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Effects of environmental contaminants on hemoglobin of larvae of aquatic midge, *Chironomus riparius* (Diptera: Chironomidae): A potential biomarker for ecotoxicity monitoring

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Abstract

The effects of environmental contaminants (i.e., nonylphenol, bisphenol A diglycidyl ether, benzo[*a*]pyrene, chlorpyriphos, paraquat dichloride, and lead nitrate) on *Chironomus* hemoglobin were investigated in the 4th instar larvae of *Chironomus riparius* (Diptera: Chironomidae), with respect to the total hemoglobin contents, individual globin gene expression, individual globin protein expression and hemoglobin oxidation. In our studies, 7 and 6 globin isoforms were preliminarily characterized by molecular weight and isoelectric point, respectively, in the 4th instar larvae of *C. riparius.* Most chemicals were unable to modify the total hemoglobin contents, however, the expression patterns of the globin transcript and proteins suggest that *C. riparius* globin exists in both inducible and consecutively expressed forms, with multiplicity that may allow this animal to better adapt toward stressful environmental conditions, including pollution stress. The oxyhemoglobin was observed to be downregulated in *C. riparius* on exposure to bisphenol A and chlorpyriphos, probably reflecting its increased autoxidation to methemoglobin. The overall results would suggest that globin can be a target molecule of environmental contaminants, and of the tested parameters, the alteration of individual globin levels (i.e., mRNA or protein levels) may have potential for the development of a biomarker for ecotoxicity monitoring.

Keywords: *Chironomus riparius*; Globin; Hemoglobin multiplicity; Ecotoxicity; Biomarker

1. Introduction

The aquatic larvae of non-biting midges (Chironomidae, Diptera) are distributed globally, and are the most abundant group of insects found in freshwater ecosystems. They hold an important position in the aquatic food chain, being a major food source for fish and other vertebrates and invertebrates (Cranston, 1995). They are sensitive to many pollutants, easy to culture and have a short lifecycle; thus, are used extensively to assess the acute and sublethal toxicities of contaminated sediments and water (Matthew and David, 1998; Choi et al., 2000, 2002; Matthew et al., 2001; Bettinetti et al., 2002; Crane et al., 2002; Lee and Choi, 2006; Lee et al., 2006).

One of the main particularities of *Chironomus*, however, is they possess hemoglobin (Hb) during their larval stage. Species of the genus *Chironomus* exhibit stage-specific and tissue-specific single-chain globin syntheses throughout the four larval stages. The Hb is synthesized in the larval fat body, and then secreted into the hemolymph (Bergtrom et al., 1976; Saffarini et al., 1991). Although Hb is widely distributed throughout the animal kingdom, its occurrence in invertebrates is restricted to only a few representatives of the large taxonomic groups, known as Insecta or Crustaceans. The Chironomid Hb members of insect respiratory proteins have been extensively studied, and found to be very attractive research materials as they offer the simplest model for more complex Hb; analysis of their structure may provide key information in understanding the action

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mechanism of haem proteins, but their high heterogeneity provides many evolutionary problems for molecular biologists (Osmulski and Leyko, 1986). From an ecotoxicological point of view, Hb possessing organisms are often considered as candidate sentinel species for biomonitoring (Osmulski and Leyko, 1986). Studies on the biomarkers of xenobiotic pollution using Hb-related parameters, have recently been expanded into include invertebrate systems (Choi and Roche, 2004; Lamkemeyer et al., 2005; Rider et al., 2005; Lee et al., 2006; Rider and LeBlanc, 2006).

Considering the potential of *Chironomus* larvae as biomonitoring species, and the physiological particularities of *Chironomus* Hb, this invertebrate respiratory pigment has considerable potential as a sensitive biomarker for environmental monitoring. In this study, the changes of the *Chironomus* Hb in 4th instar larvae of *Chironomus riparius* Mg. (Diptera: Chironomidae), due to exposure to 6 environmental chemicals, with different modes of action; namely, nonylphenol (NP), bisphenol A diglycidyl ether (BPA), benzo[*a*]pyrene (B[*a*]P) chlorpyriphos (CP), paraquat dichloride (PQ), and lead(II)nitrate (Pb) were evaluated. Nonylphenol is used in the polymer industry (European Union, 2002), BPA is an intermediate in the production of polycarbonate and epoxy resins (European Union, 2003), BaP is a ubiquitously distributed polycyclic aromatic hydrocarbons (PAH) (Juhasz and Naidu, 2000), CP is an organophosphorous insecticide, PQ is an oxygen radical generating herbicide, and Pb is commonly found heavy metal. The effects of these environmental contaminants on Hb parameters were investigated in 4th instar larvae of *C. riparius* with respect to; firstly, the total Hb contents, using a cyanometHb procedure, secondly, individual globin gene expression, with reverse transcription-polymerase chain reaction (RT-PCR), thirdly, individual globin protein expression using polyacrylamide gel electrophoresis (PAGE) and electrophoresis of isoelectric focusing (IEF), and finally, Hb oxidation employing multi-wavelength rapid-scanning spectrophotometry.

