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MULTILEVEL EVALUATION OF NONYLPHENOL TOXICITY IN FOURTH-INSTAR LARVAE OF *CHIRONOMUS RIPARIUS* (DIPTERA, CHIRONOMIDAE)

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Abstract—The multilevel biomarker approach, wherein different biological responses ranging from molecular to physiological are evaluated, is essential to determine the general health status of an organism in pollutant biomonitoring programs. Furthermore, it permits extrapolation of the relationship between responses at different levels of biological organization. The aim of this study was to develop, under laboratory conditions, a multilevel biomarker approach for evaluating the toxicological response of nonylphenol (NP) in *Chironoms riparius*. To investigate the effect of NP on *C. riparius*, an acute toxicity test was performed measuring 24-h median lethal concentration. Responses on molecular, biochemical, and physiological levels were subsequently investigated on sublethal exposure. To assess molecular-level effects, we investigated DNA damage and the expression of heat shock protein 70 (HSP70) gene, whereas biochemical-level responses were determined by investigation of various enzymes activities. Growth and development were investigated as physiological-level responses. The biomarkers found to be most sensitive to NP treatment were HSP70 gene expression and DNA strand break. After having been studied with lower concentration levels with longer exposure period, these biomarkers could be considered early warning signs of exposure to low concentrations of chemical exposure. Statistically significant correlations were observed between DNA damage and the development descriptor; however, a mechanistic study appears to be necessary to establish causal relationships. This approach could be applied in environmental biomonitoring programs, and the data obtained from this study should constitute an important contribution to knowledge of the toxicology of NP in *C. riparius*, about which little data is available.

Keywords—*Chironomus riparius* Nonylphenol Multilevel biomarkers Biomonitoring

INTRODUCTION

Nonylphenol (NP) has recently drawn attention because of its intrinsic estrogenic potential. Nonylphenol is used in the production of NP ethoxylates, and the main use of NP in the plastic industry is as a monomer in the production of phenol/formaldehyde resins. Other uses include an intermediate in the production of tri-(4-nonylphenol) phosphate and a catalyst in the curing of epoxy resins [1] (http://ecb.jrc.it/). The exposure levels of NP were reported at around 0.01 to 0.5 μg/L in surface water [1]. The major degradative product of NP, 4-nonylphenol, has been demonstrated to induce vitellogenin in male trout [2]. It was also found to cause developmental retardation in daphnids [3] and mouthpart deformity in chironomid larvae [4]. Despite the importance of NP toxicity in aquatic ecosystems, few studies have been conducted on the effect of this compound on the aquatic ecosystem components.

The aquatic larvae of nonbiting midges (Chironomidae, Diptera) are globally distributed, and they are the most abundant group of insects found in freshwater ecosystems. They hold an important position in the aquatic food chain and are a major food source for fish and other vertebrates and invertebrates [5]. Thus, they are used extensively to assess the acute and sublethal toxicity of contaminated sediments and water [6–11].

The current *Chironomus riparius* literature provides a degree of insight into the relative sensitivity of several endpoints; however, few experiments have provided a direct comparison of multiple endpoints under a common set of experimental conditions. Taking into account the frequent occurrence of NP in surface water and the ecological importance of *Chironomus* larvae in freshwater, research on the effects of NP on *C. riparius* could provide valuable information for biomonitoring or risk assessment of this compound in aquatic ecosystems. The aim of this study was to develop, under laboratory conditions, a multilevel biomarker approach for evaluating the toxicological response of NP in *C. riparius*. This multilevel approach, wherein different biological responses ranging from molecular to physiological are evaluated, is essential to determine the general health status of the organism. Moreover, it permits extrapolation of the relationship between responses at different levels of biological organization.

To investigate the effects of NP on *C. riparius*, an acute toxicity test was performed measuring 24-h median lethal concentration (LC50). Responses on molecular, biochemical, and physiological levels were subsequently investigated on sublethal exposure. To assess molecular-level effects, we investigated DNA damage and expression of the heat shock protein 70 (HSP70) gene while various enzymes activities were studied so as to determine biochemical-level responses. Antioxidant enzymes, namely, catalase (CAT), peroxidase (Px), and glutathione peroxidase (GPx), were studied as oxidative stress markers. Glutathione-*S*-transferase (GST) and acetylcholinesterase (AChE) were also studied as detoxification and neurotoxicity indicators, respectively. As physiological-level responses, growth and development were studied. Larval body dry 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 to identify any potential

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Table 1. Estimation of 10%, median, and 90% 24-h lethal concentration (LC10, LC50, and LC90, respectively) of nonylphenol in fourth-instar larvae of *Chironomus riparius*

	24-h LC (mg/L)	95% Confidence interval
LC10	0.484	0.229–0.592
LC50	0.688	0.538–0.802
LC90	0.978	0.832–1.646

difference in susceptibility between the male and female toward this compound.

