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Multi-level ecotoxicity assay on the aquatic midge, *Chironomus tentans* (Diptera, Chironomidae) exposed to octachlorostyrene

Si-Won Lee, Jinhee Choi*

Faculty of Environmental Engineering, College of Urban Science, University of Seoul, 90 Jeonnong-dong Dongdaemun-gu, Seoul, 130-743, Republic of Korea

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ABSTRACT

Octachlorostyrene (OCS) is a ubiquitously distributed compound, of which, ecotoxicological property is little known. To investigate the ecotoxicity of OCS, short- and long-term experiments were conducted under controlled laboratory conditions, using the aquatic midge, *Chironomus tentans*, as a biological model system. Ecophysiological responses using organism/population level endpoints, such as growth, reproduction and development, were investigated. Moreover, oxidative stress-related parameters and the expression of heat shock protein and hemoglobin gene were also investigated as potential biomarkers for OCS exposure. Significant disturbance in male/female adult ratio and on oviposition by chronic exposure to OCS suggests that this compound may have serious consequences on *Chironomus* population by affecting reproduction. To elucidate the mechanism of these speculated phenomena, direct experimental demonstrations on the relationships between observed ecotoxicological response and upstream-induced molecular/biochemical events may be needed.

1. Introduction

The aquatic larvae of non-biting midges (Chironomidae, Diptera) are globally distributed and are the most abundant group of insects found in freshwater ecosystems. As an insect, *Chironomus* represents the species' richest and one of the most ecologically important groups of invertebrates (Cranston, 1995). Thus, acute and chronic tests with *Chironomus* spp. are commonly used for testing and risk assessment of environmental contaminants (Kahl et al., 1997; Choi et al., 2000, 2002; Crane et al., 2002).

Among numerous aquatic contaminants, octachlorostyrene (OCS), a byproduct in the manufacture of many chlorinated hydrocarbons, was consistently found in fish and sediment samples collected near incineration areas or near potential point sources due to industrial discharges (USEPA, 2000). OCS has been found to be a major organochlorine contaminant in Europe (Chu et al., 2003), however, little is known about global emissions of OCS and its geographical distribution. OCS was only occasionally detected in some environmental investigations that had other pollutants (polychlorinated bisphenols and organochlorine pesticides, such as HCB) as target compounds. Despite its ubiquitous distribution in the environment and hence the importance of OCS toxicity in aquatic ecosystems, few ecotoxicological information has been available for this contaminant. Therefore, research on the effects of OCS on *Chironomus tentans* could provide valuable information for ecotoxicity monitoring or risk assessment of this compound in aquatic ecosystems.

The range of *Chironomus* studies in ecotoxicology focuses on organism-level endpoints, such as mortality, behavior, growth or reproduction. Although these classical test endpoints are highly relevant to population health status, more specific and sensitive endpoints than classical ecotoxicological tests need to be developed. Recently, there have been an increasing number of studies involving the direct comparison of multiple ecotoxicological endpoints under a common set of experimental conditions, but such studies are still limited. *Chironomus* is an attractive animal model for the study of chemical-induced multiple biological level responses, from molecular through population-relevant levels. This study was designed as short- and long-term experiments under controlled laboratory conditions, using *C. tentans* as a biological model system to demonstrate the ecotoxicity of OCS, a ubiquitously distributed compound with little known ecotoxicological property.

In this study, ecophysiological responses using classical ecotoxicological endpoints, such as growth, development and reproduction, were investigated upon sublethal exposure. Larval body weight was measured as a growth indicator, whereas successes in pupation and in adult emergence as well as total emergence times were examined as descriptors for development. Adult sex ratio was also studied in order to identify any potential difference in susceptibility between the male and female toward this compound. Oviposition rate and the number of egg per eggmass were used as parameters for reproduction. Oxidative stress-related

* Corresponding author. Tel.: +82 2 2210 5622; fax: +82 2 2244 2245.
E-mail address: jinhchoi@uos.ac.kr (J. Choi).

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parameters and the expression of heat shock protein (HSP) and hemoglobin (Hb) gene were investigated as potential biomarkers for OCS exposure. Chemical analysis was also performed to verify the actual exposure level of the larvae to OCS.

2. Materials and methods

2.1. Organisms

Using an original strain provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), we obtained *C. tentans* larvae from adults reared in our laboratory. The larvae, which we fed a fish flake food (Tetramin®, Tetrawerke, Melle, Germany), were reared under a 16:8 h light:dark photoperiod at room temperature (20 ± 1 °C) in a 2 L glass chamber containing dechlorinated tap water and acid-washed sand with aeration.

2.2. Sublethal exposure conditions

Using groups of the 4th instar larvae collected in the rearing aquaria, we assessed the effects of OCS exposure. OCS was purchased from Wako (Wako Pure Chemical Industries, Osaka, Japan). All larvae used in the experiment were originated from the same eggmass and being collected at the same period (30 days) after egg hatching in order to obtain age-synchronized population. At the beginning of the experiment, 1 ml of an acetonic solution of OCS was added to experimental tanks of 1 l. Prior to the main experiment, a solvent control test was conducted to compare the response of the non-solvent control group and the solvent control group, which indicated that acetone did not provoke any significant effect in all the experiments (data not shown). The controls presented in Section 3 of this paper were solvent controls. For molecular/biochemical indicator study, 10 and for chronic toxicity study, 50 larvae were then randomly introduced into each test aquarium. Exposure was carried out under constant temperature (20 ± 1 °C) and a photoperiod of 16:8 h (light:dark) was used for all the experiments. Three replicates were used for all the experiments.

