Arch Environ Contam Toxicol (2009) 57:330–337
DOI 10.1007/s00244-007-9079-0

Effects of Environmental Contaminants on Hemoglobin Gene Expression in *Daphnia magna*: A Potential Biomarker for Freshwater Quality Monitoring

Mi-Hee Ha · Jinhee Choi

Received: 24 July 2007 / Accepted: 23 October 2007 / Published online: 27 May 2009

Abstract *Daphnia* hemoglobin (Hb) is one of the widely investigated invertebrate respiratory pigment. In this study, alteration of *Daphnia magna* Hb was evaluated in terms of its gene expression, using four *D. magna* Hb open reading frames (ORFs), by exposure of various chemicals, such as nonylphenol (NP), bisphenol A (BPA), benzo[*a*]pyrene (B[*a*]P), chloropyriphos (CP), paraquat dichloride (PQ), and lead nitrate (Pb), under laboratory conditions. A *Daphnia* reproduction test was also conducted to test the ecotoxicological relevance of chemical-induced *Daphnia* Hb gene expression. *Daphnia* Hb gene expression increased by most of tested chemicals. Nonylphenol induced all four Hb ORFs, and an increase in *D. magna* hemoglobin 2 (dmhb2), dmhb3, and dmhb4 gene expression was exposure concentration dependent. Although BPA and B[*a*]P also induced most of the Hb genes, the degree of increase was less than two-fold compared to the control. For CP and Pb exposure, an increase in dmhb2 and dmhb4 gene expression was the most significant among the four *Daphnia* Hb ORFs. Each ORF might exhibit different sensitivities to chemical stress; of the four ORFs studied, an increase in dmhb2 and dmhb4 gene expression was the most significant. It seems clear that *Daphnia* Hb has a considerable potential as a biomarker for freshwater toxicity monitoring, as an increase in Hb gene expression seems to be correlated with a decrease in reproduction in this animal. The results suggest that *Daphnia* Hb could give useful information to diagnose general health conditions in a freshwater ecosystem. Considering the potential of *D. magna* as a biomonitoring species and the physiological particularities of its respiratory pigments, *Daphnia* Hb could be developed as a biomarker for ecotoxicity monitoring.

M.-H. Ha · J. Choi (✉)
Environmental Engineering, University of Seoul, Seoul 130-743, Korea
e-mail: jinhchoi@uos.ac.kr

Hemoglobin (Hb) plays a vital role in many advanced organisms in forming a constitutive part of their oxygen-transport systems. Different from vertebrate, within the invertebrates, Hb occurs sporadically in various phyla (Terwilliger 1980, 1998; Vinogradov 1985), and the concentration in the body fluids is usually lower than in vertebrates (Prosser and Brown 1961) and can vary greatly depending on environmental conditions (Fox 1955; Terwilliger 1998; Weber 1980). The presence of Hbs in invertebrates is believed to be related to the adaptation of these organisms to unfavorable environmental conditions, as these pigments help to sustain aerobic metabolism under low-oxygen conditions (Weber and Vinogradov 2001). The small-sized crustacean *Daphnia* plays a pivotal role in aquatic food webs and has been the focus of study by limnologists for well over a century. Hb in *Daphnia* was detected more than 100 years ago (Lankester 1871) and recently has been the topic of many studies (Gorr et al. 2004, 2006; Pane et al. 2003; Rider and LeBlanc 2006; Rider et al. 2005; Zeis et al. 2003a, 2003b). *Daphnia* utilizes an extracellular, multisubunit Hb as a respiratory protein. The different, di-domain subunits of Hb are encoded by several genes. *Daphnia* shows a sensitive environmental control of Hb gene expression. It was reported that levels of a *Daphnia* Hb mRNA elevated more than 10-fold when the animals were reared under oxygen-poor condition (Tokishita et al. 1997). This increased synthesis of Hb under an unfavorable hypoxic condition offers to this animal many biological advantages, which

include greater viability in hypoxic water, higher swimming activity, increased egg production, increased feeding rates, and exploitation of food resources. This remarkable flexibility in adjusting the quantity of Hb raises the question of the potential of *Daphnia* Hb as a tool for diagnosing its environment. Considering the importance of *Daphnia magna* as a bioindicator species for aquatic ecosystem monitoring, its Hb has a considerable potential as a sensitive biomarker for evaluating environmental quality.