2. Materials and methods

2.1. Organisms

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

2.2. Exposure conditions

Hemoglobin related parameters were assessed using groups of 4th instar larvae collected from the rearing aquaria. All larvae used in the experiment originated from the same egg mass, and were collected at the same time after egg hatching to obtain an age-synchronized population. From our previous experiments, 4th instar development of *C. riparius* larvae persisted for 22–38 days after the eggs had hatched, and the measurements of the cellular and extracellular proteins were conducted on the larvae during the middle of the 4th instar developmental stage (i.e., 32 days after the eggs had hatched). For the chemical treatment, 10 of the 4th instar larvae of *C. riparius* were transferred into 200 ml beakers, containing 100 ml of dechlorinated tap water, and treated with chemicals to assess the sublethal exposure. For each experiment, 0.1 ml of the test solution was added to the experimental beakers prior to larvae introduction. Acetone was used as the solvent for NP, BPA, B[*a*]P and CP, and water was used for PQ, and Pb. Our preliminary tests indicated that acetone provoked no significant effects during the experiments (data not shown). Exposure was carried at constant temperature (20 ± 1 °C), with a 16:8-h (light:dark) photoperiod for all experiments. For the analysis of each Hb parameter, 10 larvae from the control and experimental tanks were pooled, with hemolymphs withdrawn by opening the body wall, and the body fluids transferred into eppendorf cups containing ice-chilled physiological solution (NaCl 10.3 mM). Three replicates were conducted for each experimental compound for all experiments.

2.3. Acute toxicity test

Three groups of 10 larvae were exposed to four concentrations of each chemical, whereas other groups were kept as a control. Acute toxicity was determined after 24 h of exposure, using death of individuals as an endpoint. Log-Probit transformation of the data was used in order to estimate 10%, median and 90% 24 h lethal concentration (LC10, LC50 and LC90) values and the corresponding 95% confidence intervals.

2.4. Body dry weight measurement

For the measurement of body dry weights, 10 larvae collected after 24 h of exposure to the compounds. The fresh weights were immediately measured. The dry body weight of the larvae was measured after they were exposed to a temperature of 105 °C for 24 h. The weights were rounded off to the nearest 0.1 mg.

2.5. Protein and hemoglobin contents measurement

The total Hb contents of the hemolymphs were estimated via the cyanometHb procedure (Tentori and Salvati, 1981), using a plasma Hb kit (Sigma–Aldrich Chemical, St. Louis, MO, USA). The protein content was measured using the Bradford method (Bradford, 1976).

2.6. Globin gene expression analysis

The 10 larvae pooled from the control and treatment were homogenized in 700 μl of total RNA isolation reagent (Molecular Research Center, Cincinnati, OH), and the RNA was isolated according to the manufacturer's standard protocol. The RNA, resuspended in 50 μl of water treated with diethyl pyrocarbonate (DEPC–H_2O), was quantified with the aid of a spectrophotometer (Thermospectronic, Rochester, NY, USA), and was stored at −80 °C until further use. For RT-PCR, a two-step method, with RT Premix and PCR Premix kits (Bioneer, Seoul, Korea), was employed. Before the RT, 2 μg of total RNA and a random hexamer (Promega, Madison, WI, USA) were denatured at 70 °C for 5 min, and then rapidly cooled on ice. These solutions were added to the RT Premix kits, with the RT conducted at 42 °C for 60 min and at 94 °C for 5 min. These templates were then added to the PCR premix kit, containing 5 Hb open reading frames (ORFs), which were named HbA, HbB, HbC, HbD and HbE, and the actin primers. The primers were designed on the basis of sequences retrieved from GenBank™ (Supplementary Table 1). Finally, actin mRNA served for normalization of the expression of each Hb ORF level. For the comparison of mRNA transcript, semi-quantitative PCR was used. Even though this method has become questionable, and real-time-PCR is now the technique of choice, semi-quantitative PCR can provide reasonable quantification, when it performed properly. To optimize the semi-quantitative PCR conditions, a cycle number test was conducted for each ORF prior to the main experiment (Supplementary Fig. 1). Using a PTC-100 thermal cycler (MJ Research, Lincoln, MA, USA), 30 cycles of PCR were conducted at 95 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min and finally at 72 °C for 7 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Promega) and visualized with ethidium bromide (Bioneer). All the tests were repeated at least three times, and the relative densities of each band were determined with the aid of an image analyzer, a Gel documentation system (TFX-20.M UV transilluminator) (Vilber Lourmat TFX-20.M, Marne la Vallee, France), with a Kodak 1D 3.6 camera (Kodak EDAS 290, Rochester, NY, USA).

