HSP70 and HSC70 gene Expression in *Chironomus Tentans* (Diptera, Chironomidae) Larvae Exposed to Various Environmental Pollutants: Potential Biomarker for Environmental Monitoring

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ABSTRACT **: In order to identify potential biomarkers of environmental monitoring, we evaluated heat shock genes expressions as effects of various environmental pollutants (nonylphenol, bisphenol-A, 17α-ethynyl estradiol, bis(2-ethylhexyl)phthalate, endosulfan, paraquat dichloride, chloropyriphos, fenitrothion, cadmium chloride, lead nitrate, potassium dichromate, benzo[a]pyrene and carbon tetrachloride) on larvae of aquatic midge *Chironomus tentans* (Diptera, Chironomidae). Heat shock protein 70 gene expression increased in most of chemicals treated larvae compared to control. The response was rapid and sensitive to low chemical concentrations but not stressor specific. In conjunction with stressor specific biomarkers, heat shock protein 70 gene expression in *Chironomus* might be developed for assessing exposure to environmental stressors in the fresh water ecosystem. Considering the potential of *Chironomus* larvae as biomonitoring species, heat shock gene expression has a considerable potential as a sensitive biomarker for environmental monitoring in *Chironomus*.**

Key Words : ***Chironomus tentans*, heat shock protein, biomarker, environmental monitoring**

Introduction

Environmental contaminants may induce expression of certain genes in an organism. Almost without exception, gene expression is altered in toxicity, as either a direct or indirect result of toxicant exposure. Depending upon the severity and duration of the contaminant exposure, expression of certain genes may be short-term toxicological responses leading to impact on individual fitness (i.e. survival and reproduction). Although the detection of toxicants induced molecular level effects has been widely investigated, the study of gene expression and their consequences at higher level of biological organization in wildlife animal species has seldom been attempted. Recently studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates (Natalie *et al.*, 2003; Perceval *et al.*, 2004; Rotchell and Ostrander, 2003; Tatsuya *et al.*, 2002) Among aquatic invertebrates, the aquatic larvae of non-biting midges (Chironomidae, Diptera) are globally distributed, and they are the most abundant group of insects found in fresh water ecosystems. They hold an important position in the aquatic food chain and are major food sources for fish and other vertebrates and invertebrates (Cranston, 1995; Wayne, 1994). And thus they have been extensively used for assessing acute and sublethal toxicity associated with contaminated sediments and water (Bettinetti, 2002; Jinhee *et al.*, 1999, 2000, 2001, 2002, 2004; Mark, 2002; Matthew, 1998, 2001; Michael, 1997; Ralf and Torsten, 2002).

Among molecular level responses to chemical stressors, stress proteins, firstly known as heat shock proteins, are being most frequently studied in aquatic invertebrates (Arts *et al.*, 2004; Piano *et al.*, 2004; Karouna-Renier *et al.*, 2003) As stress proteins are not only induced by heat shock but also by pollutants, they are increasingly used as biomarkers. Members of heat shock protein 70 (HSP70) family of molecular chaperones are among the most highly conserved proteins in eukaryotic organisms. The HSP70 gene family contains both stress inducible and constitutively (HSC70) expressed genes that share many common structural features. The expression patterns of these proteins are quite different. Inducible heat shock genes are expressed at extremely low levels under normal conditions, but their transcription and translation increase rapidly in response to various stressors (Bernd

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et al., 1998; Carmen *et al.*, 2001; Julian *et al.*, 1998; Peter, 1991; Lindquist *et al.*, 1988). Stress proteins are promising biomarkers of exposure and probably of effects. The induction and accumulation of a stress protein following exposure of invertebrates to various pollutants has been studied (Carretero *et al.*, 1991; Karouna-Renier *et al.*, 2003; Mauro *et al.*, 2003; Qiang *et al.*, 2003; Tatsuya *et al.*, 2002) however, an application of stress protein to environmental biomonitoring is still limited (Aamir *et al.*, 2003; Carretero *et al.*, 1991; Jose Luis *et al.*, 2001; Karouna Renier *et al.*, 2003; Mauro *et al.*, 2003; Thomas *et al.*, 2003; Torsten *et al.*, 2002).

