Effects of triclosan on activity of *N*-acetyl-β-glucosaminidase in the epidermis of the fiddler crab, *Uca pugilator*

Abigail Hagen and Enmin Zou
Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA

Corresponding Author

Enmin Zou
Department of Biological Sciences
Nicholls State University
Thibodaux, LA 70310
USA
Email: em.zou@nicholls.edu

Abstract

Triclosan (TCS), an antimicrobial agent frequently found in aquatic environments, has recently been shown to inhibit crustacean molting. The present investigation sought to understand whether the molt-disrupting effect of TCS arises from disruption of molting hormone signaling. Because of the structural similarity of TCS to polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) capable of disrupting molting hormone signaling, it was hypothesized that TCS would also act through disrupting molting hormone signaling in Crustacea. Exposure of fiddler crabs, *Uca pugilator,* to TCS at 10 - 250 μg/L for six days had no effect on activity of epidermal *N*-acetyl-β-glucosaminidase (NAG), also known as chitobiase, a biomarker for molting hormone signaling. However, TCS at 2500 μg/L significantly increased enzymatic activity, suggesting that TCS at this environmentally unrealistic concentration is capable of enhancing ecdysteroid signaling in vivo. The underlying mechanism for this stimulating effect on epidermal NAG activity needs to be investigated.

Keywords

Triclosan, Crustacean, Molting, Endocrine disruption, *N*-Acetyl-β-glucosaminidase, *Uca pugilator*

Introduction

Triclosan (TCS), a chlorinated diphenyl ether (Fig. 1), is widely used as an antimicrobial product and is contained in many items, such as children's toys, hosiery, and kitchen utensils (Daughton and Ternes 1999). Entering through sewage and runoff waterways (Daughton and Ternes 1999), TCS has been found at concentrations up to11.27 μg/L in aquatic environments (Perez et. al. 2013).

Because of TCS' structural similarity to polychlorinated biphenyls (PCBs), several of which are known to disrupt endocrine processes in vertebrates (Harris et al. 2014; Bergeron et al. 1994**)**, much research attention has been attracted to the possibility that TCS is also capable of disrupting hormonal processes in vertebrates. TCS was reported to have inhibitory effects on vertebrate endocrine functions, such as inhibiting estrogen sulfotransferase, responsible for regulating estrogen activity in sheep placenta, which supplies necessary estrogen to the fetus (James et al. 2010). In the Japanese rice fish, TCS was found to increase hepatic vitellogenin concentrations in male fish, suggesting that TCS is estrogenic (Ishibashi et al. 2004). In male Wistar rats, TCS was shown to reduce thyroxine levels, which can lead to decreased gamete production if induced in fetal or postnatal rats (Zorilla et al. 2008; Francavilla et al. 1991).

Crustaceans occupy important niches in aquatic ecosystems, and many species are commercially important. However, information on the adverse effects of TCS on hormonally-regulated processes in crustaceans is scarce. Peng et al. (2013), in a 21-day toxicity test, documented a biphasic effect of TCS on growth and reproduction in *Daphnia magna*, wherein TCS at 4 – 16 μg/L increases both the body length and the total number of neonates per female while 128 μg/L TCS inhibits both parameters. Additionally, TCS administered at 16 – 128 μg/L was found to reduce the number of molts per adult *Daphnia magna*.

Molting is an important physiological process for crustaceans because it allows for growth and development of these animals bearing a rigid, confining exoskeleton. It is of interest to understand whether TCS' disrupting effect on crustacean molting stems from this pesticide's interference with molting hormone signaling. Molting is regulated by a multihormonal system but is under immediate control of ecdysteroid molting hormones (Chang et al. 1993). In decapods, ecdysteroids are produced in the Y-organs whose activity is held in acquiescence during the intermolt stage by the molt-inhibiting hormone (MIH) from the X-organ-sinus gland complexes. When the animal enters premolt stage, this inhibition of Y-organ activity by the MIH stops and ecdysteroidogenesis in the Y-organs intensifies. Ecdysteroid titer in the hemolymph is, as a result, elevated. In epidermal cells, ecdysteroids regulate gene activities at the transcriptional level through interaction with the ecdysteroid receptor (EcR), which then heterodimerizes with crustacean retinoid X receptor (RXR) (Durica and Hopkins 1996; Chung et al. 1998). This EcR/crustacean RXR dimer binds to the DNA response elements of the genes regulated by the molting hormones. Among the products of the genes regulated by the molting hormones are enzymes responsible for degradation of the old exoskeleton, such as the chitinolytic enzyme *N*-acetyl-β-glucosaminidase (NAG), also known as chitobiase. Since epidermal NAG is the terminal product of endocrine cascades, this enzyme is the biomarker for molting hormone signaling.

