 1% (v/v) DMSO. Subsequent to the exposure to TCS or TCC, *A. salina* coelomocytes were obtained as described by Zhao and Cai (2007). Individual nauplius larvae were rinsed in 1 mL volume of extrusion medium composed of 5% ethanol, 95% saline, 2.5 mg/mL EDTA, and 10 mg/mL guaiacol glyceryl ether (pH 7.3). Coelomocytes were spontaneously secreted in the medium and collected by centrifugation (9000 r/min, 4°C). Phosphate-buffered saline (PBS) was used to wash the cells prior to the comet assay. The procedure was conducted on ice.

Table 1 Retention time (R_t), precursor and product ions, and optimized electrospray ionization tandem mass spectrometry (ESI–MS/MS) parameters for TCS and TCC

Compounds	R_t (min)	Precursor ion (m/z)	Products ions (m/z)	Fragmentor voltage (V)	Collision energy (V)
TCS	15.21	299	35.1	65	1
TCC	14.93	320	163.1	107	5

The percentage of apoptotic cells was evaluated using a previously published protocol (Overbeeke et al. 1998). Surface exposure of phosphatidylserine in apoptotic cells was quantitatively detected with an annexin V-FITC/PI apoptosis detection kit (BD Biosciences Clontech) in a FACSsort flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA).

The experimental data were statistically analyzed with a two-way analysis of variance (ANOVA) followed by post hoc tests, with $p < 0.05$ being considered statistically significant between treated samples and related controls. SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA) and Software Origin 8.0 (OriginLab Corp., Northampton, MA, USA) were employed as tools to process experimental data and graphics.

Results and Discussion

A. salina was used as a biological model to reveal the potential environmental risk of TCS and TCC. Generally, the in vitro experiment was used as a preliminary screening to gain basic knowledge of the nature of the tested agents as well as the cellular response (Kirkland et al. 2011), and the in vivo approach under laboratory conditions enables us to gain toxicological information.

DRC function was determined by statistical regression analyses of experimental data sets. Unfortunately, there is no universal regression model that fits all toxicity data sets. In the median effect region, differences between regression models are usually negligible, but in the low-effect range they may become crucial for a valid prediction of toxicity. Thus, we adopted the Dixon method (Dixon 1998) to test the outliers and removed questionable values. The average corrected mortalities in different concentrations were then obtained by calculating the arithmetic average values. The scatter plot was obtained by using software Origin 8.0 with the logarithm concentration as the abscissa and the corresponding corrected mortality as the ordinate. Three

different non-linear regression models (Dose–Response, Weibull and Logit) (Faust et al. 2003) were fitted to each data set, and the best fit was selected by a robust goodness of fit criterion (sum of absolute errors). The DseResp function provided the best fit model (Table 2) and describe the dose response relationship for the data sets (Fig. 1).

Judging from the regression model parameters in Table 3, the value of average corrected mortalities could be effectively estimated both in the low and high effects regions, which were well-described with the DseResp model. It can be concluded from the good fits ($R > 0.9$) and statistical significance that *A. salina* was an appropriate model to evaluate the toxicity of TCS and TCC. Their toxicities differed largely, as their $LC_{50-24\text{ h}}$ values spanned over nearly one order of magnitude. The DRCs of the dissimilarly acting substances were sigmoid but had different shapes and steepness (Fig. 1). Typically, a non-symmetrical curvature is observed, which tends to be flat in