2.3. Sample extraction, cleanup and gas chromatography/mass spectrometry analysis for quantitative determination of octachlorostyrene

Sediment and *C. tentans* larvae samples were collected at various times after the beginning of the experiment for OCS residue analysis (i.e. 4, 24, 48, 72 and 96 h after the beginning of the experiment). The nominal concentration of OCS in water at the beginning of the study was 5 mg l^{-1} and three replicates were used. Exposure was not renewed. At each sampling time, 50 g of sediment and 25 larvae were collected in experimental tanks. Larvae were pooled, weighed and homogenized for 2 min in 5 ml of Pestipur petroleum ether containing 5 g of anhydrous sodium sulfate. The extraction of OCS from sediment and biota samples was performed by the ultrasonication method (Hong et al., 2004). The cleanup of OCS extract was carried out as previously described (Hong et al., 2004) with a slight modification. Briefly, the extract was purified by the Florisil-solid phase extraction (SPE) cartridge with elution of acetone–*n*-hexane (1:9, v/v) mixture at a flow of 1 ml min^{-1}. The sample analysis was carried out with an Agilent gas chromatography/mass spectrometry (GC/MS Palo Alto, CA, USA) equipped with DB-5MS fused-silica capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm, J&W Scientific, Folsom, CA, USA). To improve sensitivity, selected ions monitoring (SIM) mode was used, guiding by specific ions of the individual chlorostyrene. All chlorostyrenes were identified by retention time and specific ions, and quantified by the internal standard method.

2.4. Growth, development and reproduction test

As a growth indicator, body dry weights were measured on 10 larvae collected 48 h after commencing exposure. Fresh weight was immediately measured. Larval dry weight was evaluated after placing the larvae at 105 °C for 24 h and water content was calculated from the difference between dry and fresh weights. Laval ash-free dry weight was evaluated after placing the dried larvae at 540 °C for 4 h. Weighing was performed to the nearest 0.1 mg.

For the evaluation of development and reproduction, 50 of fourth instar larvae were introduced at the beginning of the experiment. Emerging adults were retained using wood cages covered with steel wire mesh until the emergence was completed in the control and experimental aquaria. As endpoints of the toxicity tests, the numbers of pupa and that of emerged adults from each vessel were counted and their sexes were determined. The two sexes could easily be distinguished by the form and length of their antennae and abdominal terminalia. Additionally, dead pupae were counted and time to completion of emergence was also investigated. For reproduction parameters, the numbers of eggmass oviposited by emerged adults and the numbers of eggs per eggmass were counted from control and OCS treated vessels. Every 2 days, 50 mg of Tetramin fish food flakes was supplied to each aquarium. Test solutions were not renewed. All data were recorded at daily intervals.

2.5. Antioxidant enzyme activities and lipid peroxidation

A total of 10 larvae were collected 24 h after treatment from the control and experimental tanks and pooled for enzyme activity measurements. Larvae were homogenized in 2.5 ml of Tris–EDTA buffer (40 mM, pH 7.8; Sigma–Aldrich, St. Louis, MO, USA) using a Potter-Elvehjem homogenizer (Wheaton Science, Milliville, PA, USA). Crude homogenate was centrifuged for 15 min at 500 × *g* (4 °C) and the supernatant was centrifuged for 30 min at 12,000 × *g* (4 °C). The resulting supernatant (post-mitochondrial fraction) was used to measure the enzyme activities. The rate of H_2O_2 disappearance (measured at 240 nm) was used to quantify catalase (CAT) activity (Beers and Sizer, 1952). Total peroxidase (Px) activity was measured using a guaiacol test (George, 1953). Enzymatic activities were calculated relative to the measured protein content of the extracts using the Bradford method (Bradford, 1976). As a lipid peoroxidation parameter, malondylaldehyde (MDA) was measured by spectrophotometry using thiobarbituric acid reactive substances (TBARs) according to Ohkawa et al. (1979) with slight modification.

2.6. Comet assay

A total of 10 larvae were collected 24 h after treatment from the control and experimental tanks and were pooled for a Comet assay. Larvae were placed in 1 ml of phosphate buffered saline (PBS) containing 20 mM ethylenediaminetetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by being pressed through gauze. A cell suspension was precipitated by vortexing, and then immediately mixed with 100 μl 1% low melting point (LMP) agarose for use in the Comet assay. An alkaline Comet assay was performed, as described by Singh et al. (1988). Briefly, 100 μl of 1% LMP agarose was spread on a normal agarose precoated microscope slide and placed at 4 °C for 5 min to allow for solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO, 10% Triton × 100, pH 10), and subsequently exposed to alkali (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow for DNA unwinding and expression of alkali-liable sites. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min. After the electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place until image analysis. Before analysis, the slides were stained with 50 μl ethidium bromide (5 μg ml^{-1}). They were then analyzed at 400× magnification using a fluorescence microscope (Nikon, kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm. Approximately, 25 cells per slide (4 slides per each treatment) were examined. The DNA damage was expressed as the Olive tail moment value ((tail mean—head mean) × tail% DNA/100) and tail moment (tail length × tail% DNA/100) using an image analysis computerized method (Komet 5.5, Kinetic Imaging, Nottingham, UK).