In this study, we investigated expression of HSP 70 and HSC 70, in *Chironomus tentans* larvae, in order to identify general biomarkers for environmental monitoring. Various classes of environmental pollutants, including alkyl phenols, pesticides, heavy metals, were examined and most of them are considered to be endocrine disruptors.

Materials and Methods

Organism

Chironomus tentans larvae were obtained from adults reared in our laboratory. The original strain was provided by Toxicology Research Center, Korea Research Institute of Chemical Technology (Daejeon, Korea). Larvae were reared under a 16 h : 8 h light : dark photoperiod at room temperature (20 ± 1°C) in a 1 *l* glass chamber containing dechlorinated tap water and acid-washed sand with aeration. The larvae were fed Tetramin.

Exposure condition

The experiment was performed at a constant temperature (20 ± 1°C) and light conditions (16 h : 8 h light : dark). The effects of physical and chemical stressors were assessed using groups 4th instar larvae collected in rearing aquaria. For heat shock treatment, larvae (10 individuals per 100 ml dichlorinated tap water) were exposed to 35°C for 1, 2, 4, 8, 12 and 24 h. Control organisms were sampled after 4 h at room temperature. For hypoxic treatment, the tanks were sealed with parafilm. Larvae were introduced 24, 48 and 96 h after the beginning of the experiment and maintained under each condition for 24 h. Control tanks were left uncovered during the duration of the experiment to ensure normal dissolution of atmospheric oxygen in water. The concentration of dissolved oxygen in water was monitored in each tank using a WTW OXI-96 oximeter (WTM GmbH, Weilheim, Germany). The mean values of dissolved oxygen concentration were 7.88 (control), 4.55, 1.88 and 0.1 mg O_2 mg/L.

For chemical treatment, sub-lethal exposure concentrations were selected from the result of acute toxicity test. Acute toxicity was determined after 24 h of exposure, using death of individuals as an end-point (data not shown). Three concentrations, corresponding to 1/1000, 1/00 and 1/10 of the 24 h LC50 were selected for each compound (Table 1). Ten of 4th instar *C. tentans* larvae were transferred into 200 ml beakers containing 100 ml dichlorinated tap water and treated with

Table 1. Chemical concentration in each treatment

Chemical	Solvent	Concentration				
nonylphenol	acetone	0	1	10	100	μg/*l*
bisphenol A	acetone	0	8	80	800	μg/*l*
ethynyl estradiol	acetone	0	8	80	800	μg/*l*
bis(2-ethylhexyl)phthalate	acetone	0	0.5	5	50	μg/*l*
endosulfan	acetone	0	0.5	5	50	μg/*l*
paraquate dichloride	acetone	0	0.25	2.5	25	mg/*l*
chloro pyriphos	acetone	0	1	10	100	μg/*l*
fenitrothion	acetone	0	7.5	75	750	μg/*l*
cadmium chloride	water	0	0.2	2	20	mg/*l*
lead(II)nitrate	water	0	0.05	0.5	5	mg/*l*
potassium dichromate	water	0	0.75	7.5	75	mg/*l*
benzo[a]pyrene	acetone	0	5	50	500	μg/*l*
carbon tetrachloride	acetone	0	0.02	0.2	2	mg/*l*

chemicals for sublethal exposure. For each experiment, 1 ml of test solution was added into experiment beakers before the introduction of larvae.

After physical and chemical treatment, the larvae were stored at -80°C until the gene expression analysis.

Gene expression analysis

Frozen larvae were homogenized in 700 μl of TRI reagent (Molecular Research Center) and RNA was isolated according to the manufacturer's standard protocol. RNA was resuspended in 50 μl water treated with diethyl pyrocarbonate (DEPC-H_2O), quantified by spectrophotometer (Biomate 3, Thermospectronic, NY, USA) and stored at -80°C until further use.

RT-PCR was performed by using two-step methods with RT Premix and PCR Premix kits (Bioneer Co., Seoul, Korea). Before reverse transcription (RT), 2 μg of total RNA and random hexamer (Promega) were denatured at 70°C for 5 min and then rapidly cooled on ice. These solutions were added to RT Premix kits and RT was carried out at 42°C for 60 min and 94°C for 5 min and these templates were then added to PCR premix kit containing each primer Primers for the detection of HSP70, HSC70 and β-actin genes were designed based on sequences retrieved from GeneBank™. (AY163157, AF448433 and AB070370, respectively). β-actin mRNA served for normalization of stress protein and hemoglobin levels. Sequences of primers used in the genes amplification were 5'CATGTGAACGAGCC AAGAGA3' and 5'TCGAGTTGATCCA CCAACAA3' for HSP70, 5'GTCTAAAGCCCCAGCCGT3' and 5'CAAAAATGGTAT TTGTTGGATTCAT3' for HSC 70 and 5'GATGAAGATCCTCACCGAACG3' and 5'C CTTACGGATATCAACGTCGC3' for β-actin.