In this study, to identify *Daphnia* Hb as a potential biomarker of environmental monitoring, we evaluated the changes in gene expression of this respiratory protein by exposure of various chemicals, such as nonylphenol (NP), bisphenol A (BPA), benzo[*a*]pyrene (B[*a*]P), chloropyriphos (CP), paraquat dichloride (PQ), and lead nitrate (Pb), under laboratory conditions. *Daphnia* Hb gene expression was carried out by the semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) method using four *D. magna* Hb open reading frames (ORFs). A conventional ecotoxicity test, such as the reproduction test, was also conducted to test the ecotoxicological relevance of chemical-induced *Daphnia* Hb gene expression.

Materials and Methods

Organism Culture

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), we obtained *D. magna* from adults reared in our laboratory. *D. magna* were individually placed in glass beakers containing a culture medium, aerated M4 media, for 2 days. Daphnids were fed on the green alga *Chlorella* sp. at a concentration of 1×10^6–10^9cells/mL every 2 days and the culture was maintained at 20 ± 1°C in a 14-h light and 10-h dark cycle photoperiod regime.

Sublethal Exposure Condition

We conducted the experiment at a constant temperature of 20 ± 1°C under light conditions of 14 h and 10 h of light and darkness, respectively. For the chemical treatment, the test solution of the chemicals (acetone was used as a solvent for NP, BPA, B[a]P, and CP, and media was used for PQ and Pb), was 1 part of which was added to 1000 parts of the medium. Solvent control did not show any statistical difference compared to the control (data not shown). Three replicates were prepared for each concentration.

An acute toxicity test was conducted to select the sublethal exposure condition for Hb gene expression and reproduction experiments. For acute toxicity, 10 neonates aged less than 24 h were individually transferred into 100-mL glass beakers filled with 50 mL of culture medium, including 0.05 mL of test solution, and incubated at 20 ± 1°C for 24 h. The 24-h EC_{50} value was determined for swimming inhibition of daphnids by the probit method (OECD 1984). The 24-h EC_{50}s of NP, BPA, B[a]P, CP, PQ, and Pb were estimated in neonates of *D. magna* (Table 1). Based on the results of the acute toxicity test, three concentrations corresponding to 1/1000, 1/100, and 1/10 of the EC_{50} were selected for sublethal exposure conditions.

Table 1 Immobility test in juvenile of *D. magna* exposed to various environmental contaminants for 24 h

Chemicals	EC_{50} (μg/L) (95% confidence interval)
Nonylphenol	303. 4 (229.6–397.1)
Bisphenol A	352.5 (167.1–498.3)
Benzo[*a*]pyrene	29.3 (13.32–242.2)
Chloropyriphos	0.953 (0.762 ~ 4.360)
Paraquat dichloride	1,126 (135.5–1834)
Lead(α)nitrate	18,153 (1080–40171)

Daphnia Reproduction Test

Ten daphnids, aged less than 24 h, were exposed to various concentrations of test chemicals, prepared in a glass jar filled with 100vmL of culture medium, including 0.1 mL of test solution, and were observed and fed daily for 21 days. Each jar was provided with *Chlorella* as food at a concentration of 5×10^5 cells/mL daily. Test animals were transferred to new medium every 2 days. Neonates were removed from the jar daily and the numbers were counted.

Hemoglobin Gene Expression Analysis

Daphnids were exposed to various concentrations of test chemicals for 24 h, in a glass jar filled with 100 mL of culture medium, including 0.1 mL of test solution. Following 24 h incubation, daphnids were homogenized in 700 μL of TRI 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) and was stored at −80°C until the analysis. For the RT-PCR, a two-step method, with RT Premix and PCR Premix kits (Bioneer Co., Seoul, Korea), was employed. Before the RT, 2 μg of total RNA and a random hexamer (Promega, Madison, WI) 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 four Hb ORFs, which were named dmhb1, dmhb2, dmhb3, and dmhb4, and the actin primers. The primers were designed on the basis of sequences retrieved from GenBank™ (Table 2). Finally, actin mRNA served for normalization of the expression of each Hb ORF level.

Using a PTC-100 thermal cycler (MJ Research, Lincoln, MA), 30 cycles of PCR were conducted at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min, and, finally, 72°C for 7 min. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Promega, Madison, WI) and visualized with ethidium bromide (Bioneer Co., Seoul, Korea). All of 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).

Chemicals

The NP and CP were purchased from Riedel-deHaen (Sigma Corp. St. Louis, MO), BPA from Fluka (Buchs SG, Switzerland), and B[*a*]P, PQ, and Pb from Sigma-Aldrich Chemical (St. Louis, MO).

Data Analysis

The data passed the normality test and the equal variance test. Statistical differences between the control and treated *Daphnia* were examined using variation analysis with Dunnett's multiple comparison test. A parametric Pearson test was conducted to study correlations between the parameters. All the statistical tests were performed using SPSS® 12.0KO (SPSS, Chicago, IL).