2.7. Gene expression analysis

A total of 10 larvae were collected 24 h after treatment from the control and experimental tanks and pooled for gene expression analysis. Larvae were homogenized in 700 μl of TRI reagent (Molecular Research Center, Cincinnati, OH, USA) and RNA was isolated according to the manufacturer's standard protocol. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted with RT Premix and PCR Premix kits (Bioneer, Seoul, Korea). The primers were designed on the basis of sequences retrieved from GenBank™ (Supplementary Table 1). To optimize the semi-quantitative PCR conditions, a cycle number test was conducted for each gene prior to the main experiment (data not shown). All the PCR experiments were conducted using an optimized cycle number and a template amount. Finally, actin mRNA was used for the normalization of the expression of HSP and Hb levels. The tests were replicated in three times, and the relative densities of each band were determined with the aid of an image analyzer, a Gel documentation system (Vilber Lourmat TFX-20.M, Marne la Vallee, France) with a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

2.8. Data analysis

The data were passed normality test and equal variance test. Statistical differences between the control and treated larvae were examined using analysis of variation with Dunnett's multiple comparison test. All statistic tests were performed using SPSS® 12.0 KO (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. OCS analysis

Despite its associated difficulties and limitations, the use of the internal body concentration of chemical contaminants may have distinct advantages over using environmental concentrations as a toxicological index. In terms of toxicity, bioaccumulation data can be explained more meaningfully, and they make a better connection between the accumulated dose and the toxicological effect, thus permitting better interpretation of the hazard associated with complex exposure routes (Callaghan et al., 2002). In this study, prior to ecotoxicity assays, analysis of OCS was conducted using the

GC/MS method to verify the actual level of OCS in test media and biota (Supplementary Fig. 1). Results of the 96-h long study show that OCS concentrations increased in the sediment compartment rapidly during the first 24 h and this tendency continued until 72 h. OCS concentration in sediment decreased after 96 h. A similar pattern was observed for the kinetics of OCS concentration in sediment and in the larvae during the experiment. Chemical analysis revealed that the changes with time in the concentration of OCS in sediment and larvae exhibited a similar pattern with the rapid absorption of this compound by the sediment and by the biotic compartment (larvae). This phenomenon may suggest a probability of bioaccumulation, which was probable as OCS is known to have a high partition coefficient (log octanol–water partition coefficient (K_{ow}) = 6.29; http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB). Indeed, the presence of OCS in sediment or in the tissues of aquatic organisms, including fish, was frequently reported in aquatic environments (Russell and Gobas, 1989; Bester et al., 1998; Vorkamp et al., 2004). From the results of the present study and the physico-chemical property of this compound (i.e., high lipid solubility), long-term bioaccumulation of OCS in *Chironomus* may be expected.

3.2. Growth, reproduction and development

Lethal toxicity experiment was conducted after 24 h of exposure, using death of individuals as an endpoint. However, 24 h LC50 of OCS in the 4th instar larvae of *C. tentans* could not be estimated, as OCS did not cause mortality on *C. tentans* larvae up to 5 mg l^{-1} of concentration (data not shown). Mortality is a reliable ecotoxicological endpoint, however, such a high level of exposure hardly occurred in the real environment. Therefore, more sensitive testing endpoints are needed to diagnose environmental quality. Physiological-level alterations, such as growth, reproduction, movement, and behavior, have been used as endpoints for chemical-induced toxicity testing in *C. tentans.* Sublethal ecotoxicity study was conducted on OCS using three concentrations, that is, 0.05, 0.5 and 5 mg l^{-1}.

Table 1 shows the growth and reproduction parameters investigated in OCS-exposed *C. tentans*. As a growth indicator, we measured the body fresh weight and ash-free dry weight in the larvae of *C. tentans* 48 h after treatment. Ash-free body dry weight decreased in the larvae, which had been exposed to 5 mg l^{-1} of OCS. The effect of OCS on the reproduction of *C. tentans* was investigated by counting the numbers of eggmass oviposited by emerged adults and the numbers of eggs per eggmass. Complete failure in oviposition was observed in the emerged adults of *C. tentans*, which had been exposed to 5 mg l^{-1} of OCS. Total numbers of eggmass were 2, 2, 5 and 0 at control, 0.05, 0.5 and 5 mg l^{-1} of OCS exposure, respectively. Whereas the average numbers of eggs per eggmass were 532.5, 666.5 and 541 at the control, 0.05 and 0.5 mg l^{-1} of OCS exposure, respectively.