In view of the structural similarity of TCS to PCB and polybrominated diphenyl ether PBDE (Fig. 1) and the reports that molt-inhibiting PCBs (Zou and Fingerman 1997) and PBDEs (Davies and Zou 2012) act through inhibiting epidermal NAG activity (Zou and Fingerman 1999a, b; Gismondi and Thomé 2014), it was hypothesized that TCS would disrupt molting hormone signaling, thereby altering epidermal NAG activity in Crustacea. We tested this hypothesis using the fiddler crab, *Uca pugilator*, as the model crustacean.

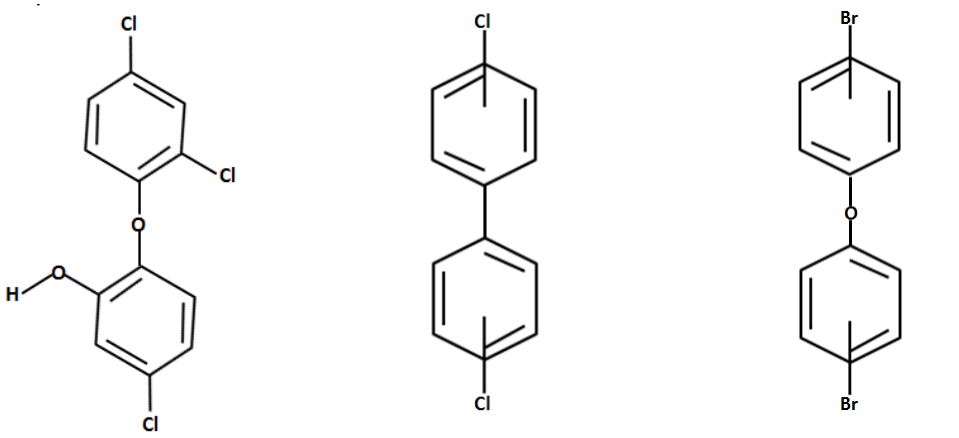

Figure 1. Structures of triclosan, polychlorinated biphenyl and polybrominated diphenyl ether.

Methods and Materials

Female fiddler crabs, *Uca pugilator*, were purchased from the Gulf Specimen Marine Laboratories (Panacea, FL) and stored in artificial seawater made with Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH). Crabs were molt-staged according to the methods and criteria developed by Vigh and Fingerman (1985). Only intermolt (Stage C) crabs were used in the exposure experiment.

Five groups of 20 crabs each were placed into clean polyethylene bins, each containing 1 L artificial seawater. The control group was exposed to 0.01% v/v ethanol since ethanol was used to dissolve TCS, and the remaining four groups were exposed to differing concentrations of TCS (AccuStandard, New Haven, CT): 10, 50, 250 and 2500 μg/L, respectively. Crabs were exposed for six days at room temperatures of 19-21°C. After exposure for six days, the carapaces with attached epidermal tissues were harvested from all the survivors, snap-frozen with liquid nitrogen, and stored at -80°C until the enzymatic assays. Over a six-day period, control mortality rate was 15% while a mortality rate ranging from zero to 20% was registered for TCS-treated groups.