Table 2 Sequence of primers used in the amplification of *D. magna* hemoglobin and actin cDNA

ORF (GenBank accession No.)	Primer sequencea
dmhb1 (AF255951)	5′-TGA ACG TCG TCA TCC AGT CC-3′
	5′-GCG ATG CCA GCA ACC AAA GC-3′
dmhb2 (AB021136)	5′-GCT TTG GTT GCT GGC GTA TC-3′
	5′-AGA ATC AAC TGC GAC ACT GG-3′
dmhb3 (AB021137)	5′-TGG AAG AAC GGA CTT ACT GC-3′
	5′-AGA ATC AAC AGC AAC ATT AG-3′
dmhb4 (AY737794)	5′-ACC AGA AGA TGT CCA AGT CC-3′
	5′-TCC ACG GGG TTT ATG GGC AC-3′
actin (AJ292554)	5′-GAT GAA GAT CTT GAC TGA ACG-3′
	5′-CCT TAC GGA TGT CGA CGT CGC-3′

Results and Discussion

Daphnia is one of the most preferred and proven standard invertebrate for ecotoxicity testing. Recently, an international *Daphnia* Genomics Consortium (http://www.daphnia.cgb.indiana.edu) has formed to develop *Daphnia* (*D. pulex*) as a model system for ecological genomics. However, not much is currently known about the genomic responses of this species toward pollution stress. Environmental contaminants might 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 might 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 to and the effects of environmental chemicals is currently receiving significant attention. The recent sequencing of the entire genome on environmentally relevant aquatic organisms will heighten the use of these toxicity test organisms in molecular-level biomonitoring. Recently, several studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates (Lee et al. 2006; Rotchell and Ostrander 2003; Yoshimi et al. 2002)

We therefore address the interesting question of whether the globin molecules of the species *D. magna* show any differential gene regulation after application of environmentally hazardous chemicals. *Daphnia* Hb is one of the widely investigated invertebrate Hb, as *Daphnia* plays a pivotal role in the aquatic ecosystem and its Hb confers to this animal many physiological particularities (*i.e.*, acclimation to hypoxia; Paul et al. 2004). Most of studies on *Daphnia* Hb regulation have been conducted using hypoxic stress; however, few studies have been done about how chemicals affect the regulation of *Daphnia* Hb.

In this study, the regulation of *Daphnia* Hb was investigated following chemical treatments. An increase in the hemolymph Hb concentration was reported in *Daphnia* exposed to a hypoxic environment (Kobayashi and Hoshi 1982). However, in our results, looking at the chemical effects on the Hb concentrations, the response of the total Hb contents was not sensitive enough to prove a chemical-induced modification (data not shown). It is believed that globin multiplicity in *Daphnia* might be related to their high level of tolerance to extreme environmental condition, such as hypoxia.

Kimura et al. (1999) reported four Hb genes in *D. magna* constituting a cluster in the order dmhb4, dmhb3, dmhb1, and dmhb2 on a single chromosome. Three of them have been sequenced completely (dmhb1, dmhb2, and dmhb3) and one only partially (dmhb4). Expression of the *Daphnia* Hb genes is believed to be controlled at the

transcriptional level (Tokishita et al. 1997), which led us to investigate the expression patterns of individual globin transcripts (mRNA) following chemical treatments. In order to study the effects of chemicals on the expression patterns of the Hb transcript, the levels of Hb mRNA were assessed in *D. magna* using four different *Daphnia* ORFs of Hb (Fig. 1). *Daphnia* Hb gene expression increased by most of the tested chemicals. NP induced all four Hb ORFs, and an increase in dmhb2, dmhb3, and dmhb4 gene expression was exposure concentration dependent. Although BPA and B[*a*]P also induced most of the Hb genes, the degree of increase was less than two-fold compared to the control. For CP and Pb exposure, an increase in dmhb2 and dmhb4 gene expression was the most significant among the four *Daphnia* Hb ORFs at the highest concentrations. Each ORF might exhibit different sensitivities to chemical stress, as of the four ORFs studied, an increase in dmhb2 and dmhb4 gene expression was the most significant. Expression of dmhb1 gene seems to be hardly induced by chemical stressors; rather, it seems to be constitutionally expressed compared to other forms.