As development indicators, pupation and emergence of the larvae of *C. tentans* were investigated in control and OCS-exposed samples (Fig. 1). At a high concentration of OCS (5 mg l^{-1}), about 60% and 90% of pupation and emergence failure occurred. Average time to completion of emergence was 24, 28.5, 28 and 27.5 days after the beginning of the experiment for the control and 0.05, 0.5 and 5 mg l^{-1} of OCS exposure, respectively. The effects of OCS on the kinetics of emergence of male and female adults in *C. tentans* were also studied (Fig. 1 B and C). Among emerged adults, males and females were identified to verify whether there is any difference in susceptibility to OCS exposure. Serious alteration was observed on sex ratio at 5 mg l^{-1} of OCS exposure: the ratio of male/female adult was about 1.5, 1.2, 1 and 0.3, at control and 0.05, 0.5 and 5 mg l^{-1} of OCS exposure, respectively.

The effects of xenobiotics on the growth, reproduction and development of the test organisms are broadly accepted test parameters and were found to be more sensitive indicators of toxicity than lethality, as also shown in this study (Table 1 and Fig. 1). The decrease of pupation and the emergence rate in 30-day long experiment, at the highest concentration of OCS, suggests that the alteration of this parameter might be considered as a chronic toxicity, such as a consequence of a serious progression of the toxic effect on animal development. One of the most distinguished results in this study is OCS-induced disturbance on the male/female adult ratio. It is widely accepted that sex-specific effects, effects on sexual differentiation, and/or alteration in sex ratios induced by a chemical compound hint at the potential of the substance to disturb hormonal processes (Hahn et al., 2001). Bogart (1987) suggested that sexual differentiation processes in animals, including arthropods are based on the ratio of male and female sex hormones. Vitellogenesis has become a well-established biomarker for the effects of environmental chemicals with estrogenic activity in the aquatic environment. Most research has been conducted on fish, and by now encompasses a wide variety of species (Jones et al., 2000). In insects, vitellogenesis is under hormonal control and the hormones involved are ecdysteroids and juvenile hormones, which, in the adult insect, do not trigger molting processes but play a new role in gonadal maturation (Gäde et al., 1997). Thus, the potential of vitellogenesis as a biomarker for endocrine disruption is probably not restricted to vertebrates. Hahn et al. (2002) used vitellogenesis as a potential marker for possible effects of endocrine disrupting agents on *C. riparius*, using tebufenozise, bisphenol and 4-*n*-nonylphenol. OCS may alter invertebrate sex hormone-related pathways, as a strikingly serious alteration on sex ratio was observed with 5 mg l^{-1} of OCS exposure. Such a significant disturbance on male/female adult ratio by a high level of OCS exposure suggests that this compound may provoke serious consequences on *Chironomus* reproduction and, in turn, on its population. As shown in Table 1, the complete inhibition of eggmass oviposition by 5 mg l^{-1} of OCS exposure provides an experimental evidence to support this hypothesis. Further studies on the eventual role of vitellogenesis on the male/female differentiation in *C. tentans*, as a mechanism by which OCS altered sex ratio of emergence are warranted to better understand the OCS-induced differential male/female susceptibility in *Chironomus*.

Table 1
Growth and reproduction parameters. Body fresh weight, and ash-free body dry weight were measured as growth indicators, whereas, total number of eggmass oviposited and the average numbers of egg per eggmass were counted as reproduction indicators, in the fourth instar larvae of *Chironomus tentans* exposed to 0, 0.05, 0.5 and 5 mg l^{-1} of octachlorostyrene (OCS) for 24 h (n = 3, mean ± standard error of mean).

OCS (mg l^{-1})	Growth		Reproduction	
	BFW (mg/larva)	AFDW (mg/larva)	Total number of eggmass	Average number of egg per eggmass
0 (control)	7.46 ± 1.80	0.11 ± 0.08	2	532.5
0.05	6.95 ± 1.38	0.09 ± 0.03	2	666.5
0.5	7.74 ± 1.73	0.10 ± 0.05	5	541.8
5	7.56 ± 2.00	0.07 ± 0.04*	0	0*

BFW: body fresh weight, AFDW: ash-free dry weight.
* Indicates statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