MATERIALS AND METHODS

Organisms

Using an original strain provided by the Toxicology Research Center of the Korea Research Institute of Chemical Technology (Daejeon, Korea), we obtained *C. riparius* 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.

Exposure conditions

Using groups of fourth-instar larvae collected in the rearing aquaria, we assessed the effects of NP exposure. All larvae used in the experiment originated from the same egg mass and were collected during the same period (30 d) after egg hatching to obtain age-synchronized population. At the beginning of the experiment, 1 ml of an acetonic solution of NP was added to experimental tanks of 1 L. Our preliminary test indicates that acetone did not provoke any significant effects for all type of experiments (data not shown). Ten (acute toxicity) to 50 larvae (sublethal toxicity) 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 experiments. Three replicates were used for all the experiments.

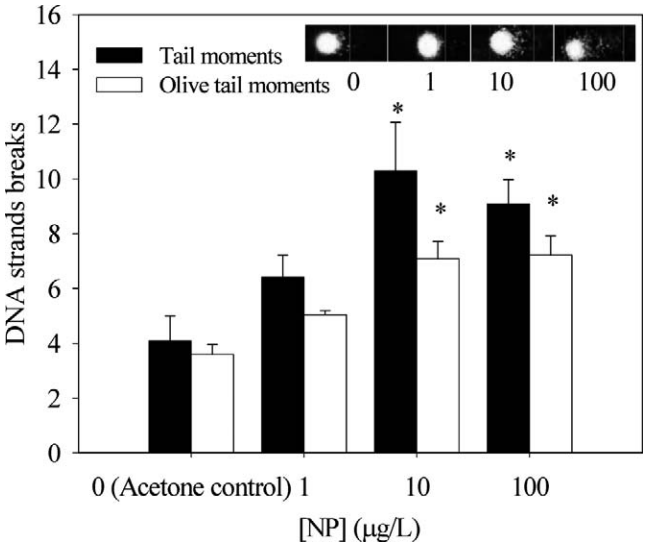
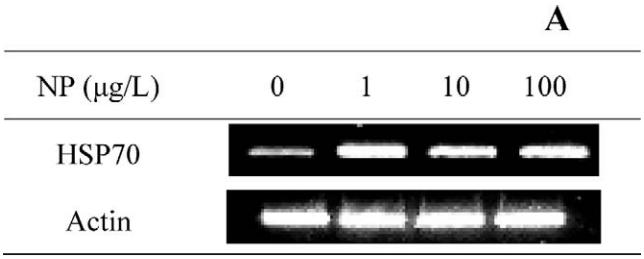


Fig. 1. DNA damage measured in the fourth-instar larvae of *Chironomus riparius* exposed to sublethal concentrations of nonylphenol (NP) for 24 h with the use of single-cell gel electrophoresis ($n = 3$, mean ± standard error of the mean, * $p < 0.05$).



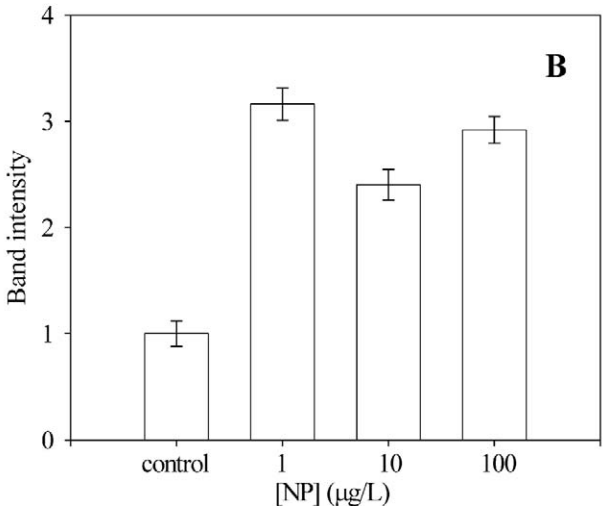


Fig. 2. Expression of heat shock protein 70 genes in the fourth-instar larvae of *Chironomus riparius* exposed to sublethal concentrations of nonylphenol (NP) for 24 h (**A**). Data are expressed in densitometric values normalized by actin mRNA (**B**) ($n = 3$, mean ± standard error of the mean, * $p < 0.05$).

