TOXICITY OF TRICLOSAN AND TRICLOCARBAN IN MYSID CRUSTACEA (*AMERICAMYSIS BAHIA*)

Koji ARIZONO[1], Masaya UCHIDA[2,3], Masashi HIRANO[4], Sonoko MIURA[1,3], Remi YOSHIDU[1], Hiroshi NAKAMURA[3], Yoshihiro KAGAMI[3], Teruhiko KUSANO[3], Norihisa TATARAZAKO[5], Yasuhiro ISHIBASHI[1], Minoru KOGA[1]

[1]Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Higashi-ku, Tsukide, Kumamoto, 862-8502 JAPAN; [2]Graduate School of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Higashi-ku, Tsukide, Kumamoto, 862-8502 JAPAN; [3]Ecogenomics, Inc. , 1-1 Hyakunenkouen, Kurume, Fukuoka, 839-0864 JAPAN; [4]Center for Marine Environmental Studies (CMES), Ehime University, Bunkyo-cho 2-5, Matsuyama 790-8577, JAPAN; [5]National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki, 305-8506, JAPAN

Introduction

Personal care products (PCPs) are a diverse group of compounds used in soaps, lotions, toothpaste, fragrances, and sunscreens, to name a few. The primary classes of PCPs include disinfectants, fragrances, insect repellants, preservatives and UV filters. Unlike pharmaceuticals which are intended for internal use, PCPs are products intended for external use on the human body and thus are not subjected to metabolic alterations; therefore, large quantities of PCPs enter the environment unaltered through regular usage (Ternes *et al*., 2004). Many of these compounds are used in large quantities, and recent studies have indicated many are environmentally persistent, bioactive, and have the potential for bioaccumulation (Peck, 2006; Mackay and Barnthouse, 2010).Triclosan (5‑chloro−2-(2,4-dichlorophenoxy)phenol: TCS) is widely used as an antibacterial agent in liquid toothpaste, soap, shampoo, and cosmetics (Black *et al*., 1975; Yuge, 1983). Water samples collected near the outfall of a wastewater treatment plant in Rhode Island, USA, showed 10–20 g/l of TCS in the effluent and 80–100 g/g of TCS in the sediment (Lopez-Avila and Hites, 1980). TCS and its chlorinated derivatives are readily converted into various chlorinated dibenzo-p-dioxins by heat and ultraviolet irradiation (Kanetoshi et al., 1987, 1998a, 1998b). TCS has high toxicity on the early life stages of medaka, and that the metabolite of TCS may be a weak estrogenic compound with the potential to induce vitellogenin in male medaka (Ishibashi *et al.*, 2004).Triclocarban (3,4,4'-Trichlorocarbanilide: TCC) is an antimicrobial agent that is widely used in soaps and other personal care products, with some absorption occurring through the skin (EPA, 2002). Several studies have shown that TCC can bioaccumulate, raising concerns about potential effects on soil microorganisms, wildlife and humans (Clarke BO and Smith SR, 2011). TCC increases hormonestimulated transcription of androgen receptor (AR) and estrogen receptor (ER) reporter constructs in mammalian tissue culture cells by up to 30% (Ahn KC, *et a*l., 2008). In vivo, TCC strongly amplifies effects of testosterone on male sex organs in castrated male rats (Duleba AJ *et al*., 2011). TCC by itself stimulates *Cytochrome P450 19* expression only slightly, but TCC strongly enhances the overexpression of *Cytochrome P450 19* that is induced by exogenous estrogen (Chung E *et al*., 2011). In this study, we sought to evaluate potential biological effects of TCS and TCC on the mysid (*Americamysis bahia*). In addition, the aim of this study was to investigate toxicologically significant effects of TCS and TCC exposure on gene expression in mysid (*Americamysis bahia*) using DNA microarray.

Materials and methods

2.1. Test chemicals

5–chloro-2-(2,4-dichlorophenoxy)phenol (TCS) and 3,4,4'-Trichlorocarbanilide (TCC) was obtained from Tokyo Chemical Industry Co., Ltd., (Tokyo, Japan). These compounds were Extra Pure grade. Test solutions were prepared by dissolving the reagents in Dimethyl Sulfoxide (DMSO, Wako Pure Chemical Industries, Ltd.).