Zeis et al. (2003b) also observed that oxygen deficiency hardly impacts concentrations of dmhb1 transcripts, whereas it rapidly (1 h) and markedly (up to five-fold) elevates dmhb2 and particularly dmhb3 mRNA levels. Numerous researchers reported that hypoxia regulates globin genes in *D. magna* at the mRNA level (Hebert et al. 1999; Kimura et al. 1999; Tokishita et al. 1997). It has also been reported that at least the dmhb2 gene is hypoxically stimulated through a hypoxia response elements (HRE) promoter, which explains the dramatic induction of Hb expression when daphnids are challenged by hypoxia (Gorr et al. 2004). The promoter regions of *Daphnia*'s globin genes each contain numerous potential binding sites for hypoxia-inducible transcription factors (HIFs). It has been also reported that, at the protein level, these globin genes respond differentially to hypoxia, with rates of induction ranging from 5-fold (dmhb1) to 14-fold (dmhb2) and even 20-fold (dmhb3), suggesting that the preferential expression of the dmhb2 and dmhb3 genes at a low oxygen level could contribute to the increased O_2 affinity seen in hypoxic hemolymphs (Gorr et al. 2004).

As such, it seems clear that *Daphnia* Hb is regulated by ambient oxygen concentrations; *Daphnia* Hb might also be regulated by chemical stress. However, the study on chemical-induced Hb regulation is limited. Rider and LeBlanc (2006) found that the herbicide atrazine elevated Hb levels in *D. magna* through dmhb2 gene induction by the oxygen-sensing pathway, not by the hormonal regulatory pathway. The expression of Hb genes, as well as heat shock protein genes, were studied in the sediment-dwelling aquatic macroinvertebrate *Chironomus* as a response system for exposure to various environmental pollutants, with the results suggesting the possible use of *Chironomus* Hb gene expression as a biomarker for assessing the general health conditions of freshwater ecosystems (Lee et al. 2006). Even though our result suggests that dmhb2 and dmhb4 genes seem to respond to chemical stresses, it seems that the induction rate is more important to hypoxia than chemical stress (Fig. 1b; slightly above two-fold). Moreover, experimental evidence is still very much limited to draw any general conclusion about the response of *Daphnia* Hb to chemical stress, especially substance class-specific response. Therefore, to identify *Daphnia* Hb as a chemical-specific biomarker for toxicity monitoring, further studies with a broad range of chemicals might be needed. *Daphnia* Hb seems to impart some physiological and ecological particularities to *Daphnia*, which make them an interesting biological model for ecotoxicology studies. In some cases, the presence of Hb appears to be connected with an animals' resistance to extreme environments, as attested in Hb possessing fresh water macroinvertebrates, which are usually highly tolerant to environmental pollution (Osmulski and Leyko 1986). A good supply of oxygen might help in the active and rapid removal of toxic compounds by accelerating metabolic reactions.

It has been reported that *Daphnia* Hb induction has been regulated by at least two distinct molecular pathways: not only by an oxygen-sensing pathway involving the HIF but also by an endocrine pathway stimulated by terpenoid hormones (Gorr et al. 2004, 2006; Rider and LeBlanc 2006; Rider et al. 2005). The terpenoid hormone is a major hormone responsible for transducing environmental signals in crustaceans. It has been associated with a variety of physiological processes in crustaceans related to reproduction and is structurally similar to juvenoid hormones of insects and retinoid hormones of vertebrates (see in Rider et al. 2005). Recently, putative juvenoid response elements (JREs) were identified by Gorr et al. (2006), and they found that a specific gene, such as the dmhb2 gene, was induced by juvenoids and JRE might mediate this induction. However, our results were not sufficient enough to provide experimental evidence that the oxygen-sensing pathway or the hormonal pathway were involved in the observed induction of daphnid Hb. More mechanism studies on the chemical-induced *Daphnia* Hb gene expression have to be simultaneously conducted, which will be the subject of our further research.

Although, the molecular-level response of environmental species, such as gene expression, has a potential as an early warning system for environmental quality monitoring, biomarker response alone does not seem to be sufficient to assess environmental quality. Pollutant-induced molecular effects might potentially have consequences at higher levels of biological organization (Caquet and