Acute toxicity test

Groups of 10 larvae were exposed to four concentrations of NP, whereas other groups were kept as a control. Acute toxicity was determined after 24 h of exposure, with death of individuals as an endpoint. Log-probit transformation of the data was used to estimate 24-h LC10, LC50, and LC90 values and the corresponding 95% confidence intervals.

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 containing 20 mM ethylenediaminetetraacetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by pressing through gauze. A cell suspension was precipitated by vortexing and then was mixed immediately with 100 μl of 1% low–melting point (LMP) agarose for use in the Comet assay. An alkaline Comet assay was performed as described by Singh et al. [12]. 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 M NaCl, 10% DMSO, 10% Triton X-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 electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides

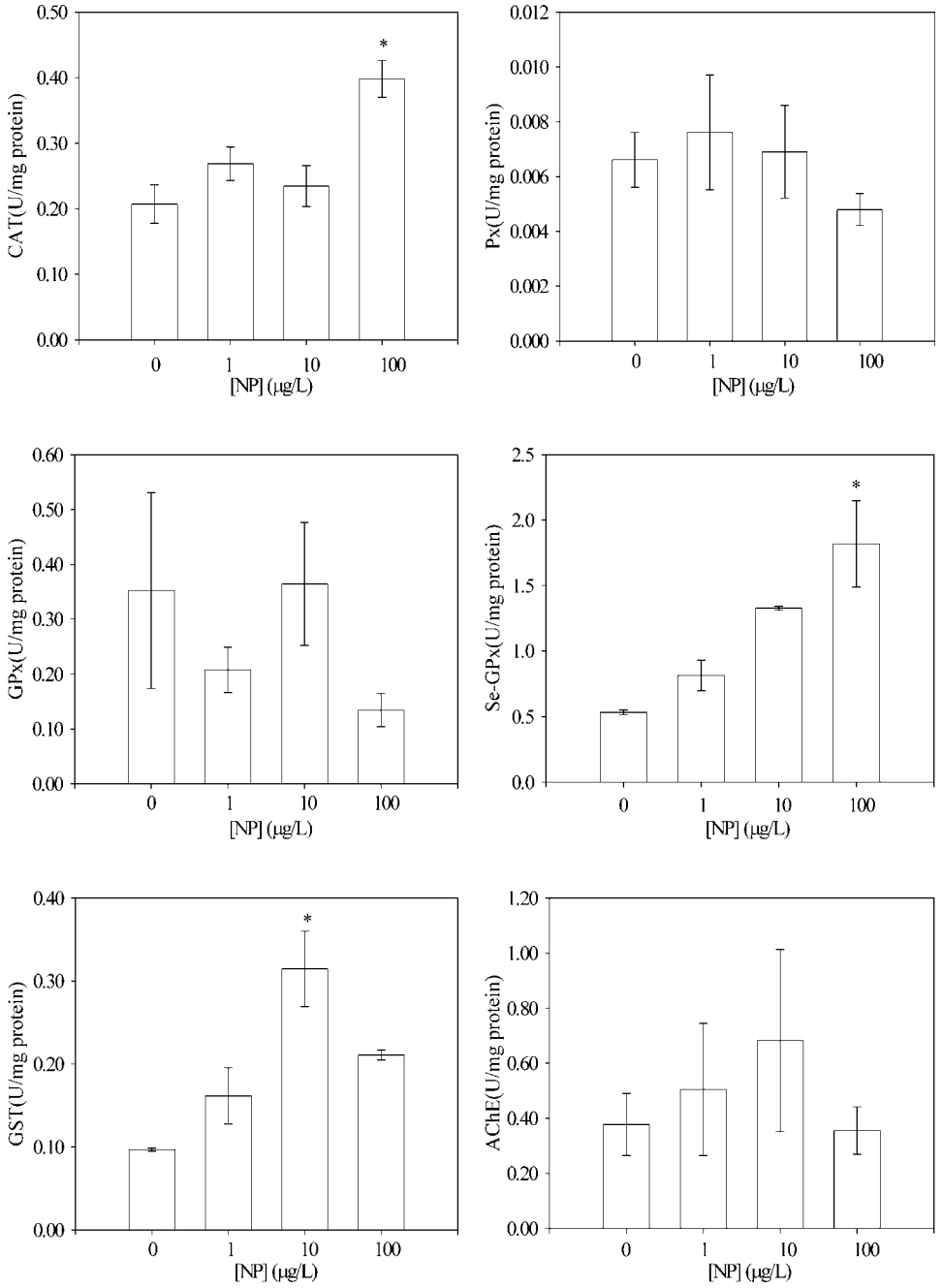


Fig. 3. Catalase (CAT), peroxidase (Px), glutathione peroxidase (GPx), selenium-dependent glutathione peroxidase (Se-GPx), glutathione-*S*-transferase (GST), and acetylcholine esterase (AChE) activities measured in the fourth-instar larvae of *Chironomus riparius* exposed to sublethal concentrations of nonylphenol [NP] for 24 h ($n = 3$, mean ± standard error of the mean, * $p < 0.05$).

were stored in a dry place until image analysis. Before analysis, the slides were stained with 50 μl of ethidium bromide (5 μg/ml). They were then analyzed at ×400 magnification with a fluorescence microscope (Nikon, Kanagawa, Japan). Approximately 25 cells per slide (four slides per 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) with an image analysis computerized method (Komet 5.5, Kinetic Imaging, Nottingham, UK).

HSP70 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™, specifically, 5′CATGTGAACGAGCCAAGAGA3′ and 5′TCGAGTTGATCCACCAACAA3′ for HSP70 (AY163157) and 5′GATGAAGATCCTCACCGAACG3′ and 5′CCTTACGGATATCAACGTCGC3′ for actin (AB070370). Finally, actin mRNA was used for normalization of the HSP70 levels. The tests were replicated three times, and the relative densities of each band were determined with the aid of an image ana-