2.7. Non-denaturing polyacrylamide gel electrophoresis

Ten larvae from the control and experimental tanks were pooled, hemolymphs withdrawn by opening the body wall, and the body fluids subjected to electrophoresis. Non-denaturing polyacrylamide gel electrophoresis was performed on hemolymph protein samples to determine their molecular weights. After the electrophoresis, the gels were photographed and stained with brilliant Blue G (Sigma–Aldrich). Molecular weights were determined in a non-denaturing system, using a non-denatured protein molecular weight marker kit (Sigma–Aldrich), based on modifications to the methods developed by Davis (1964) and Bryan (1977). Briefly, the proteins were subjected to electrophoresis on a set of gels, containing various polyacrylamide concentrations (from 5% to 12%), and the electrophoretic mobility (Rf) of the protein in each gel was determined relative to the tracking dye, the 100 Log(-Rf × 100) was plotted against the percentage gel concentration for each marker protein, with the slope of the plot taken as the retardation coefficient. The individual slopes for each protein were determined from the logarithm of the negative slope plotted against the logarithm of the molecular weight of each marker protein. This produced a linear plot from which the molecular weight of a *Chironomus* hemolymph protein could be determined. Following the determination of the molecular weight of the Hb isomer in the control larvae, using the above-mentioned procedure, a 12% gel was used to investigate the effects of chemicals on individual components of *Chironomus* Hb, due to the ease of identification of the individual Hb bands.

2.8. Non-denaturing electrophoreses of isoelectric focusing

Electrophoreses of isoelectric focusing (IEF) in non-denaturing systems were used to characterize the *Chironomus* Hb, which allowed separation of the proteins according to their isoelectric points (pI). As with the PAGE analysis, hemolymphs prepared from control and exposed larvae were used for IEF. The IEF was performed as described previously (Choi and Roche, 2004). Briefly, a 12% acrylamide gel was prepared using ampholyte mix (range of pH 3.5–10; Fluka, Buchs SG, Switzerland), with a pH gradient formed on the gel by ampholyte pre-migration of the gel for 20 min at 300 V. The electrophoresis was performed on non-denatured hemolymph protein samples for 3 h at 300 V. After the electrophoresis, the gels were photographed and stained with brilliant Blue G (Sigma–Aldrich). The pI was determined from a standard curve prepared from marker proteins (range of pI 3.6–9.3; Sigma–Aldrich).

2.9. Multi-wavelength rapid-scanning spectrophotometry

To measure the oxyHb, 100 μl of the hemolymph samples from the control and treated larvae were diluted with 500 μl of ultra pure water, and multi-wavelength rapid-scanning spectrophotometry immediately performed between 500 and 650 nm (Thermospectronic). Three replicates were conducted.

2.10. Chemicals

The NP (92%) was purchased from Riedel-de Haen, BPA was from Fluka and B[*a*]P (97%), CP (99.2%), PQ (98%), and Pb (>99%) were from Sigma–Aldrich.

2.11. Data analysis

Statistical differences between the control and treated larvae for all analyses were examined with the aid of a

parametric *t*-test, using SPSS 12.0KO (SPSS Inc., Chicago, IL, USA).

3. Results

Acute toxicity was studied using LC50s derived through Probits analysis (Table 1). The 24 h LC50s of NP, BPA, B[*a*]P, CP, PQ and Pb in *C. riparius* were 0.688, 6.030, 31.59, 2.774, 1325 and 6693 mg l^{-1}, respectively. Based on the results of the acute toxicity test, 3 concentrations—corresponding to 1/1000, 1/100, and 1/10 of the 24 h LC50 or LC10—were selected for the Hb analysis.

Table 1
Estimation of 10%, median and 90% 24 h lethal concentration (LC10, LC50 and LC90 respectively) of nonylphenol (NP), bisphenol A (BPA), benzo[*a*]pyrene (B[*a*]P), paraquate dichloride (PQ), chlorpyriphos (CP) and lead(II)nitrate (Pb) in 4th instar larvae of *Chironomus riparius*

Chemicals		24 h LC (mg l^{-1})	95% Confidence interval
NP	LC10	0.484	0.229–0.592
	LC50	0.688	0.538–0.802
	LC90	0.978	0.832–1.646
BPA	LC10	3.732	0.913–5.041
	LC50	6.030	3.788–7.410
	LC90	9.742	7.840–21.84
B[*a*]P	LC10	10.03	1.390–17.34
	LC50	31.59	18.38–141.1
	LC90	99.54	45.06–6189
CP	LC10	0.668	0.017–1.566
	LC50	2.774	0.861–5.188
	LC90	11.52	5.955–125.9
PQ	LC10	917.3	181.3–1139
	LC50	1325	1008–2121
	LC90	1916	1502–14753
Pb	LC10	1174	80.63–2344
	LC50	6693	3559–39879
	LC90	38157	12830–8306797