2.2. Test organisms

Mysids (*A. bahia*) (Chesapeake Cultures, Inc., Virginia, USA) were obtained from Japan Pulp and Paper Research Institute, Inc. (Tsukuba, Japan). The culture originated at the USEPA (Gulf Breeze, FL, USA) and was maintained in our laboratory for more than 8 years. Culture medium was prepared using artificial seawater (SEA LIFE, marinetech Co.,Ltd., Japan), diluted with aerated deionized tap water to a final salinity of 25‰. A 16 h

light: 8 h dark photoperiod was used during culturing and water temperature was maintained at 25 °C. These mysids were fed twice daily with brine shrimp (within 24hr post-hatch) (Hirano *et al.*, 2009).

2.3. Survival and growth test

Survival and growth test was performed from a procedure of the USEPA using 7-day old juveniles (USEPA, 1994). Briefly, this test consisted of three different concentrations of TCS, TCC and solvent control treatments under static renewal conditions. The selected range of concentrations for the test was based on the results of the 96 h acute toxicity test performed previously by present study. The toxicity data were processed by Probit method, and 96 h LC_{50} of TCS and TCC were 70.3μg/L, and 12.3 μg/L, respectively. Mysids were randomly separated into groups of 5 per chamber in 8 chambers (for a total of 40 mysids total per treatment type) and exposed to 250 mL of TCS at concentrations of 0.5, 5.0 and 50 μg/L in 300-mL glass beakers, TCC at 0.05, 0.5and 5.0 μg/L in 300-mL glass beakers for 14 days. The solvent control group was exposed only to the solvent carrier (DMSO 0.01 mL/L). Test solutions were exchanged every 24 h and the mysids were subjected to a 16:8 light:dark photoperiod. The pH and salinity were 8.0 and 25‰, respectively. The test water temperature was maintained at 25 °C. Mysids were fed a diet of approximately one hundred fifty brine shrimp (within 24hr post-hatch) per mysid per day. Daily maintenance included removal of dead organisms and cleaning of test chambers. During the 14 day testing period, the number of the shedding of the old exoskeleton (exuviae) and mortality in each treatment group was counted daily, and the total number of molts was calculated. At the end of the 14 day exposure period, body length, body weight and carapace length (the base of the eyestalk to the posterior mid-dorsal margin of the carapace) was measured for mysids in each treatment group. The sex ratio, based on the appearance of informative secondary sex characteristics, and a cumulative rate of mortality were also determined. Identification of mature male, mature female and immature animals was decided according to USEPA (2002).

2.4. Exposure design for microarray analysis

Based on LOEC (Lowest Observed Effect Concentration) data obtained from the mysid survival and growth test, chemical exposure concentration in the test for microarray analysis was determined. Juvenile mysid exposed to each chemicals at nominal concentration (0.5 μg/L of TCS and 0.05μg/L of TCC) in 1-L glass beakers for 24 hours at 24 ± 1°C. None of the tested mysid was fed during the entire exposure period. After 24 hours of exposure, the tested mysids were sampled (whole body). These samples were collected and stored in RNAlater (inhibition of RNA decomposition solution; Sigma-Aldrich Corporation, USA) solution at -30 ºC until total RNA extraction was carried out.

2.5. Mysid DNA microarray gene expression analysis

Total RNA samples were extracted from the exposed mysid samples with an RNeasy Mini Kit (Qiagen, Hilden, Germany). Integrity of the total RNA was evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and the qualified total RNA samples were used in the further DNA microarray analyses. For DNA microarray hybridization, antisense RNA (aRNA) was our choice, prepared by Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, Austin, TX, USA), and a fluorescent dye, Cy5 (GE Healthcare, Little Chalfont, UK), was used to label the aRNA samples. The DNA microarray is the Mysid DNA micorarray manufactured by Ecogenomics Inc. which has 24,556mysid oligo DNA probes (35bases). Each probe is spotted in duplicate on the microarray. Cy5-labeled aRNA samples were used in hybridization with the oligo DNA probes on the microarray, and it was carried out for 16 hours at 45ºC in a hybridization solution (6x SSPE, 0.05% Tween-20, 20mM EDTA, 25% formamide, 100ng/μL salmon sperm DNA, 0.04% SDS). After the hybridization reaction, washing was performed in the following order and frequency of wash solutions: twice with 6x SSPE and 0.05% Tween-20 at 45ºC; twice with 3x SSPE and 0.05% Tween-20 at room temperature; twice with 0.5x SSPE and 0.05% Tween-20 at room temperature; twice with 2x PBS and 0.1% Tween-20 at room temperature; and finally twice with 2x PBS at room temperature. Each of the post-wash microarrays was scanned with GenePix 4000B scanner at 5 μm resolution (Axon Instruments, Union city, CA, U.S.A.), and expression signal intensities were digitally processed using Microarray Imager (Combimatrix Corp., WA, USA).