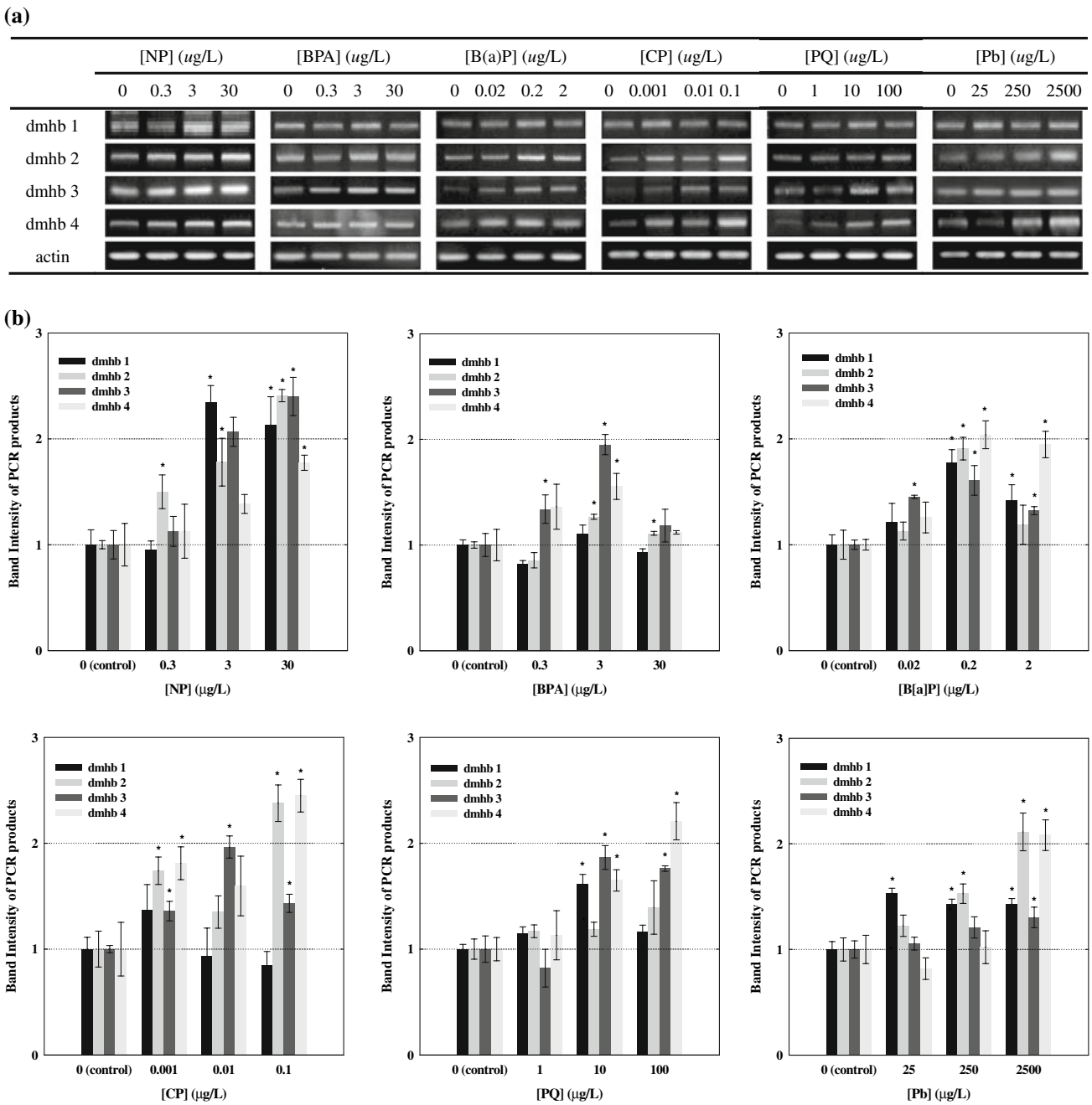

Fig. 1 Expression of hemoglobin genes in the *D. magna* exposed to various environmental contaminants for 24 h (**a**). Densitometric values normalized using those of actin mRNA (**b**); $n = 3$, mean ± SEM, $^*p < 0.05$

Lagadic 2000; Depledge et al. 1993). Therefore, a chronic toxicity test using reproduction as an endpoint was conducted in order to validate the ecotoxicological relevance of *Daphnia* Hb response (Fig. 2). A 21-day reproduction test revealed that the number of neonates per female decreased significantly in *D. magna* that had been exposed to NP, B[*a*]P, PQ, and Pb. The decrease was concentration dependent in NP- and PQ-exposed *D. magna*, whereas BPA and CP did not seem to affect the reproduction of *D. magna*. Generally, an increase in Hb gene expression was associated with a decrease in reproduction potential. The most significant case was in NP exposure, in which a NP-induced concentration-dependent decrease in the *Daphnia* reproduction parameter occurred concomitantly with an increase in Hb gene expression, which suggests that the increase in Hb gene expression might be considered a toxicity that might lead to physiological consequences in *Daphnia*, rather than a homeostatic response. The experiments with B[*a*]P-, PQ-, and Pb-exposed *Daphnia* show that long-term (21-day) effects on