Protein and Hb contents, as well as, body dry weight were measured 24 h after exposure to sublethal concentrations of the 6 chemicals in 4th instar larvae of *C. riparius* (Table 2). Larval growth investigated by the measurement of body dry weight, was not affected by chemical exposure. In control, the hemolymph protein was found to constitute about 50% of the total protein (data not shown), with the main property proven to be Hb (more than 90%). No statistically significant alteration in the Hb/protein ratio was observed after any of the treatments.

In order to study the effects of pollutants on the expression patterns of the globin transcript, the levels of globin mRNA were assessed in 4th instar larvae of *C. riparius* using 5 different *Chironomus* ORFs of Hb retrieved from Genbank™ (Fig. 1). NP and BPA induced HbA and HbB gene expressions, but a decrease in the HbE gene expression was observed at the highest concentrations. Especially, the increase in HbA due to exposure to NP and to BPA occurred in concentration-dependant manners. B[*a*]P and CP induced HbA and HbB gene expressions, respectively, whereas, CP and PQ down-regulated the HbD gene expression. Each globin ORF exhibited different sensitivities to stressors. Among 5 ORFs, the expressions of HbA and HbB seem to be increased by all tested chemicals, except HbB by Pb exposure, where statistically significant change was not observed. On the other hand, the expression of

Table 2
Body dry weight (BDW), protein and hemoglobin contents in the 4th instar larvae of *Chironomus riparius* measured 24 h after exposure to nonylphenol (NP), bisphenol A (BPA), benzo[*a*]pyrene (B[*a*]P), paraquate dichloride (PQ), chlorpyriphos (CP) and lead(II)nitrate (Pb) ($n = 3$; mean ± standard error of mean)

Chemicals	Concentrations (μg l^{-1}, *mg l^{-1})	BDW (μg $larva^{-1}$)	Protein (μg $mgBDW^{-1}$)	Hemoglobin (μg $mgBDW^{-1}$)	Hemoglobin $protein^{-1}$
Control	0	0.711 ± 0.020	113 ± 4.34	104 ± 2.84	0.923
NP	1	0.733 ± 0.423	126 ± 6.4	117 ± 5.24	0.923
	10	0.716 ± 0.414	149 ± 5.48	135 ± 1.07	0.901
	100	0.706 ± 0.407	134 ± 6.88	122 ± 0.09	0.911
BPA	5	0.728 ± 0.045	115 ± 9.06	97 ± 5.44	0.844
	50	0.729 ± 0.004	120 ± 8.34	108 ± 3.13	0.903
	500	0.744 ± 0.028	132 ± 15.36	112 ± 9.65	0.849
B[*a*]P	10	0.724 ± 0.039	136 ± 13.41	107 ± 13.69	0.787
	100	0.707 ± 0.049	135 ± 8.24	121 ± 3.97	0.891
	1000	0.753 ± 0.003	121 ± 7.53	109 ± 2.28	0.9
CP	2	0.661 ± 0.020	140 ± 2.19	134 ± 3.63	0.958
	20	0.694 ± 0.078	159 ± 4.18	154 ± 4.14	0.970
	200	0.694 ± 0.036	116 ± 1.72	114 ± 1.95	0.976
PQ	1*	0.739 ± 0.029	143 ± 15.58	118 ± 8	0.821
	10*	0.707 ± 0.059	158 ± 3.7	146 ± 0.94	0.923
	100*	0.653 ± 0.048	158 ± 1.96	125 ± 7.23	0.795
Pb	5*	0.706 ± 0.034	132 ± 10.55	116 ± 6.16	0.882
	50*	0.712 ± 0.040	137 ± 1.58	135 ± 1.96	0.981
	500*	0.761 ± 0.039	115 ± 6.66	106 ± 5.08	0.927

Hemoglobin $protein^{-1}$ ratio was also calculated.