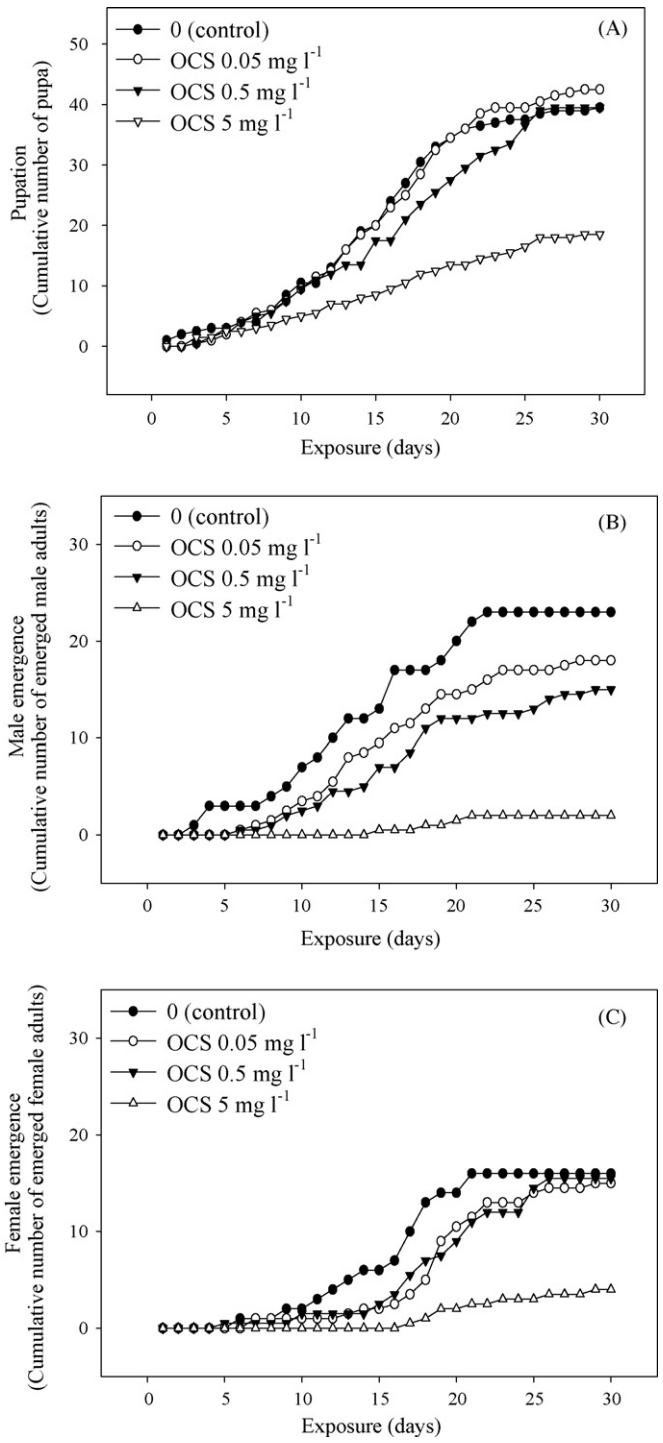

Fig. 1. Kinetics of cumulative pupation (A) and male (B) and female adult emergence (C) measured in control and octachlorostyrene (OCS) exposed *Chironomus tentans*.

3.3. Oxidative stress and DNA damage

The decreases in larval ash-free dry weight, pupation, adult emergence and failure in oviposition observed after OCS exposure may induce alteration in the growth, development and reproduction of the *Chironomus* population in the long term. However, due to the low concentration of xenobiotics in the environment, it is hard to establish any correlation between the occurrence of contaminants and physiological effects of test organisms in the environment, even when using reliable ecotoxicological endpoints such as growth or reproduction. This situation emphasizes the need for understanding the sublethal effects at molecular/biochemical levels where the toxicant-induced responses are initiated. Various molecular/biochemical parameters measured in *Chironomus* larvae, such as heat shock protein and hemoglobin genes expression, acetylcholine esterase (AChE), superoxide dismutase (SOD), glutathione-*S*-transferase (GST), electron transport system, and energy-yielding substrates, have already demonstrated a high sensitivity to environmental pollutants (Choi et al., 2000, 2001; Olsen et al., 2001; Lee and Choi, 2006; Lee et al., 2006).

Chironomus seems to have an efficient biochemical defense mechanism, which may contribute to the organism's tolerance of various environmental stresses, including chemical pollutions. Previous studies have shown that enzymatic radical scavengers, including SOD, CAT, Px, and GPx could be developed as non-stressor-specific biomarkers in *C. riparius* larvae (Choi et al., 1999, 2000). Oxidative stress-related parameters were, therefore, evaluated in the 4th instar larvae of *C. tentans* exposed to OCS for 24 h (Table 2), as they are well-known general stress-response mechanism in aquatic organisms (Di Giulio et al., 1995; Livingstone, 2001; Valavanidis et al., 2006), and specifically well studied in *Chironomus* from our previous studies (Choi et al., 1999, 2000; Lee and Choi, 2006, 2007). CAT and Px activities decreased in the larvae of *C. tentans* that had been exposed to OCS, except for peroxidase at 0.05 mg l^{-1} of OCS, where a slight increase of the activity was observed. Lipid and DNA damage indicators, MDA and (Olive) tail moment did not increase significantly by any tested concentrations of OCS. Our experiment only deals with some antioxidant enzyme activities and damage on DNA and lipid, which are not sufficient to provide a clear explanation for the described phenomenon. If related oxidative stress parameters (i.e., measurement of free radicals, non-enzymatic antioxidant levels, such as GSH) had concomitantly been investigated, this could probably be evaluated and explained to a greater extent.