Table 2. Body dry weight and water content measured in the fourth-instar larvae of *Chironomus riparius* exposed to nonylphenol (NP) for 24 h (n = 3, mean ± standard error of the mean)

NP (μg/L)	Body dry wt (mg/larvae)	Water content (mg/larvae)
0 (control)	0.601 ± 0.347	3.609 ± 2.084
1	0.733 ± 0.423	4.195 ± 2.422
10	0.716 ± 0.414	3.908 ± 2.256
100	0.706 ± 0.407	4.096 ± 2.236

lyzer: 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).

Enzyme activities

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, Saint Quentin Fallavier, France) with 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 (postmitochondrial fraction) was used to measure the enzyme activities. The rate of H_2O_2 disappearance (measured at 240 nm) was used to quantify catalase activity [13]. Total Px activity was measured with a guaiacol test [14]. For the determination of glutathione peroxidase (GSH-Px) activity, we employed the method described by Paglia and Valentine [15], whereas GST activity was assessed spectrophotometrically through a measurement of glutathione-1-chloro-2,4-dinitro benzene conjugate production [16]. Acetylcholinesterase activity was measured by the method of Elmann et al. [17]. Enzymatic activities were calculated relative to the measured protein content of the extracts according to the Bradford method [18].

Water content and body dry weight measurement

Water content and body dry weight 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 respective dry and fresh weights. Weighing was performed to the nearest 0.1 mg.

Adult emergence rate

For the measurement of adult emergence rate, 50 fourth-stage larvae were introduced at the beginning of the experiment. Emerging adults were retained by wood cages covered with steel wire mesh until emergence was completed in the control and experimental aquaria. As endpoints of the toxicity tests, the numbers 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. Every 2 d, 50 mg of Tetramin fish food flakes was supplied to each aquarium. Test solutions were not renewed. All data were recorded at daily intervals.