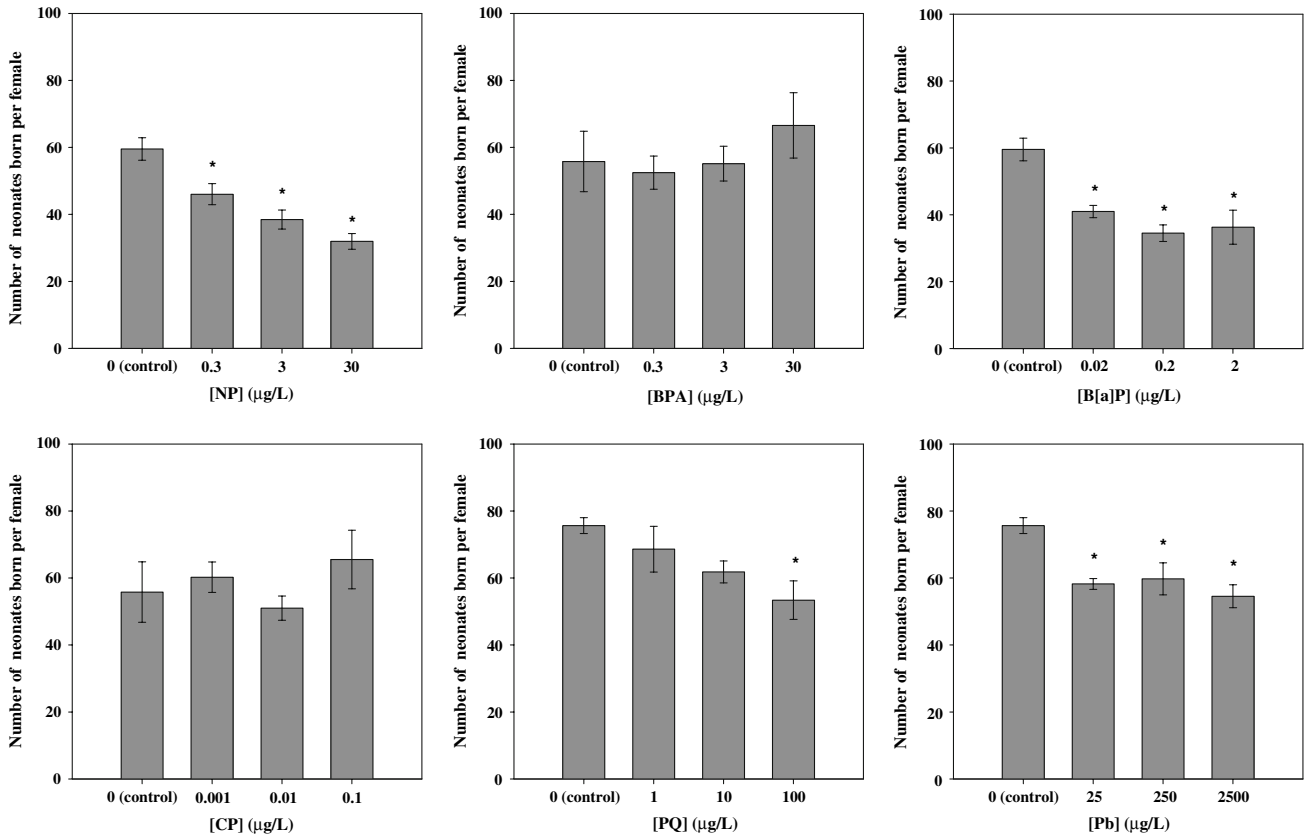

Fig. 2 Numbers of neonate per female of *D. magna* exposed to various environmental contaminants for 21 days ($n = 3$, mean ± SEM, $^*p < 0.05$)

reproduction were significant, whereas 24- h effects on Hb gene expression were not very significant (an increase in expression was mostly less than two-fold compared to the control). This might be an example of a false-negative result from the biomarkers' perspective. It is clear that this type of error can occur; however, this result could be interpreted as a mechanism other than Hb-induction might be involved in *Daphnia* reproduction failure. On the other hand, significant effects in Hb gene expression in CP-exposed *D. magna* were not related to a degree of impairment of reproduction. False-positive results from the Hb biomarker obtained in CP-exposed *D. magna* make it more difficult to use Hb gene expression as an early warning biomarker.

It is widely accepted that to be an ideal biomarker, effects at the population level can be predicted from the biomarker changes measured in a sample of individuals (Hyne and Maher 2003). To relate the effects measured at the individual level to higher levels of biological organization, the biomarker response should be related to an impairment of growth, reproduction, or metabolic function that directly affects the survival of the organism and that can be attributed to exposure to a known amount of specific contaminants (Depledge and Fossi 1994).