3.4. Gene expression

Environmental contaminants may induce the expression of certain genes in an organism. Depending on the severity and duration of exposure to the contaminant, the expression of certain genes may be linked to short-term toxicological responses that impact on individual fitness (i.e., survival and reproduction). The basic premise that changes in gene expression can be harnessed to diagnose exposure and the effects of environmental chemicals is currently receiving significant attention. With respect to responses of gene expression to chemical stressors, HSPs are the most frequently studied aspect of aquatic invertebrates (Yoshimi et al., 2002; Karouna-Renier and Zehr, 2003; Arts et al., 2004; Piano et al., 2004). Hemoglobin, which constitutes one of the main particularities of *Chironomus*, has a considerable potential as a biomarker for the biomonitoring of chemical contamination in *Chironomus*, which has been investigated in our previous studies (Lee et al., 2006). The expression of HSP70 and HSC70 and that of Hb gene were investigated in OCS-exposed 4th instar larvae of *C. tentans* (Fig. 2). The expression of both HSP70 and HSC70 gene increased in OCS-treated larvae. Of the five ORFs of Hb, the expression of HbB, HbD and HbE genes increased significantly at all OCS treatments. The increase of HSP70 gene expression in *C. tentans* larvae by OCS exposure was expected, as HSP70 is well known to be inducible by various environmental stressors. In contrast to HSP70, HSC70 is known to be constitutively expressed and not inducible by environmental stressors (Juliann and George, 1998). Yet we observed that the expression of HSC70 genes increased in response to OCS exposure, which was also observed in our previous study (Lee et al., 2006). These results suggest that, as with HSP70, HSC70 might be inducible in response to environmental stressors. OCS-induced

Table 2
Catalase (CAT) and peroxidase (Px) activities and malondylaldehyde (MDA) and DNA strand breaks measured in the fourth instar larvae of *Chironomus tentans* exposed to 0, 0.05, 0.5 and 5 mg l^{-1} of octachlorostyrene (OCS) for 24 h.

OCS (mg l^{-1})	CAT (U/mg protein)	Px (U/mg protein)	MDA (nM/mg protein)	DNA strand breaks (Comet assay)	
				Tail moment	Olive tail moment
0 (control)	60.407 ± 1.951	0.434 ± 0.005	11.203 ± 1.914	4.267 ± 0.255	3.297 ± 0.295
0.05	53.941 ± 1.585	0.487 ± 0.008	11.996 ± 1.603	3.587 ± 0.397	3.033 ± 0.334
0.5	45.097 ± 2.914*	0.317 ± 0.018*	16.911 ± 1.901*	4.501 ± 1.279	3.455 ± 0.455
5	48.825 ± 0.918*	0.394 ± 0.004*	16.292 ± 2.146*	5.716 ± 0.858	4.258 ± 0.274

DNA strand breaks were expressed as tail and olive tail moment obtained from Comet assay ($n = 3$, mean ± standard error of mean).
* Indicates statistically significant differences between treatments and the corresponding control group, $p < 0.05$.

Hb gene expression, observed in this study, suggests that Hb might be a target molecule of OCS exposure and/or can be involved in the toxicity of this compound.

The early response of HSP and Hb gene expression to low concentrations of OCS may be considered a homeostasis-maintaining process rather than an indicator of permanent adverse effects of this compound. Homeostatic responses of HSP and Hb gene expression are known to have little impact at higher levels of biological organization; they may be used as an early warning signal however, before the physiological-level deterioration starts. Although exposure concentration–response relationship was not established in HSP and Hb gene expression, physiological disturbance at a high level of OCS, which occurred concomitantly with increased gene expression, could be considered as toxicity by OCS exposure. Functional genomics study, using mutant or RNAi, can provide clear experimental evidence on causal relationships between gene expression and altered physiological indicators. However, this kind of approach is technically difficult to apply to *Chironomus*. We can only speculate the response at the different biological levels and deduct correlations between these levels relying on statistical analysis. Nevertheless, our study on HSP and Hb gene expression analysis is able to show that the OCS-induced gene expression can be a tool to detect the response to pollutants on the transcriptional level in *C. tentans*.