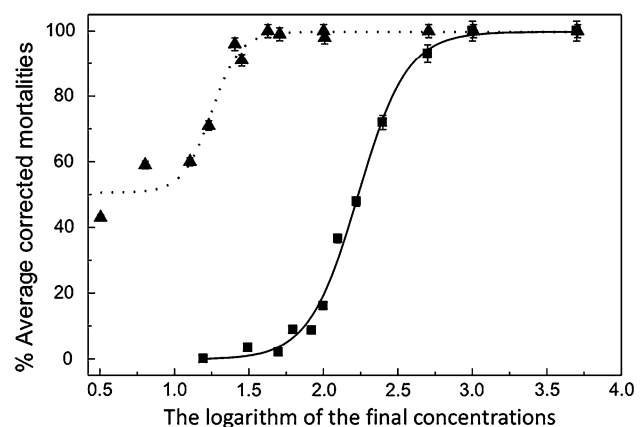


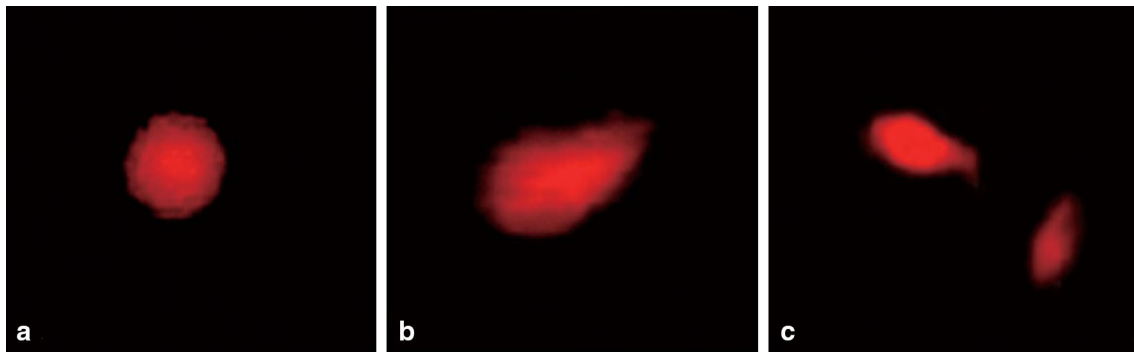
Fig. 1 Dose–response curves for TCS and TCC acute toxicity tests with *A. salina*. Experimental data (filled triangle): corrected mortalities in different concentrations of TCC. Experimental data (filled square): corrected mortalities in different concentrations of TCS. Curve (dotted line): dose–response curve of the toxic effect of TCC. Curve (solid line): dose–response curve of the toxic effect of TCS

Table 2 Selected non-linear fitting functions describing dose–response curves (DRC)

Name	Function (F)	Function (F^{-1})
DseResp	$E = A_1 + (A_2 - A_1)/(1 + 10 \exp((\log x_0 - c) \cdot p))$	$c = (\log(E - A_1) - \log(A_2 - E))/p - \log x_0$

Table 3 Collective parameters for toxicities of TCS and TCC to *A. salina*

Substance name	Fitting model parameters					<i>R</i>	LC _{50–24 h} (µg/L)	95 % CI for LC _{50–24 h} (µg/L)
	<i>A</i> ₁	<i>A</i> ₂	log <i>x</i> ₀	<i>p</i>	Span			
TCS	−0.14	99.78	2.23	2.64	99.92	0.997	171.1	162.5–179.7
TCC	50.61	99.76	1.25	4.51	49.16	0.978	17.8	16.9–18.7

**Fig. 2** **a** Negative control (artificial marine solution with 1 % (v/v) of DMSO); **b** 171.0 µg/L of TCS; **c** 18.0 µg/L of TCC

the low effects region compared with the high effects region. However, intersections between the curves clearly indicated that they were not parallel in a strict mathematical sense. The effects of these two chemicals were assessed as having non-linear curve change trends, presenting the typical s-shaped dose–effect relationship. As for the LC_{50–24} value of *A. salina*, a much stronger toxicity was found for TCC (approximately 17.8 µg/L), and this value was one order of magnitude lower than the value for TCS (approximately 171.1 µg/L). For all subsequent experiments, 171.0 µg/L of TCS and 18.0 µg/L of TCC were used for weighing convenience. Based on toxicity data, the acute toxicity of TCC to the aquatic invertebrates, *A. salina*, was greater than that of TCS, which was consistent with the results reported by TCC Consortium (2002).

The DNA damage of *A. salina* nauplii coelomocytes was compared with control coelomocytes after exposure to TCS and TCC for 24 h. Figure 2a shows a typical comet image of undamaged DNA from *A. salina* coelomocytes. Figure 2b, c show typical comet images of damaged DNA of *A. salina* coelomocytes induced by TCS and TCC, respectively.