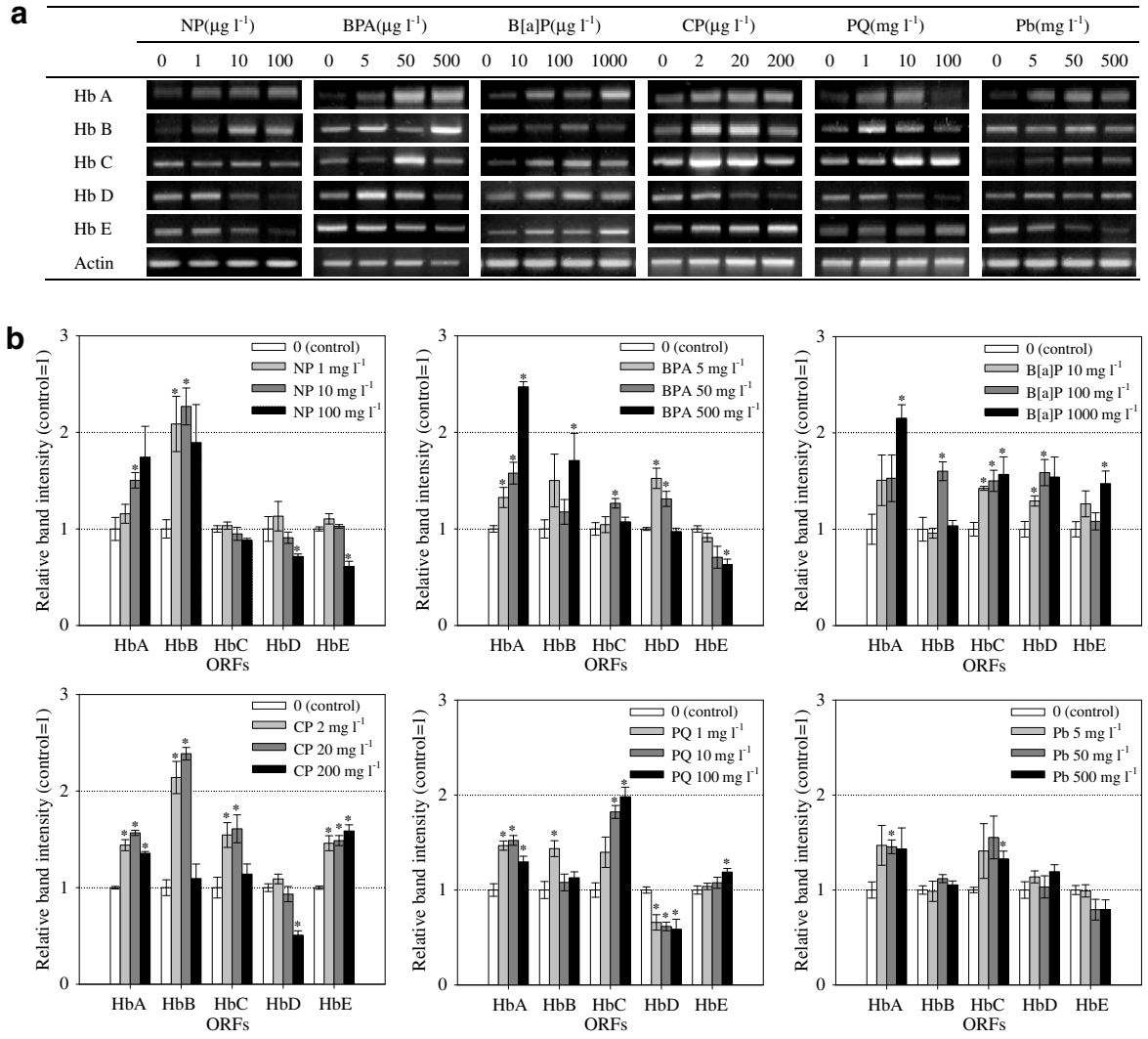

Fig. 1. Expression of hemoglobin genes in the 4th instar larvae of *Chironomus riparius* exposed to environmental pollutants for 24 h (a). Densitometric values were normalized using actin mRNA (b) ($n = 3$, mean ± standard error of mean, $^{*}p < 0.05$).

HbD was rather decreased, especially by NP, CP and PQ exposure, whereas, that of HbE was also decreased by NP and BPA.

As well as, the level of mRNA, the expression pattern of *Chironomus* globin was also examined in the protein level of 4th instar *C. riparius* larvae. The effects of 6 environmental pollutants on the multiplicity of globin were investigated on treated and control larvae using PAGE and IEF (Fig. 2). Preliminary characterization of the multiplicity of *Chironomus* globin protein was concomitantly conducted. The globin of *C. riparius* was separated by non-denaturing PAGE and by non-denaturing IEF into 7 and 6 different components, respectively. As a red color was visible on the bands of the gel prior to the staining procedure, the properties of the hemolymph proteins were presumed to be those of globin. However, additional bands in a PAGE gel, which gave positive result with blue staining, were not seen prior to staining; these were probably minor non-globin proteins associated with *C. riparius* hemolymph (Supplementary Fig. 2). The ranges of molecular weights and pI of the observed globins varied widely, from 7 to 26 kDa, and 3.5 to 6.2, respectively.