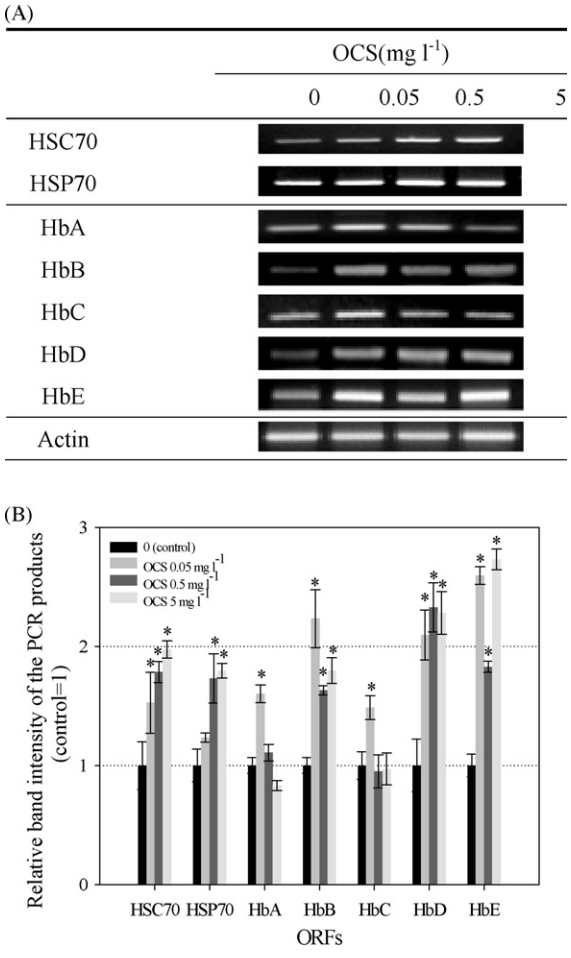

Fig. 2. Expression of heat shock protein (HSP) and hemoglobin (Hb) genes in the fourth instar larvae of *Chironomus tentans* exposed to 0.05, 0.5 and 5 mg/l of octachlorostyrene (OCS) for 24 h (A). Data are expressed in densitometric values normalized using actin mRNA (B) (number = 3, mean ± standard error of mean). Asterisks (*) indicate statistically significant differences between treatments and the corresponding control group, $^*p < 0.05$.

The exposure concentrations were based on the result from lethal toxicity experiment, thus, were generally higher than those found in the field, as *C. tentans* showed a high tolerance to acute OCS exposure. Although the concentrations used were still rather high and may not be environmentally relevant, molecular indicators, such as gene expression, have the potential to be a more sensitive approach than classical ecotoxicity indicators, like reproduction or mortality. The present study focused on the investigation of ecotoxicities across the biological organizations in OCS-exposed *C. tentans* under the laboratory condition. The *in situ* calibration and validation of the identified biomarkers using environmentally relevant exposure conditions will be addressed in future studies.

4. Conclusion

Multiple biological level toxicity descriptors were investigated in OCS-exposed *C. tentans*. This approach could be applied in ecotoxicity monitoring programs and the data obtained from this study can comprise a contribution to the knowledge of the ecotoxicity of OCS in *C. tentans*, about which little data are available. Significant disturbance on male/female adult ratio and on oviposition by chronic exposure of OCS suggests that this compound may have serious consequences on *Chironomus* population through alteration on reproduction. Gene expression response could be used as an early warning signal for ecotoxicity monitoring, but this endpoint alone does not seem to be sufficient in the diagnosis of environment quality; simultaneous measurements of biomarkers at different levels of biological organization (multibiomarkers) may help to fully understand the effect of toxicant on organisms. Moreover, to elucidate the mechanism of these speculated phenomena, direct experimental demonstrations on the relationships between observed ecotoxicological response and upstream-induced molecular/biochemical events (i.e., enzyme activities, gene expression and DNA damage) may be needed.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.etap.2009.05.004.

References

Arts, M.J., Schill, R.O., Knigge, T., Eckwert, H., Kammenga, J.E., Kohler, H.R., 2004. Stress protein(HSP70, HSP60) induced in isopods and nematodes by field exposure to metals in a gradient near Avonmouth, UK. Ecotoxicology 13, 739.

Beers, J.R., Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide. J. Biol. Chem. 195, 133.

Bester, K., Biselli, S., Ellerichmann, T., Huhnerfuss, H., Moller, K., Rimkus, G., Wolf, M., 1998. Chlorostyrenes in fish and sediment samples from the river Elbe. Chemosphere 37, 2459.

Bogart, M.H., 1987. Sex determination: a hypothesis based on steroid ratios. J. Theor. Biol. 128, 349.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein–dye binding. Anal. Biochem. 72, 248.

Callaghan, A., Fisher, T.C., Grosso, A., Holloway, G.J., Crane, M., 2002. Effect of temperature and pirimiphos methyl on biochemical biomarkers in *Chironomus tentans* Meigen. Ecotoxicol. Environ. Saf. 52, 128.

Choi, J., Caquet, T., Roche, H., 2002. Multilevel effects of sublethal fenitrothion exposure in *Chironomus tentans* Mg. (Diptera, Chironomidae) larvae. Environ. Toxicol. Chem. 21, 2725.

Choi, J., Roche, H., Caquet, T., 1999. Characterisation of superoxide dismutase activity in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae—a potential biomarker. Comp. Biochem. Physiol. C 124, 73.

Choi, J., Roche, H., Caquet, T., 2000. Effects of physical (hypoxia, hyperoxia) and chemical (potassium dichromate, fenitrothion) stress on antioxidant enzyme activities in *Chironomus riparius* Mg. (Diptera, Chironomidae) larvae: potential biomarkers. Environ. Toxicol. Chem. 19, 495.

Choi, J., Roche, H., Caquet, T., 2001. Hypoxia, hyperoxia and exposure to potassium dichromate or fenitrothion alter the energy metabolism in *Chironomus riparius* Mg. (Diptera: Chironomidae) larvae. Comp. Biochem. Physiol. C 130, 11.

Chu, S., Covaci, A., Voorspoels, S., Schepens, P., 2003. The distribution of octachlorostyrene (OCS) in environmental samples from Europe. J. Environ. Monit. 5, 619.