2.6. Statistical analysis

For a statistical analysis of the microarray data, the expression signal data collected from each of the 24,556 genes probes were normalized by the median of all signal strength. Then, they were statistically analyzed using the ArrayStat z-test (Imagin Research, Piscataway, NJ, USA) with offset correction and $p<0.05$ (significance cutoff) to obtain an expression level ratio of the exposure group to the solvent control group for each of the genes on the microarray.

Results and discussion

3.1. Effect of TCS and TCC on survival, growth, sexual development and molting of mysid

The mortality after exposure to 0.5, 5.0 and 50 μg/L TCS for 14 days were 15, 10 and 35%, respectively. Average body length in mysid exposed to 0.5, 5.0 and 50 μg/L TCS for 14 days was significantly shorter than to controls (Table 1). Moreover, carapace length was significantly shorter in the 0.5, 5.0 and 50 μg/L TCS treatment groups, and body weights of mysids exposed to 50 μg/L TCS for 14 days were also significantly lower than controls (Table 1). Although no significant differences were observed in the sex ratio as measured after the appearance of secondary sex characteristics, the number of immaturemysids 0.5, 5.0 and 50 μg/L TCS for 14 days were 14.7, 11.1 and 50%, treatment were significantly higher than the control groups (Table 1). The total number of molts in animals exposed to TCS for 14 days was significantly lower in the treatment groups exposed to 0.5, 5.0 and 50 μg/L TCS relative to the controls. The mortality after exposure to 0.05, 0.5 and 5.0 μg/L TCC for 14 days were 0, 22.5 and 7.5%, respectively. Average body length in mysid exposed to 5.0 μg/L TCC for 14 days was significantly shorter than to controls (Table 2). Moreover, carapace length was significantly shorter in the 5 μg/L TCC treatment groups, and body weights of mysids exposed to 0.05 and 5.0 μg/L TCC for 14 days were also significantly lower than controls (Table 2). Although no significant differences were observed in the sex ratio as measured after the appearance of secondary sex characteristics, the number of immaturemysids (13.5%) in the 5.0 μg/L TCC treatment were significantly higher than the control groups (Table 2). The total number of molts in animals exposed to TCC for 14 days was significantly lower in the treatment groups exposed to 0.5 and 5.0 μg/L TCC relative to the controls. These results clearly indicate that subchronic exposure to TCS and TCC affected growth of bahia as measured by several traits, including body and carapace length and measures of reproductive fitness.

Table 1. Total number of molting, body length, carapace length, body weight and sexual maturation of mysids after exposure to TCS for 14 d

TCS (μg/l)	N	Total number of molting	Total length (mm)	Carapace length (mm)	Body weight (mg)	Immature (%)
Solvent Control	31	13.38 ± 2.39	5.11 ± 0.20	1.88 ± 0.20	2.79 ± 0.43	0.0
0.5	34	10.13 ± 2.59 *	4.83 ± 0.26 **	1.73 ± 0.21 **	2.67 ± 0.41	14.7
5	36	8.13 ± 2.59 **	4.93 ± 0.16 **	1.74 ± 0.18 **	2.74 ± 0.38	11.1
50	26	6.88 ± 1.81 **	4.32 ± 0.38 **	1.53 ± 0.23 **	2.04 ± 0.38 **	50.0

*$p<0.05$ **$p<0.01$

Table 2. Total number of molting, body length, carapace length, body weight and sexual maturation of mysids after exposure to TCC for 14 d

TCC (μg/l)	N	Total number of molting	Total length (mm)	Carapace length (mm)	Body weight (mg)	Immature (%)
Solvent Control	29	19.13 ± 3.98	5.08 ± 0.28	1.92 ± 0.15	3.20 ± 0.47	3.4
0.05	37	16.25 ± 2.87 *	5.00 ± 0.24	1.84 ± 0.24	2.83 ± 0.40 *	0.0
0.5	31	15.50 ± 2.20 **	5.01 ± 0.18	1.91 ± 0.15	3.00 ± 0.40	0.0
5	37	12.88 ± 2.64 **	4.47 ± 0.45 **	1.66 ± 0.22 **	2.34 ± 0.46 **	13.5

*$p<0.05$ **$p<0.01$

3.2. Mysid gene expression analysis

We used DNA microarray analysis to assess molecular toxicological effects of TCS and TCC exposure in the juvenile mysid (7-day old). Juvenile mysid were exposed to 0.5 μg/L of TCS and 0.05μg/L of TCC for 24 hours,

and mRNA expression profiles in their whole bodies were analyzed. We identified statistically significant (p<0.05) regulation (expression ratio of exposed to control > 3) in 312 genes responding to 0.5 μg/L of TCS and 264 genes responding to 0.05μg/L of TCC. There were merely 231 genes found responding to both exposures of TCS and TCC. The up-regulation in response to TCS and TCC was observed in vitellogenin and clottable protein. These genes increased more than 30 times in both experimental plot. Possibly it may be the biomarker of antibacterial agent in mysid. We will be investigate this gene in detail in further study. These results suggested that endocrine disrupting effect of TCS and TCC would be potent for marine crustacean.