Exposure of *C. riparius* larvae to CP and PQ induced alterations in the expressions of individual globin proteins. CP exposure induced the expression of globins, of which molecular weights correspond to 10 and 14 kDa, whereas, PQ exposure (100 mg l^{-1}) induced the expression of globins of 26 and 17 kDa. Only scarce toxicant-induced variations in the individual globin components were found on the IEF gels: the expressions of globin of pI 6.2 increased, whereas, that of globin of pI 4.3 rather decreased by 100 μg l^{-1} of B[*a*]P exposure.

Fig. 3 shows the absorption spectra of the oxyHb of 4th instar *C. riparius* larvae, measured 24 h after exposure to various environmental pollutants, which indicated that BPA and CP reduced the peaks corresponding to oxyHb (i.e., 542 and on 576 nm) in 4th instar larvae of *C. riparius*. Concentration-dependant decreases were observed with CP exposure; whereas, these were only observed with high concentration of BPA.

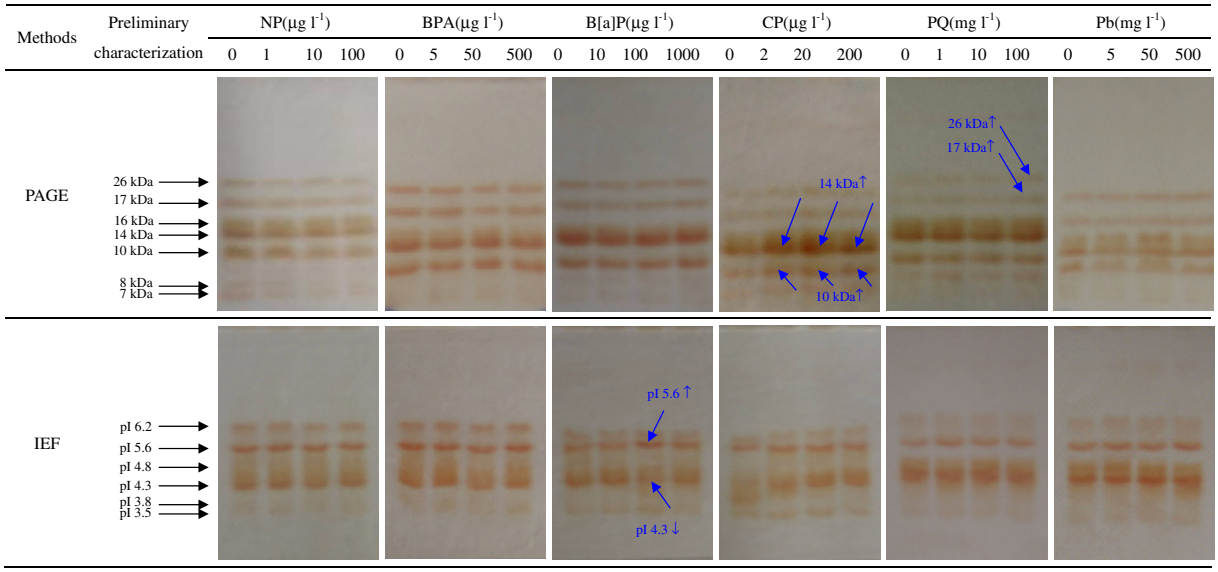

Fig. 2. Expression of molecular weight and charge isoforms of hemoglobin protein in the 4th instar larvae of *Chironomus riparius* exposed to environmental pollutants for 24 h.