In this study, to identify correlations between the *Daphnia* Hb gene expression and a higher-level effect (reproduction), Pearson correlation tests were performed on the parameters studied (Table 3). Statistically significant positive correlations were observed between the exposure concentration of BPA and reproduction, and the exposure concentration of Pb and dmhb4 gene expression. Our dataset established a negative correlation between Hb gene expression and reproduction outcomes (dmhb2 for NP; dmhb2 and hmhb4 for PQ exposure), which could suggest the possible protective role of Hb toward these chemicals' stress in *Daphnia.* The experimental evidence provided by this study, however, especially at the molecular/biochemical level, is not sufficient to demonstrate a causal relationship between Hb gene expression responses and higher biological-level effects.

In conclusion, the investigation on contaminants-induced gene expression and its higher-level consequences seems to be a powerful tool to determine the general health status of an organism and, thus, to assess environmental quality more efficiently. As proven by many ecotoxicity studies, *D. magna* seems to be a good biological model for this kind of approach. Even though our result could not provide any concrete conclusion about the chemical class-

Table 3 Pearson coefficients of correlation estimated between hemoglobin gene expression and reproduction parameters in *D. magna* exposed to various environmental contaminants

	dmhb1	dmhb2	dmhb3	dmhb4	Reproduction
Nonylphenol	0.556	0.875	0.786	0.922	−0.731
dmhb1		0.762	0.945	0.807	−0.811
dmhb2			0.923	0.980(*)	−0.970(*)
dmhb3				0.956(*)	−0.916
dmhb4					−0.922
Bisphenol A	−0.122	0.282	−0.212	−0.303	0.974(*)
dmhb1		0.875	0.534	0.250	0.009
dmhb2			0.353	0.624	0.661
dmhb3				0.053	0.703
dmhb4					0.559
Benzo[a]pyrene	0.221	−0.100	0.010	0.581	−0.444
dmhb1		0.938	0.848	0.922	−0.844
dmhb2			0.781	0.746	−0.642
dmhb3				0.701	−0.900
dmhb4					−0.870
Chloropyriphos	−0.596	0.858	0.078	0.834	0.744
dmhb1		−0.135	−0.231	−0.171	0.023
dmhb2			0.165	0.985(*)	0.839
dmhb3				0.334	−0.397
dmhb4					0.732
Paraquat dichloride	−0.084	0.879	0.579	0.904	−0.851
dmhb1		0.238	0.697	0.349	−0.417
dmhb2			0.628	0.926	−0.969(*)
dmhb3				0.846	−0.799
dmhb4					−0.977(*)
Lead(II)nitrate	0.271	0.929	0.829	0.991(**)	−0.572
dmhb1		0.513	0.527	0.142	−0.942
dmhb2			0.972(*)	0.895	−0.770
dmhb3				0.800	−0.761
dmhb4					−0.463

See text for definitions of abbreviations. Asterisks indicate significant correlations (*$p < 0.05$; **$p < 0.01$)

specific response of *Daphnia* Hb, it seems clear that *Daphnia* Hb gene expression has a considerable potential as a biomarker for freshwater toxicity monitoring, as an increase in Hb gene expression (*i.e.*, dmhb2 and dmhb4) seems to be correlated with the decrease in reproduction potential in this animal.

Acknowledgment This work was supported by the Korean Ministry of Environment through the Ecotechnopia 21 project.

References

Caquet T, Lagadic L (2000) Consequences of individual-level alterations on population dynamics and community structure and function. In: Lagadic L, Caquet T, Amiard JC, Ramade F (eds) Use of biomarkers for environmental quality assessment. Science Publishers, Enfield, UK, pp 79–111

Depledge MH, Amaral-Mendes JJ, Daniel B, et al. (1993) The conceptual basis of the biomarker approach. In: Peakall DG, Shugart LR (eds) Biomarkers: Research and application in the assessment of environmental health. Springer-Verlag, Berlin, pp 15–29

Depledge MH, Fossi MC (1994) The role of biomarkers in environmental assessment (2). Invertebrates. Ecotoxicology 3:161–172

Fox HM (1955) The effect of oxygen on the concentration of haem in invertebrates. Proc R Soc B 143:203–214

Gorr TA, Cahn JD, Yamagata H, Bunn HF (2004) Hypoxia induced synthesis of hemoglobin in the crustacean *Daphnia magna* is HIF dependent. J Biol Chem 279:36,038–36,047

Gorr TA, Rider CV, Wang HY, Olmstead AW, LeBlanc GA (2006) A candidate juvenoid hormone receptor *cis*-element in the *Daphnia magna* hb2 hemoglobin gene promoter. Mol Cell Endocrinol 247:91–102