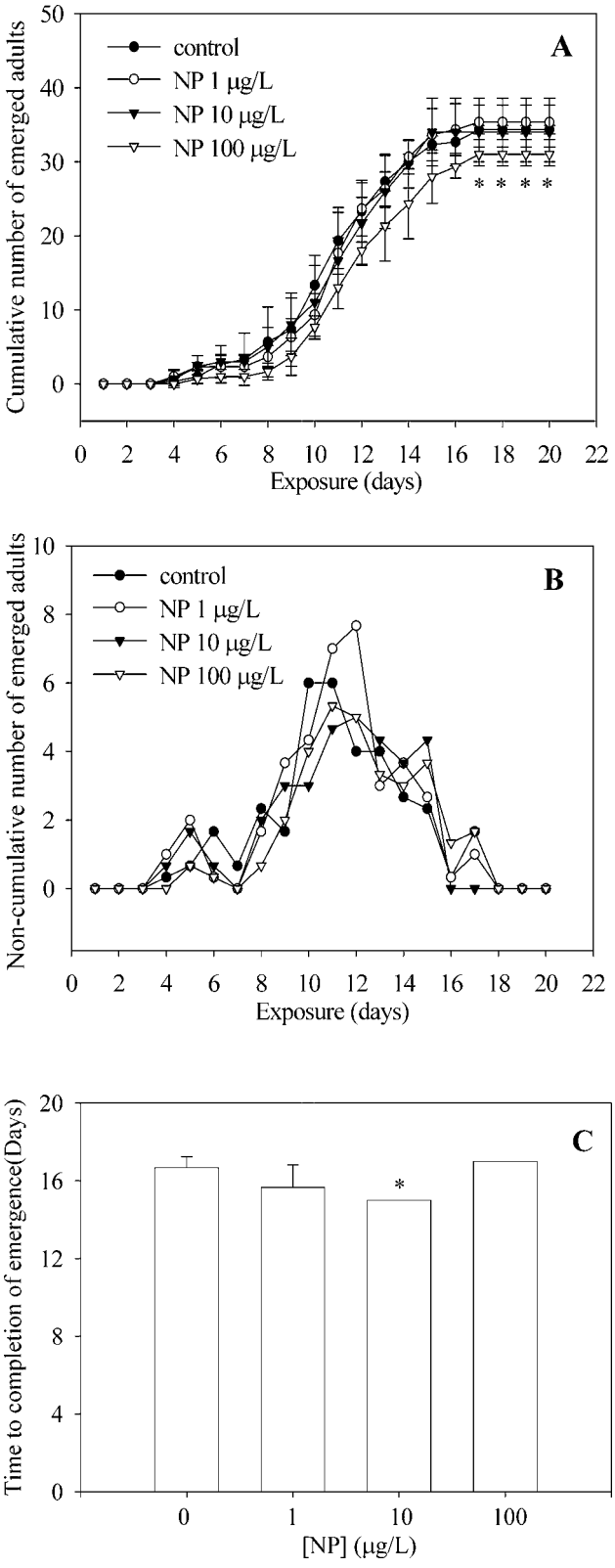


Fig. 4. Kinetics of cumulative (**A**) and noncumulative adult emergence (**B**) and time to completion of emergence (**C**) of *Chironomus riparius* exposed to sublethal concentrations of nonylphenol (NP) (n = 3, mean ± standard error of the mean, * $p < 0.05$).

Table 3. Failure in pupation and adult emergence[a] measured in nonylphenol (NP)-exposed *Chironomus riparius* ($n = 3$, mean ± standard error of the mean)

NP (μg/L)	Pupation failure (%)	Emergence failure (%)	
		From pupa	From larva
0 (control)	26 ± 1.15	5.33 ± 1.76	31.4 ± 0.67
1	24.67 ± 0.67	11.33 ± 3.33	36 ± 4.0
10	20.67 ± 4.81	11.33 ± 0.67	32 ± 5.29
100	20 ± 2.0	18 ± 2.31*	38 ± 1.15

[a] Results are expressed as percentage of the total number of larvae introduced at the beginning of the experiment.
* Value is significantly different from the control ($p < 0.05$).

Chemicals

Nonylphenol and biochemicals were purchased from Sigma (Sigma).

Data analysis

The data passed the normality test and equal variance test. Statistical differences between the control and treated larvae were examined by analysis of variation with Dunnett's multiple comparison test. A parametric Pearson test was used to study correlations between parameters. All statistical tests were performed with SPSS® 12.0KO (SPSS).

RESULTS

Twenty-four–hour LC50 of NP in fourth-instar larvae of *C. riparius* was estimated (Table 1). Twenty-four–hour LC10, LC50, and LC90 were 0.484, 0.688, and 0.978 mg/L, respectively, indicating that NP has considerable potential for acute toxicity to *C. riparius* larvae. On the basis of the results of the acute toxicity test, three concentrations corresponding to a thousandth, a hundredth, and a tenth of the 24-h LC90 were selected for sublethal exposure conditions (i.e., 1, 10, and 100 μg/L).