NAG activity was quantified using an assay described by Zou (2009). Briefly, harvested epidermal tissue was homogenized in 0.15 M pH 5.5 citrate-phosphate buffer with 5 µL 0.04% v/v EDTA-free (100x) Halt Proteinase Inhibitor Cocktail (Thermo Scientific, Rockford, IL). The

mixture was centrifuged at 10,000*g* for 3 minutes with an Eppendorf Centrifuge 5415 D (Hamburg, Germany). To measure NAG activity, a 20 µL portion of the supernatant was incubated with 100 µL of its substrate, 2 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO), in microcentrifuge tubes with an Eppendorf ThermoStat plus incubator (Hamburg, Germany), at 25°C for 15 minutes. To stop the reaction, 0.9 mL 0.5 M NaOH was added to each sample reaction. The amount of nitrophenol liberated in each reaction was measured at 405 nm with a Bio-Rad Benchmark Plus spectrophotometer against a nitrophenol standard curve. The Bradford method was used to determine sample protein content. NAG activity was expressed in nmol nitrophenol liberated • (µg protein)$^{-1}$ • (15 min)$^{-1}$**.** The data was transformed using a $\log_{10}$ function for normalization, and a one-way analysis of variance (ANOVA) and the post-hoc Tukey's test (IBM SPSS Statistics Version 21) were used to determine the significant difference in enzyme activity between TCS-exposed and control groups. A probability value of less than 0.05% was considered significant.

Results and Discussion

Exposure of *Uca pugilator* to TCS at 10, 50, and 250 μg/L had no effect on epidermal NAG activity (Fig. 2). As aforementioned, the highest TCS concentration measured in aquatic environments is 11.27 μg/L. Our data show that TCS at environmentally realistic concentrations does not impact ecdysteroid signaling as reflected by epidermal NAG activity in fiddler crabs. Interestingly, epidermal NAG activity in crabs treated with TCS at 2500 μg/L was significantly higher than that in control crabs ($p = 0.002$), suggesting that TCS at this environmentally unrealistic concentration is capable of enhancing molting hormone signaling.

The stimulating effect of TCS on molting hormone signaling in *Uca pugilator* observed in the present investigation does not appear to be in agreement with TCS' inhibitory effect on molting of *Daphnia magna* reported by Peng et al. (2013). This discrepancy could arise from interspecific differences in sensitivity to TCS. It must be noted that Peng et al. (2013) did register a biphasic effect of TCS on body length of *Daphnia magna,* with TCS at low concentrations capable of increasing the body length. This stimulating effect of TCS on growth of *Daphnia magna* could be underpinned by enhanced molting hormone signaling as a result of exposure to TCS since crustacean growth is known to be a function of molting due to the rigid, confining exoskeleton. Presumably, the biphasic effect of TCS would also occur in fiddler crabs, with the inhibitory effect on molting hormone signaling becoming apparent if TCS is given at concentrations exceeding 2500 μg/L.

How could TCS stimulate molting hormone signaling? Zou (2005, 2010) outlined the action routes of a xenobiotic capable of disrupting crustacean molting. A xenobiotic can disrupt molting hormone signaling through directly or indirectly altering ecdysteroidogenesis in the Y-organs and/or interfering with ecdysteroid signaling in the epidermis. Whether TCS' stimulating effect on molting hormone signaling can actually translate into increased molting frequency, along with the underlying mechanisms, awaits future investigations.

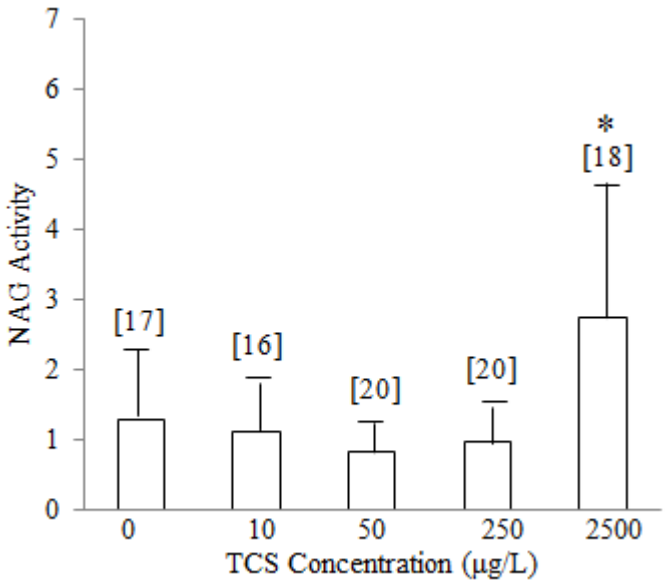

Figure 2. Effect of triclosan on *N*-acetyl-β-glucosaminidase (NAG) activity in the epidermis of the fiddler crab, *Uca pugilator*. Enzymatic activity is expressed in nmol nitrophenol liberated•(15 min)$^{-1}$•(µg protein)$^{-1}$. Sample sizes are shown in brackets. *p = 0.002 relative to control.