Hebert PDN, Um YM, Prokopowich CD, Taylor DJ (1999) Gene conversion and evolution of daphniid hemoglobins (Crustacea, Cladocera). J Mol Evol 49:769–779

Hyne RV, Maher WA (2003) Invertebrate biomarkers: links to toxicosis that predict population decline. Ecotoxicol Environ Safety 54:366–374

Kimura S, Tokishita S, Ohta T, Kobayashi M (1999) Heterogeneity and differential expression under hypoxia of two-domain hemoglobin chains in the water flea, *Daphnia magna*. J Biol Chem 274:10,649–10,653

Kobayashi M, Hoshi T (1982) Relationship between the haemoglobin concentration of *Daphnia magna* and the ambient oxygen concentration. Comp Biochem Physiol 72A:247–249

Lankester ER (1871) Ü ber das Vorkommen von Haemoglobin in den Muskeln der Mollusken und die Verbreitung desselben in den lebendigen Organismen. Pflügers Arch Ges Physiol 4:315–320

Lee SM, Lee SB, Park CH, Choi J (2006) Expression of heat shock protein and hemoglobin genes in *Chironomus tentans* (Diptera, Chironomide) larvae exposed to various environmental pollutants: a potential biomarker of freshwater monitoring. Chemosphere 65:1074–1081

OECD (1984) Guidelines for testing of chemicals, Vol. 1, Section 2: Effects on biotic systems, *Daphnia magna* acute immobilization Test 202. OECD, Paris

Osmulski PA, Leyko W (1986) Structure, function and physiological role of *Chironomus* haemoglobin. Comp Biochem Physiol B: Biochem 85:701–722

Pane EF, Smith C, McGeer JC, Wood CM (2003) Mechanisms of acute and chronic waterborne nickel toxicity in the freshwater cladoceran, *Daphnia magna*. Environ Sci Technol 37:4382–4389

Paul RJ, Zeis B, Lamkemeyer T, Seidl M, Pirow R (2004) Control of oxygen transport in the microcrustacean *Daphnia*: regulation of haemoglobin expression as central mechanism of adaptation to different oxygen and temperature conditions. Acta Physiol Scand 182:259–275

Prosser CL, Brown FA (1961) Comparative animal physiology. Saunders, Philadelphia

Rider CV, Gorr TA, Olmstead AW, Wasilak BA, LeBlanc GA (2005) Stress signaling: coregulation of hemoglobin and male sex determination through a terpenoid signaling pathway in a crustacean. J Exp Biol 208:15–23

Rider CV, LeBlanc GA (2006) Atrazine stimulates hemoglobin accumulation in *Daphnia magna*: Is it hormonal or hypoxic? Toxicol Sci 93:443–449

Rotchell JM, Ostrander GK (2003) Molecular markers of endocrine disruption in aquatic organisms. J Toxicol Environ Health B 6:453–496

Terwilliger RC (1980) Structure of invertebrate hemoglobins. Am Zool 20:53–67

Terwilliger NB (1998) Functional adaptations of oxygen-transport proteins. J Exp Biol 201:1085–1098

Tokishita S, Shiga Y, Kimura S (1997) Cloning and analysis of a cDNA encoding a two-domain hemoglobin chain from the water flea *Daphnia magna*. Gene 189:73–78

Vinogradov SN (1985) The structure of invertebrate extracellular hemoglobins (erythrocruorins and chlorocruorins). Comp Biochem Physiol 82B:1–15

Weber RE (1980) Functions of invertebrate hemoglobins with special reference to adaptations to environmental hypoxia. Am Zool 20:79–101

Weber RE, Vinogradov SN (2001) Nonvertebrate hemoglobins: functions and molecular adaptations. Physiol Rev 81:569–628

Yoshimi T, Minowa K, Karouna-Renier NK, Watanabe C, Sugay AY, Miura T (2002) Activation of a stress-induced gene by insecticides in the midge *Chironomus yoshimatsui*. J Biochem Mol Toxicol 16:10–17

Zeis B, Becher B, Goldmann T, et al. (2003b) Differential haemoglobin gene expression in the crustacean *Daphnia magna* exposed to different oxygen partial pressures. Biol Chem 384:1133–1145

Zeis B, Becher B, Lamkemeyer T, Rolf S, Pirow R, Paul RJ (2003a) The process of hypoxic induction of *Daphnia magna* hemoglobin: subunit composition and functional properties. Comp Biochem Physiol B: Biochem Mol Biol 143:243–252