PCR was performed for 30cycles of 95°C for 1 min, 60°C for 1 min and 72°C, 1 min, and a final extension at 72°C for 7 min using a PTC-100 Thermal cycler (MJ Research, Lincoln, USA). PCR products were separated by electrophoresis on a 1.5% agarose gel (Promega) and visualized with ethidium bromide (Bioneer). All tests were replicated at least three times. Relative densities of band were determined using the image analyser, Gel doc (Vilber Lourmat TFX-20.M, Marne la Vallee, France), with of Kodak 1D 3.6 camera (Kodac EDAS 290).

Chemicals

Nonylphenol (Ca. No. 46018), 17α-ethynyl estradiol (Ca. No. 46263), chloro pyriphos (Ca. No. 49395) and fenitrothion (Ca. No. 45487) were purchased from Riedel-deHaen (German). Bisphenol-A diglycidyl ether (Ca. No. 15138), bis(2-ethylhexyl)phthalate (Ca. No. 36735) and endosulfan (Ca. No. 36750) were obtained from Fluka (Switzerland) and paraquat dichloride (Ca. No. M2254), cadmium chloride (Ca. No. 287652), lead (II)nitrate (Ca. No. 228621), potassium dichromate (Ca. No. P2588), benzo[a]pyrene (Ca. No. B1760) and carbon tetrachloride (Ca. No. 270652) were purchased from Sigma-aldrich (USA).

Data analysis

Statistical differences between control and treated larvae were examined using the parametric t test. All analyses were performed using Sigmaplot 8.0 (Microsoft Co., USA).

Results and Discussion

We investigated heat shock gene expression by various classes of environmental pollutants in *Chironomus* larvae. As expected, HSP gene expression increased in most of chemicals treated larvae, as well as heat shock and hypoxia treated ones. Since hypoxic conditions frequently occur in polluted natural environment, it seems relevant to investigate stress protein gene expression as general biomarker of environmental quality.

Heat shock and hypoxia treatments-induced HSP70 and HSC70 genes expression change was evaluated in 4th instar larvae of *C. tentans* (Fig. 1). Heat shock treatment was used as a positive control, and as expected, we observed increase in HSP70 by thermal stress. Hypoxia induced increases in HSP70 mRNA, on the contrary, HSC70 levels decreased by oxygen depletion. Both responses exhibited oxygen content dependant patterns.

The effect of nonylphenol and bisphenol A on HSP70 and HSC70 expression was studied in *C. tentans*. As shown in Fig. 2, two alkyl phenolic compounds induced remarkable increases in both genes expression in *C. tentans*. Statistically significant increases were observed at all treatments, except for HSP70 at the highest concentration of bisphenol A treatment due to high experimental variation.

HSP70 and HSC70 genes expression increased in *C. tentans* larvae exposed to ethynyl estradiol and bis(2-

ethylhexyl)phthalate (Fig 3). Stress protein mRNA levels increased markedly at the lowest concentrations of both compounds (8 μg/*l* and 0.5 μg/*l* for ethynyl estradiol and bis(2-ethylhexyl) phthalate, respectively), and these increases were maintained at higher concentrations for ethynyl estradiol exposure. For bis(2-ethylhexyl) phthalate exposure, the pick was observed at the lowest concentration of exposure, but the expression levels of treated larvae still remained higher than control larvae.

HSP70, as well as HSC70, expression increased in *C. tentans* larvae exposed to pesticides, except for fenitrothion, where a concentration-dependant decrease was observed. Statistically significant increase was observed only at high level of exposure for endosulfan (5 and 50 μg/*l* for HSP70 and 50 μg/*l* for HSC70), whereas for paraquat and chloro pyriphos, increases were significant at the concentrations as low as that corresponding 1/1000 of 24 h LC50.