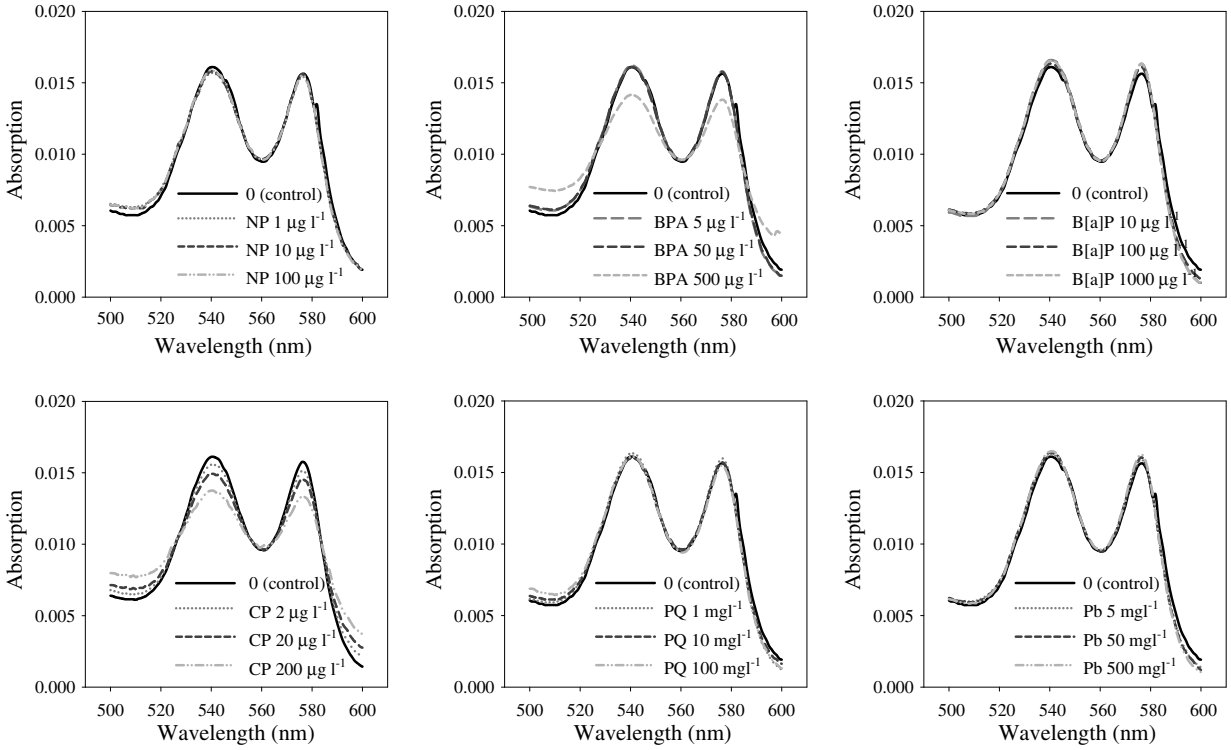

Fig. 3. Absorption spectrum of oxyhemoglobin of 4th instar larvae of *Chironomus riparius* measured 24 h after exposure to various environmental pollutants.

4. Discussion

Chironomus Hb shows many interesting features, such as a high affinity for oxygen, an extracellular localization and a high degree of polymorphism (Osmulski and Leyko, 1986); they also seem to impart some physiological and ecological particularities to *Chironomus*, which make them an interesting biological model for ecotoxicology studies. *Chironomus* Hb appears to fulfill clear physiological roles in the transportation and storage of oxygen in larvae that burrow in polluted and hypoxic muds. According to Weber (1980) and Cranston (1995), extracellular Hb enhances the exploitation of hypoxic oxygen. Moreover, a possible, but as yet undefined, role has been proposed for *Chironomus* Hb in the metabolism of xenobiotics in frequently polluted environments, where *Chironomus* flourish (Osmulski and

Leyko, 1986; Weber and Vinogradov, 2001). In this study, however, the response of the total Hb contents was not sensitive enough to prove a chemical-induced alteration, as no statistically significant alteration in the Hb/protein ratio was observed in most of the larvae exposed to chemicals (Table 2). It is believed that globin multiplicity in *Chironomus* may be related to their high level of tolerance to pollution. Therefore, the expression patterns of globin transcripts (mRNA) and proteins were investigated following chemical treatments.

As with other *Chironomus* species striking heterogeneity was also observed in *C. riparius* Hb (Weber et al., 1985; Fukuda et al., 1993; Kao et al., 1995; Bergtrom, 1999; Gruhl et al., 2000). In the larvae of 4th instar *C. riparius*, 5 Hb ORFs were investigated at mRNA level (Fig. 1), while, 7 and 6 globin isoforms were preliminary characterized (by molecular weight and pI, respectively) at protein level (Fig. 2). Each globin ORF might exhibit different sensitivities to chemical stress, as of the 5 ORFs studied, the expressions of HbA and HbB seem to increase; whereas, those of HbD and HbE were rather decreased by chemical stressors. According to Weber and Vinogradov (2001), *Chironomus* Hb exists in monomeric or dimeric forms; there are exclusively monomeric Hbs (~17–18 kDa) in *C. tentans* and no evidence of subunit aggregation, whereas, *C. thummi thummi* Hbs are in monomeric and dimeric forms. Taken into account these previous observations, molecular isoform corresponding to 26 kDa in *C. riparius* may be dimeric form of Hb. The investigation of *Chironomus* globin, using PAGE and IEF analyses, however, can only provide preliminary characterization; therefore, 2-dimensional analysis (proteomics approach) may be needed for more detailed identification of individual globin, which is ongoing project in our laboratory.