As observed in Table 4, the values of olive tail moment (OTM) and of the % tail DNA (TDNA) in all of the treatment groups are higher than those of the controls at the same time point and are increasing with exposure time. OTM values of TCS ranged from 0.015 to 1.796, with the highest value observed after 24 h of exposure. OTM values of TCC ranged from 0.055 to 5.651, with the highest value observed at the same time point. TDNA values of TCS

ranged from 0.053 to 12.659 and TCC ranged from 0.059 to 27.814, with the same temporal trend. At the tested concentrations, TCS induced significant ($p < 0.05$) primary genetic damage in the *A. salina* coelomocytes at the end of exposure (24 h) while TCC induced significant ($p < 0.05$) damage after 12 h as well as after 24 h ($p < 0.01$). TCC increased the levels of DNA damage (OTM values) over three times that of TCS at all time points. The primary DNA damage by both chemicals seemed to follow the same trend, and a clear time/effect relationship ($F = 736.6$; $p < 0.01$) was obtained for TCS, as well as a significant ($F = 511.4$; $p < 0.01$) time/effect correlation for TCC.

The same temporal trend was also obtained for the second measured end point, showing a significant increase ($p < 0.05$) in DNA fragments in the comet tails after 12 h of exposure to both chemicals and a significant increase ($p < 0.001$) at 24 h of exposure to TCC. The percentages of tail DNA fragmentation of 12.659 % (TCS) and 27.814 % (TCC) were already obtained after 24 h of exposure, indicating that both TCS and TCC have fast genotoxic effects on *A. salina* coelomocytes.

Many studies have shown that reactive oxygen species (ROS) are the major sources of DNA damage. ROS cause DNA damage by causing strand breaks, removing nucleotides and modifying the nucleotide bases (Cooke et al. 2003). In this study, as indicated by the Comet test (Fig. 2; Table 3), enhanced DNA damage may result from oxidative stress, indicating that the increase in TCS and TCC may cause ROS accumulation leading to subsequent DNA

Table 4 DNA damages of *A. salina* coelomocytes exposed to TCS and TCC

Group	OTM (% μm)	TDNA (%)
Controls		
0 h	0.013 (0.001–0.125)	0.047 (0.003–1.012)
6 h	0.020 (0.002–0.379)	0.053 (0.007–0.099)
12 h	0.023 (0.001–0.195)	0.066 (0.004–1.003)
24 h	0.029 (0.001–0.142)	0.085 (0.002–1.118)
TCS (171.0 $\mu\text{g/L}$)		
0 h	0.015 (0.001–0.180)	0.053 (0.004–0.082)
6 h	0.025 (0.004–0.142)	0.067 (0.002–1.108)
12 h	0.089 (0.002–2.108)	0.781 (0.001–2.358)
24 h	1.796 (0.004–20.388)*	12.659 (0.026–54.187)*
TCC (18.0 $\mu\text{g/L}$)		
0 h	0.055 (0.001–2.808)	0.059 (0.001–19.449)
6 h	0.117 (0.004–2.418)	0.155 (0.002–4.496)
12 h	3.424 (0.001–28.208)*	19.851 (0.001–48.708)*
24 h	5.651 (0.004–44.059)**	27.814 (0.002–74.601)**

Temporal trend (h) of the medians (95 % CI) of OTM and TDNA calculated for *A. salina* coelomocytes for controls and treated samples with TCS and TCC. Significant values (two-way ANOVA, Bonferroni posthoc test, * $p < 0.05$, ** $p < 0.001$) refer to the comparison between treated samples

damage. The time-dependent dramatic increase in the amount of genetic damage found in the SCGE assay can be partially overcome by the defense mechanisms of *A. salina*.

The translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface is a characteristic occurrence in early apoptotic cells (Overbeeke et al. 1998). We investigated cell apoptosis when the cells were incubated with TCS and TCC at the LC_{50-24} h for 24 h. Cells undergoing apoptosis will first express phosphatidylserine on the outer leaflet of the cell

membrane, which is marked by annexin-V-FITC binding. The membrane becomes compromised, followed by PI intercalation into the cellular DNA and RNA. The results showed that the apoptotic rates all increased after being treated with TCS ($p < 0.05$) and TCC ($p < 0.001$) at the designated concentrations after 24 h compared with the control. As indicated in Fig. 3, the apoptotic rate in untreated cells was 4.3 %. After treatment with TCS and TCC for 24 h, the corresponding rates were 22.3 % and 45.8 %, respectively, suggesting that these cells were expressing phosphatidylserine on the outer leaflet of the membrane and that these cells may have been undergoing apoptosis. Moreover, a concentration of TCS (171.0 $\mu\text{g/L}$) was able to produce an apoptotic frequency that was about five times higher than the baseline level. The highest percentage of apoptosis (45.8 %), was approximately 10 times that of controls. It was observed with TCC at a concentration of 18.0 $\mu\text{g/L}$.

Here, for the first time, we demonstrated that TCS ($p < 0.05$) and TCC ($p < 0.001$) actively induced apoptosis in *A. salina* coelomocytes after treatment with the two chemicals at the designated concentrations for 24 h. The first mechanism is a possible increase in intracellular oxidative stress, which could arise from a pathological increase in the production of oxyradicals (mainly H_2O_2 , which is produced in mitochondria by dismutation of O_2^-) through the cellular mitochondrial enzymatic complex chain (Moore et al. 2006). Alternatively, TCS and TCC may act together as a DNA adduct and/or DNA intercalant to directly exert genotoxic effects. Additionally, TCS has been reported to act as a precursor of 2,8-dichlorodibenzo-*p*-dioxin (Aranami and Readman 2007), which is a well-known DNA-intercalating agent.

Numerous uncertainties remain including the evaluation of antioxidant enzymes, the possible interference with

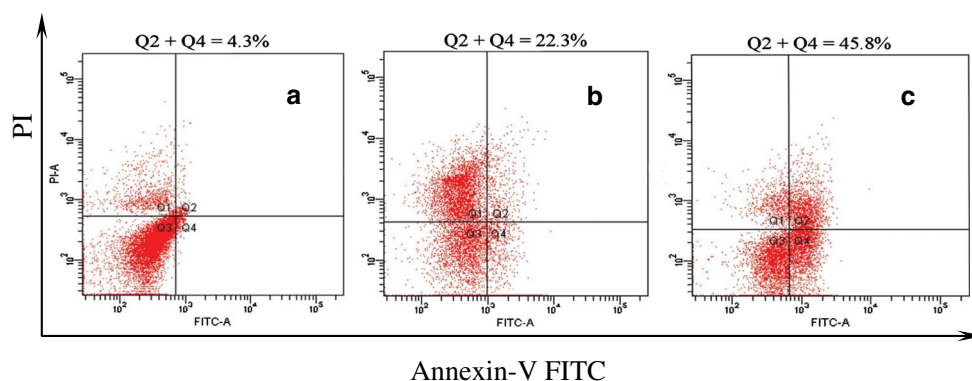


Fig. 3 Flow cytometric analysis of *A. salina* coelomocytes treated with TCS and TCC **a** Control; **b** 171.0 $\mu\text{g/L}$ of TCS for 24 h; **c** 18.0 $\mu\text{g/L}$ of TCC for 24 h. Different subpopulations were distinguishable: the necrotic cells were in the Quadrant 1 (Q1, annexin $\text{V}^- \text{PI}^+$); the cells at the late apoptosis were in the Quadrant 2

(Q2, annexin $\text{V}^+ \text{PI}^+$); the viable cell population was in the Quadrant 3 (Q3, annexin $\text{V}^- \text{PI}^-$); the ones at the early apoptosis were in the Quadrant 4 (Q4, annexin $\text{V}^+ \text{PI}^-$). The apoptotic rate was determined as the percentage of Q2 + Q4

hormone metabolism and the role played by the metabolites of TCS and TCC. It is necessary to fully understand the biological toxic effect of TCS and TCC on this sentinel organism. As these questions continue to be addressed, more definitive and comprehensive aquatic risk assessments should be developed to raise greater public awareness about the potential risk of TCS and TCC released into aquatic environments.

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