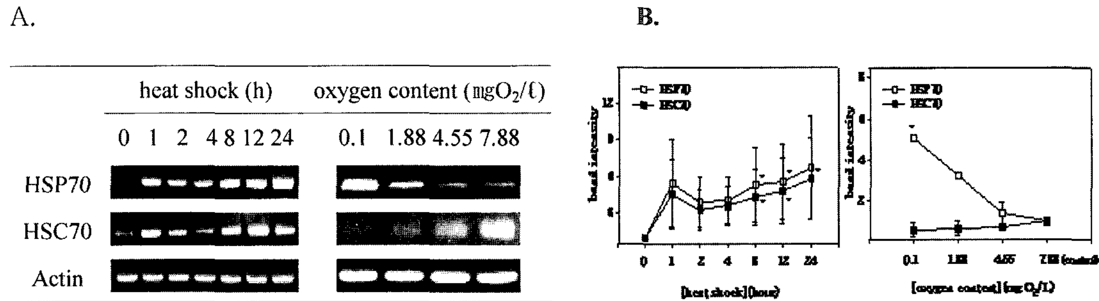

Fig. 1. Effects of heat shock and hypoxia on the expression of HSP70 and HSC70 mRNA in the 4th instar larvae of *C. tentans* (A). Densitometric values were normalized using actin mRNA expression (B) (n=3, mean ± SEM (standard error of mean), $*p < 0.05$).

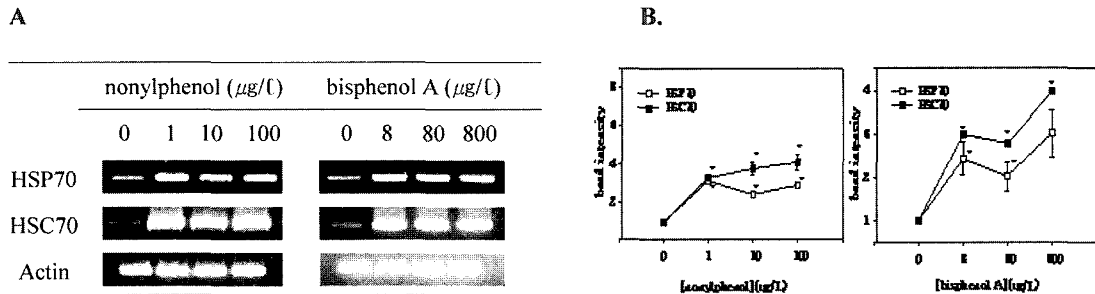

Fig. 2. Expression of HSP70 and HSC70 genes in the 4th instar larvae of *C. tentans* exposed to nonylphenol and bisphenol A for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) (n=3, mean ± SEM, $*p < 0.05$).

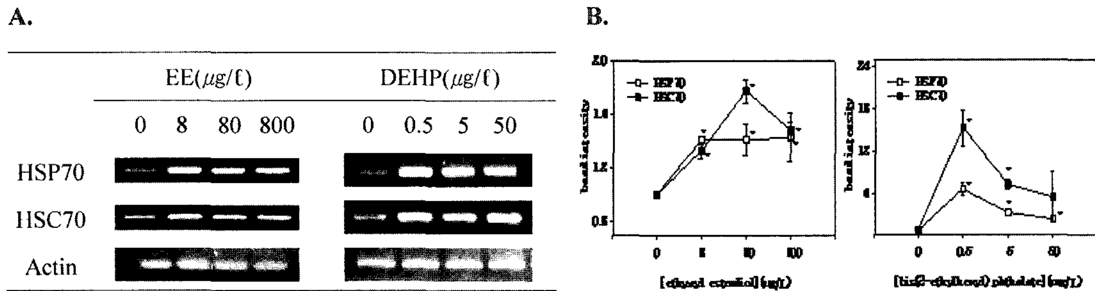

Fig. 3. Expression of HSP70 and HSC70 genes in the 4th instar larvae of C. tentans exposed to ethynyl estradiol (EE) and bis(2-ethylhexyl)phthalate (DEHP) for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) (n=3, mean ± SEM, $*p < 0.05$).