The DNA damage, particularly DNA strand breaks, was measured by a single-cell gel electrophoresis (Comet) assay to evaluate whether NP induces any genetic toxicity in *C. riparius* (Fig. 1). Both tail moment and Olive tail moment increased significantly by 10 and 100 μg/L of NP exposure, respectively.

The expression of HSP70 gene, a well-known general stress indicator, was investigated in NP-exposed fourth-instar larvae of *C. riparius* (Fig. 2). The expression of HSP70 gene increased in NP-treated larvae.

Similar to the biochemical parameters, enzyme activities were measured in the fourth-instar larvae of *C. riparius* ex-

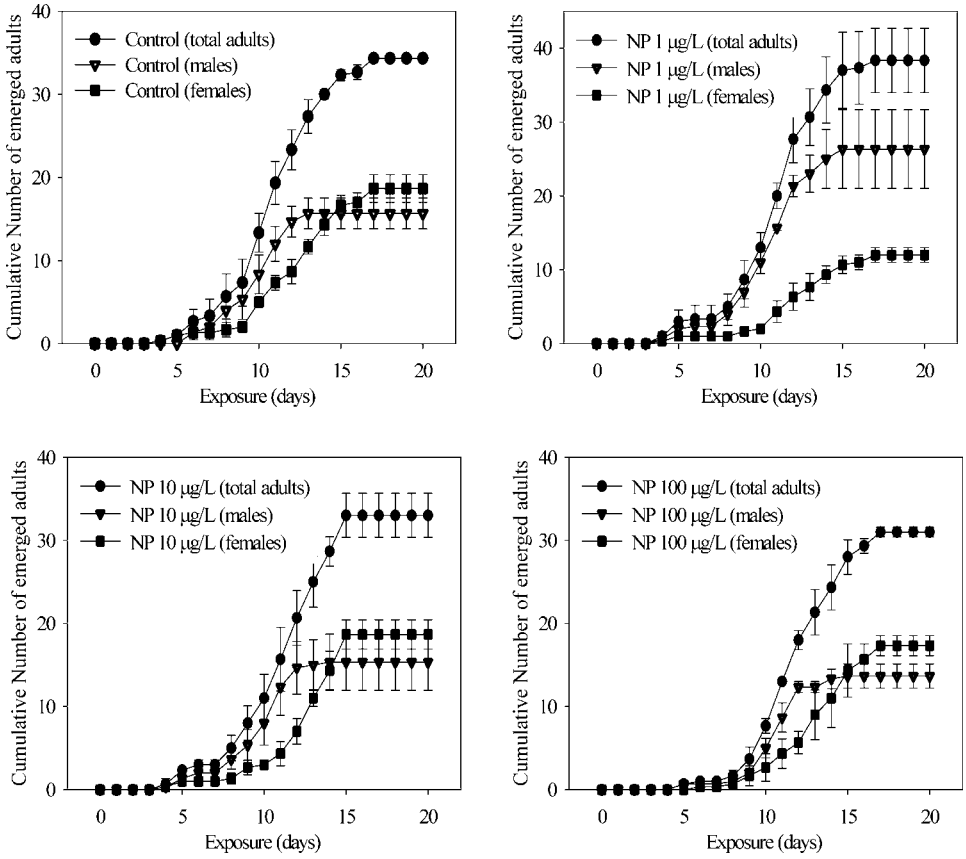


Fig. 5. Cumulative emergence measured in nonylphenol (NP)-exposed *Chironomus riparius* ($n = 3$, mean ± standard error of the mean). Results are expressed as percentage of the total number of larvae introduced at the beginning of the experiment.

posed to NP for 24 h (Fig. 3). Catalase, selenium-dependent glutathione peroxidase (Se-GPx), and GST activities increased by NP exposure, whereas no significant change was observed in Px, total GPx, and AChE activities.

To assess physiological markers, we measured the body dry weight and water content of *C. riparius* larvae 48 h after treatment (Table 2). As a growth indicator, body dry weight and water content of the larvae did not change by NP exposure.

To assess development parameters, effects of NP on the kinetics of emergence in *C. riparius* were studied via cumulative and noncumulative methods (Fig. 4A and B). Total adult emergence rate decreased by 100 μg/L of NP exposure. The lowest concentration of NP (1 μg/L) showed an approximately 3% higher emergence rate than the control. However, the emergence pick appeared 2 d later than control. Time to completion of emergence was 17, 16, 15, and 17 d after the beginning of the experiment for the control and 1, 10, and 100 μg/L of NP exposure, respectively (Fig. 4C).