Our result indicated that chemical exposure induced the expression in selected globin genes and proteins (Figs. 1 and 2) rather than in total Hb content (Table 2). As with transcriptional level, chemical-induced individual globin synthesis was also observed, at translational level (Fig. 2). Fine regulation of globin synthesis on mRNA and protein levels seems to confer a high tolerance to some pollutants in *Chironomus*, as severe disturbance on larval physiology was not observed (e.g., larval growth, Table 2). In relation to the different sensitivities of globin mRNA expression to chemical exposure, increased synthesis of individual components of globin at the protein level suggests that *C. riparius* globin consist of a mixture of inducible and consecutively expressed forms. Globin multiplicity might contribute to their flexibility toward various environmental conditions and thus, may allow this animal to better adapt to exogenous stresses, potentially acting as a fine tuning system toward stressful environmental conditions (Osmulski and Leyko, 1986). Although, increases in certain globin genes does not necessarily induce an increase in the total Hb concentration, an increased number of globin genes was positively selected as a mechanism for achieving the high Hb concentration presumed desirable for the survival of the larvae. Previously, the expression of globin, as well as, heat shock protein genes, were studied in *C. tentans*, as a response for exposure to various environmental pollutants, with the results suggesting the possible use of *Chironomus* globin gene expression as a biomarker for assessing the general health conditions of freshwater ecosystems (Lee et al., 2006). From our data, it is speculated that *Chironomus* globin is regulated at both the protein and mRNA levels, but without providing experimental evidence on the relationship between transcriptional and translation regulations, which will need to be elucidated using more sophisticated techniques. The expression of each ORF may be related to each globin protein component identified by PAGE and IEF.

Hemoglobin undergoes spontaneous oxidation of the iron in its haem groups, forming metHb, which cannot transport oxygen. Hemoglobin reacts with a wide variety of redox active compounds, with numerous studies having suggested that environmental pollutants can give rise to Hb oxidation (Medeiros et al., 1983; Winterbourn, 1985). Autoxidation of oxyHb to metHb is considered a major source of oxyradicals (Misra and Fridovich, 1972; Weber et al., 1973; Abele-Oeschger and Oeschger, 1995). Hemoglobin from lower animals is known to be more rapidly auto-oxidized than the corresponding mammalian proteins (Abele-Oeschger and Oeschger, 1995). In our study, of the 6 chemicals tested, exposure to BPA and CP induced decreases in oxyHb, probably reflecting the increased autoxidation of oxyHb to metHb (Fig. 3), as previously observed with chromium treated *C. riparius* larvae (Choi and Roche, 2004). The presence of highly active respiratory pigments implies these organisms possess efficient antioxidant enzymatic systems. Our present and unpublished studies showed that *C. riparius* larvae were able to survive under severely stressed conditions, with little disturbance at the organism (Table 2) or population levels (data not shown). Such a tolerance suggests these animals possess efficient protection equipment against oxidative stress, at either the biochemical or physiological level, as elucidated in our previous studies (Choi et al., 2000, 2002).

Shortcomings in the present study about not dealing with non-protein part in Hb synthesis and regulation, as well as, about the exposure concentrations, should be mentioned. First, the focus of this study was exclusively on the protein side of Hb synthesis and regulation, however, chemicals, such as, Pb and B[*a*]P, may affect on haem synthase and iron metabolism. It is well known that organic chemicals, including, B[*a*]P, and some metals induce δ-aminolevulinic acid synthase, whereas, Pb and to a lesser extent, other metals inhibit δ-aminolevulinic acid dehydratase; both are involved in poryphyrin synthesis (Hodgson, 2004). Therefore, these need to be investigated, in relation to the effects on globins, to better understand the effects of chemicals on *Chironomus* Hb. Second, the present study focused on the identification of Hb parameters as potential biomarkers for chemical contamination under laboratory conditions. The exposure concentrations were based on

the result from acute toxicity (Table 1), using mortality as an endpoint, which were generally higher than those found in the field, as *Chironomus* showed high tolerance to environmental stresses, including chemical contamination. However, the present study focused on the identification of Hb-related parameters as potential biomarkers for environmental contamination under laboratory conditions. The *in situ* calibration and validation of the identified biomarkers using environmentally relevant exposure conditions will be addressed in future studies.

Summarizing all of the results obtained from this study, as well as the findings and hypotheses from numerous other previous studies, it is clear that *Chironomus* larvae possessing haem respiratory proteins have become pre-adapted to different extreme environmental conditions, including hypoxia and/or pollution, due to qualitative and quantitative compensation phenomena and the exploration of new niches. The flexibility of Hb in response to the environment allows for the best possible adaptation of *Chironomus* larvae. In conclusion, our study revealed the high multiplicity of *C. riparus* globin, and suggests that *Chironomus* Hb could be a target molecule for chemical exposure. Of the Hb parameters tested, the expression patterns of globin transcripts and proteins may have greater potential than the total Hb contents for the development of a biomarker for ecotoxicity monitoring. Simultaneous use of a suite of biomarkers from various environmental species would be ideal for effective ecotoxicity monitoring, and Hb parameters can be considered as a promising species-specific biomarker in *Chironomus*.

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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.chemosphere.2008.01.018.

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