HSP70 and HSC70 mRNA levels were assessed in *C. tentans* larvae exposed to cadmium, lead and chromate (Fig. 5). Stress protein genes expression increased by these metals exposure. Especially, cadmium chloride and potassium dichromate induced concentration-dependent increases in HSC70 mRNA (6, 9 and 16-fold expression at 0.2, 2, and 20 mg/*l* of cadmium exposure and 1.5, 1.8 and 2.1-fold expression at 0.075, 0.75, and 7.5 mg/*l* of chromate exposure, respectively).

The effects of benzo[a]pyrene and carbon tetrachloride on expression of stress protein were presented in Fig. 6. Benzo[a]pyrene induced increases of both HSP70 and HSC70 mRNA expression. For carbon tetrachloride exposure, HSC70 mRNA expression increased, whereas,

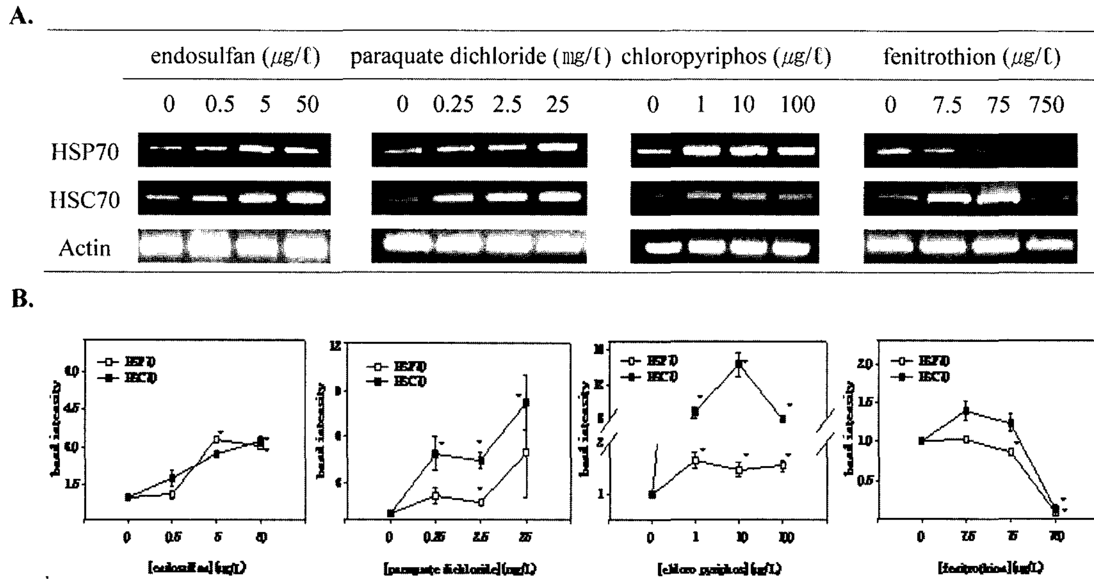

Fig. 4. Expression of HSP70 and HSC70 mRNA in the 4th instar larvae of *C. tentans* exposed to pesticides for 24 h (A). Densitometric values were normalized using those of actin mRNA(B) (n=3, mean ± SEM, $*p < 0.05$).