Crane, M., Sildanchandra, W., Kheir, R., Callaghan, A., 2002. Relationship between biomarker activity and developmental endpoints in *Chironomus tentans* Meigen exposed to an organophosphate insecticide. Ecotoxicol. Environ. Saf. 53, 361.

Cranston, P.S., 1995. Introduction. In: Armitage, P., Cranston, P.S., Pinder, L.C.V. (Eds.), The Chironomidae—The Biology and Ecology of Non-biting Midges. Chapman & Hall, London, p. 1.

Di Giulio, R.T., Benson, W.H., Sanders, B.M., Van Veld, P.A., 1995. Biochemical mechanisms: metabolism, adaptation and toxicity. In: Rand G. (Ed.), Fundamentals of aquatic toxicology. Effects, environmental fate, and risk assessment, second ed. London.

Gäde, G., Hoffmann, K.H., Spring, J.H., 1997. Hormonal regulation in insects: facts, gaps, and future directions. Physiol. Rev. 77, 963.

George, P., 1953. Intermediate compound formation with peroxidase and strong oxidizing agents. J. Biol. Chem. 201, 413.

Hahn, T., Liess, M., Schulz, R., 2001. Effects of the hormone mimetic insecticide tebufenozide on *Chironomus tentans* larvae in two different exposure setups. Ecotoxicol. Environ. Saf. 49, 171.

Hahn, T., Schenk, K., Schulz, R., 2002. Environmental chemicals with known endocrine potential affect yolk protein content in the aquatic insect *Chronomus riparius*. Environ Pollut 120, 525.

Hong, J.K., Kim, H.Y., Kim, D.G., Seo, J.J., Kim, K.J., 2004. Rapid determination of chlorinated pesticides in fish by freezing-lipid filtration, solid-phase extraction and gas chromatography/mass spectrometry. J. Chromatogr. A 1038, 27.

Jones, P.D., DeCoen, W.M., Tremblay, L., Giesy, J.P., 2000. Vitellogenin as a biomarker for environmental estrogens. Water Sci. Technol. 42, 1.

Juliann, G.K., George, C.T., 1998. Heat shock protein 70 kDa: molecular biology, biochemistry and physiology. Pharmacol. Ther. 80, 183.

Kahl, M.D., Makynen, E.A., Kosian, P.A., Ankley, G.T., 1997. Toxicity of 4-nonylphenol in a life-cycle test with the midge *Chironomus tentans*. Ecotoxicol. Environ. Saf. 38, 155.

Karouna-Renier, N.K., Zehr, J.P., 2003. Short-term exposures to chronically toxic copper concentrations induced HSP70 protein in midge larvae (*Chironomus tentans*). Sci. Total. Environ. 312, 267.

Lee, S.B., Choi, J., 2006. Multiple level evaluation of nonylphenol toxicity in 4th instar larvae of *Chironomus riparius* (Diptera, chironomidae). Environ. Toxicol. Chem. 25, 3006.

Lee, S.B., Choi, J., 2007. Effects of bisphenol A and ethynyl estradiol exposure on enzyme activities, growth and development in the fourth instar larvae of *Chironomus riparius* (Diptera, Chironomidae). Ecotoxicol. Environ. Saf. 68, 84.

Lee, S.M., Lee, S.B., Park, C.H., Choi, J., 2006. Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, chironomidae) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere 65, 1074.

Livingstone, D.R., 2001. Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. Mar. Pollut. Bull. 42, 656.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95, 351.

Olsen, T., Ellerbeck, L., Fisher, T., Callaghan, A., Crane, M., 2001. Variability in acetylcholinesterase and glutathione S-transferase activities in *Chironomus riparius* Meigen deployed in situ at uncontaminated field sites. Environ. Toxicol. Chem. 20, 1725.

Piano, A., Valbonesi, P., Fabbri, E., 2004. Expression of cytoprotective proteins, heat shock protein 70 and metallothioneins, in tissues of *Ostrea edulis* exposed to heat and heavy metals. Cell Stress Chaperon. 9, 134.

Russell, R.W., Gobas, F.A., 1989. Calibration of the freshwater mussel, *Elliptio complanata*, for quantitative biomonitoring of hexachlorobenzene and octachlorostyrene in aquatic systems. Bull. Environ. Contam. Toxicol. 43, 576.

Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp. Cell. Res. 175, 184.

USEPA (United States Environmental Protection Agency), 2000. Draft PBT National Action Plan for Octachlorostyrene, Draft report. http://www.epa.gov/oppt/pbt/pubs/octaaction.htm.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Saf. 64, 178.

Vorkamp, K., Riget, F., Glasius, M., Pecseli, M., Lebeuf, M., Muir, D., 2004. Chlorobenzenes, chlorinated pesticides, coplanar chlorobiphenyls and other organochlorine compounds in Greenland biota. Sci. Total. Environ. 331, 157.

Yoshimi, T., Minowa, K., Karouna-Renier, N.K., Watanabe, C., Sugay, A.Y., Miura, T., 2002. Activation of a stress-induced gene by insecticides in the midge *Chironomus yoshimatsui*. J. Biochem. Mol. Toxicol. 16, 10.