Acknowledgements:

This study was supported in part by funds from the Ministry of the Environment, Japan. This work was supported by Arizono Laboratory and Koga Laboratory (Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto) members and Ecogenomics, Inc. members.

References:

Ahn KC, *et al*., 2008. In vitro biologic activities of the antimicrobials triclocarban, its analogs, and triclosan in bioassay screens: Receptor-based bioassay screens. Environ Health Perspect 116:1203–1210.

Black, J.G., Howes, D., Rutherford, T., 1975. Percutaneous absorption and metabolism of Irgasan DP300. Toxicology 3, 33–47.

Clarke BO, Smith SR, 2011. Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. Environ Int 37:226–247

Duleba AJ, et al., 2011. Effects of triclocarban on intact immature male rat: Augmentation of androgen action. Reprod Sci 18:119–127.

EPA, 2002. Robust Summaries and Test Plans: Triclocarban. <http://www.epa.gov/hpv/pubs/summaries/tricloca/c14186tp.pdf.>

Chung E, Genco MC, Megrelis L, Ruderman JV, 2011. Effects of bisphenol A and triclocarban on brain-specific expression of aromatase in early zebrafish embryos. Proc Natl Acad Sci U S A. 2011 Oct 25;108(43):17732-7.

Hirano M, Ishibashi H, Kim JW, Matsumura N, Arizono K.., 2009. Effects of environmentally relevant concentrations of nonylphenol on growth and 20-hydroxyecdysone levels in mysid crustacean, Americamysis bahia. Comp Biochem Physiol C Toxicol Pharmacol. 149(3):368-73.

Ishibashi H, Matsumura N, Hirano M, Matsuoka M, Shiratsuchi H, Ishibashi Y, Takao Y, Arizono K. 2004. Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. Aquat Toxicol 67:167–179.

Kanetoshi, A., Ogawa, H., Katsura, E., Kaneshima, H., 1987. Chlorination of Irgasan DP300 and formation of dioxins from its chlorinated derivatives. J. Chromatogr. 389, 139–153.

Kanetoshi, A., Ogawa, H., Katsura, E., Kaneshima, H., Miura, T., 1998a. Formation of polychlorinated dibenzo-p-dioxins upon combustion of commercial textile products containing 2,4,4'-trichloro-2'-hydroxydiphenyl ether (Irgasan DP300). J. Chromatogr. 442, 289–299.

Kanetoshi, A., Ogawa, H., Katsura, E., Kaneshima, H., Miura, T., 1998b. Formation of polychlorinated dibenzo-p-dioxins from 2,4,4'-trichloro-2'-hydroxydiphenyl ether (Irgasan DP300) and its chlorinated derivatives by exposure to sunlight. J. Chromatogr. 454, 145–155.

Peck, A.M., 2006. Analytical methods for the determination of persistent ingredients of personal care products in environmental matrices. Anal. Bioanal. Chem. 386, 907–939.

Ternes, T.A., Joss, A., Siegrist, H., 2004. Scrutinizing pharmaceuticals and personal care products in wastewater treatment. Environ. Sci. Technol. 38, 392A–399A.

USEnvironmental ProtectionAgency, 1994. Short-termmethods for estimating the chronic toxicity of effluents and receivingwaters tomarine and estuarine organisms. EPA/600/4-91/003. Cincinnati, OH.

US Environmental Protection Agency, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms, 3rd edn. EPA-821-R-02-014. Washington, DC

Lopez-Avila, V., Hites, R.A., 1980. Organic compounds in an industrial wastewater. Their transport into sediments. Environ. Sci. Technol. 14, 1382–1390.

Mackay, D., Barnthouse, L., 2010. Integrated risk assessment of household chemicals and consumer products: addressing concern about triclosan. Integr. Environ. Assess. Manage. 6, 390–392.

Yuge, O., 1983. The antimicrobial treatments for fabrics. J. Antibact. Antifung. Agents 11, 76–81.