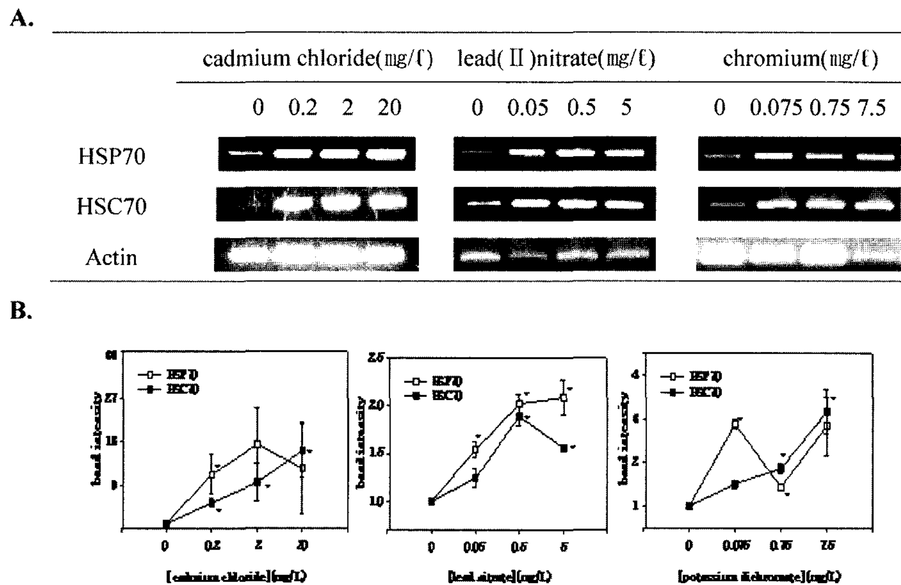

Fig. 5. Effects of heavy metals on the expression of HSP70 and HSC70 genes in the 4th instar larvae of *C. tentans* (A). Densitometric values were normalized using those of actin mRNA (B) (n=3, mean ± SEM, $*p < 0.05$).

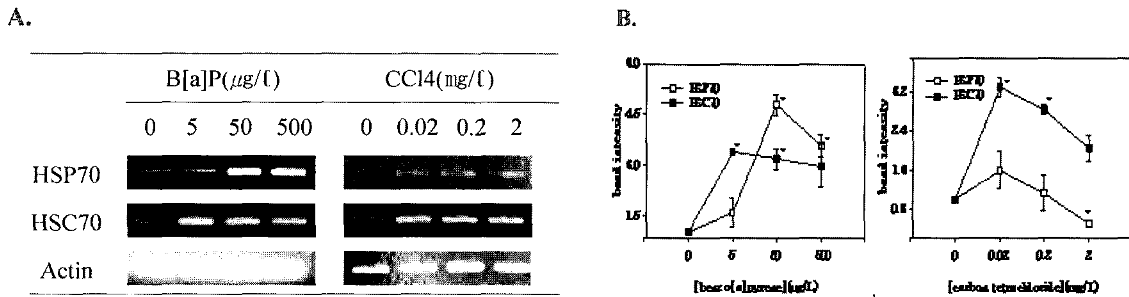

Fig. 6. Expression of HSP70 and HSC70 mRNA in the 4th instar larvae of *C. tentans* exposed to benzo[a]pyrene and carbon tetrachloride for 24 h (A). Densitometric values were normalized using those of actin mRNA (B) (n=3, mean ± SEM, *$p > 0.05$).

a decrease of HSP70 was observed at the highest concentration of exposure (2 mg/*l* of carbon tetrachloride).

Although HSP70 was initially identified for their response to heat shock (Osmulski *et al.*, 1986) members of this family are expressed in response to a variety of physical or chemical stresses, or as a result of normal changes associated with development and cellular differentiation (Carretero *et al.*, 1991; Peter *et al.*, 1991; Jonathan, 2003; Karouna Renier and Jonathan, 2003; Karouna Renier *et al.*, 2003; Tatsuya *et al.*, 2002) Most of tested chemicals induced expression of two stress protein genes (HSP70 and HSC70) at sublethal concentrations, except for fenitrothion and carbon tetrachloride, where expressions of those genes were proved to decrease. The response was rapid and sensitive to low chemical concentrations but not stressor specific. For its being responsive even to minor assaults, HSP70 and HSC70 expression may prove useful as a molecular indicator for adverse biological effects, including chemical toxicity in *Chironomus*. Different from HSP70, HSC70 is known to be constitutively expressed, and not inducible by environmental stressors (Bernd *et al.*, 1998; Caremen *et al.*, 2001; Juliann *et al.*, 1998; Lindquist *et al.*, 1988) However, in this study, we observed increase of HSC70 gene expression in response to variety of chemical exposure. These results suggest, as HSP70, HSC70 might be inducible in response to environmental stressors. However, further studies with broad range of chemicals are needed to elucidate the mechanism.

It would be ideal for environmental monitoring purposes to have a limited set of specific biomarkers indicating the exposure and assessing the hazards of all major classes of pollutants, as well as non-specific biomarkers that assess accurately and completely health condition of organisms and the ecosystem (Peakall *et al.*, 1994). Therefore, in conjunction with stressor specific biomarkers, stress protein expression in *Chironomus* might be useful for assessing fresh water quality. Taken into account overall results, *Chironomus* larvae seem to be a good model for studying molecular biomarker based environmental monitoring and HSP70 and HSC70 gene expression in *Chironomus* might be developed as non specific biomarker for assessing general health condition of ecosystem.

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