Acknowledgements

This study was sponsored by the Louisiana Sea Grant Undergraduate Research Opportunities Program (UROP).

References

Bergeron JM, Crews D, McLachlan JA (1994) PCBs as environmental estrogens: turtle sex determination as a biomarker of environmental contamination. Environ Health Perspect 102:780-781

Chang ES, Bruce MJ, Tamone SL (1993) Regulation of crustacean molting: a multihormonal system. Am Zool 33:324-329

Chang ES, Chang SA, Mulder EP (2001) Hormone in the lives of crustaceans: an overview. Am Zool 41:1090-1097

Daughton CG, Ternes TA (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ Health Perspect 107:907-938

Davies R, Zou E (2012) Polybrominated diphenyl ethers disrupt molting in neonatal Daphnia magna. Ecotoxicology 21:1371-1380

Durica DS, Hopkins PM (1996) Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*. Gene 171:237-241

Francavilla S, Cordeschi G, Properzi G, Di Cicco L, Jannini EA, Palermo S, Fugassa E, Loras B, D'Armiento M (1991) Effect of thyroid hormone on the pre- and post-natal development of the rat testis. J Endocrinol 129:35-42

Harris JB, Eldridge ML, Sayler G, Menn F, Layton AC, Baudry J (2014) A computational approach predicting CYP450 metabolism and estrogenic activity of an endocrine disrupting compound (PCB-30). Environ Toxicol Chem 33:1615-1623

Ishibashi H, Matsumrua 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 *Or*yzias *latipes* and induction of hepatic vitellogenin. Aquat Toxicol 67:167-179

James MO, Li W, Summerlot DP, Rowland-Fox L, Wood CE (2010) Triclosan is a potent inhibitor of estradiol and estrone sulfonation in sheep placenta. Environ Int 36:942-949

Peng Y, Kuo Y, Nie X, Liao W, Yang Y, Ying G (2013) Toxic effects of triclosan on the detoxification and breeding of *Daphnia magna.* Ecotoxicology 22:1384-1394

Perez AL, De Sylor MA, Slocombe AJ, Lew MG, Unice KM, Donovan EP (2013) Triclosan occurrence in freshwater systems in the United States (1999-2012) a meta-analysis. Environ Toxicol Chem 32:1479-1487

Vigh DA, Fingerman M (1985) Molt staging in the fiddler crab *Uca pugilator*. J Crust Biol 5:386-396

Zou E (2005) Impacts of xenobiotics on crustacean molting: the invisible endocrine disruption. Integr Comp Biol 45:33-38

Zou E (2009) Effects of hypoxia and sedimentary naphthalene on the activity of N-acetyl-*β*-glucosaminidase in the epidermis of the brown shrimp, *Penaeus aztecus*. Bull Environ Contam Toxicol 82:579-582

Zou E (2010) Aquatic invertebrate endocrine disruption. In: Breed MD, Moore J (eds) Encyclopedia of animal behavior. Academic Press, Oxford, Vol 1, pp 112-123

Zou E, Fingerman M (1997) Effects of estrogenic xenobiotics on molting of the water flea, *Daphnia magna*. Ecotoxicol Environ Saf 38:281-285

Zou E, Fingerman M (1999a) Effects of estrogenic agents on chitobiase activities of the epidermis and hepatopancreas of the fiddler crab, *Uca pugilator*. Ecotoxicol Environ Saf 42:185-190

Zou E, Fingerman M (1999b) Effects of exposure to diethyl phthalate, 4-tert-octylphenol, and 2,4,5-trichlorobiphenyl on activity of chitobiase in the epidermis and hepatopancreas of the fiddler crab, *Uca pugilator*. Comp Biochem Physiol 122C:115-120