Nonylphenol-induced failure rate in pupation and emergence was studied in *C. riparius* (Table 3). Nonylphenol exposure appeared to have no significant effect on pupation, whereas it induced emergence failure at high concentration (100 μg/L of NP). The degree of failure was more significant when counted from the pupa to adult than when counted from larva to adult.

Among emerged adults, males and females were identified to verify whether susceptibility to NP exposure differs (Fig. 5). No significant difference was found from group to group, except for 1 μg/L of NP exposure, in which a high male to female ratio was observed relative to the control.

To identify any correlations between molecular-/biochemical-level and physiological-level effects, Pearson correlation tests were performed on the 16 parameters studied, including nominal concentration of NP (Table 4). Statistically significant correlations were observed between nominal concentration of NP and CAT/Px activities, between DNA damage and GST activity, and between DNA damage/GPx and development descriptors.

DISCUSSION

We studied the possible use of *C. riparius* as a sentinel organism in a freshwater ecosystem, as well as the utility of molecular/biochemical parameters as biomarkers in this species, to demonstrate the effectiveness of the multibiomarker approach in the assessment of NP exposure in freshwater ecosystem quality. Twenty-four–hour LC50 of NP on *C. riparius* was determined to be around 0.7 mg/L, which suggests this compound has relatively high acute toxicity to this species. Moreover, sublethal effects, particularly molecular effects, which were produced at 100- to 1,000-fold lower concentrations (i.e., DNA strand breaks and HSP70 expression) could have considerable potential as biomarkers of chemical stress in *C. riparius*.

Although pollutants can influence the genetic constitution of populations by causing direct damage to DNA molecules within the individual cell nuclei, the ecological relevance of changes in single cells within some tissues of some individual organisms is extremely difficult to assess. Nonetheless, sensitive detection of DNA damage in wildlife species is necessary because pollutant-induced DNA damage could influence the genetic constitution of populations [19]. The Comet assay is considered a sensitive and rapid technique for the detection of DNA strand break, which is ideally suited as a nonspecific biomarker of genotoxicity in aquatic species [20]. As such, it has seen widespread use in environmental monitoring programs [20–22]. Nonylphenol could exert a genotoxic effect on *C. riparius*, given that DNA strand breaks increased in *C. riparius* exposed to 10 and 100 μg/L of this compound.

With respect to molecular-level responses to chemical stressors, HSPs are the most frequently studied aspect of aquatic invertebrates and are used as biomarkers because they are induced not only by heat shock but also by pollutants [23–25]. Increase of HSP70 gene expression by NP exposure was expected because HSP70 is well known to be inducible by various environmental stressors. The early response of HSP70 to low concentrations of NP could be considered a homeostasis-maintaining process rather than an indicator of permanent adverse effects of this compound. Its response at high NP concentrations likely reflects an adverse effect of exposure because perturbations of physiological parameters were observed at these concentrations. Homeostatic responses of HSP70 appear to have little effect at higher levels of biological organization. Therefore, HSP70 perhaps should be considered as an early warning biomarker of pollution, rather than as a specific biomarker of adverse effect.

Environmental exposure can lead to various pathologies. In particular, on the basis of vertebrate studies, compelling evidence has emerged for oxidative stress–related toxicity from numerous environmental contaminants. Previous studies have shown that enzymatic radical scavengers, including superoxide dismutase (SOD), are abundant in *C. riparius* larvae [26] and that early response of antioxidant enzymes, including SOD, could be considered biomarkers in *C. riparius* [7]. Various biochemical parameters measured in *C. riparius* larvae, such as AChE, Cu, Zn-SOD, GST, the electron transport system, and energy-yielding substrates, have already demonstrated high sensitivity toward environmental pollutants [7,27,28]. However, the response of antioxidant enzymes does not appear to be sensitive to NP exposure. Oxidative stress might not be directly or indirectly involved in NP toxicity in *Chironomus*. Increased GST activity at the highest concentration of NP exposure might represent possible involvement of the GSH conjugation pathway in detoxification of this compound. However, it appears that the GST activity response is not sensitive to NP exposure in *C. riparius*. Stegeman et al. [29] noted conflicting results in studies on induction of GST in various aquatic invertebrates, and the sensitivity of this biomarker in *C. riparius* has been questioned elsewhere [11,30,31]. A contradictory result was also reported from our previous study, in which *Chironomus* GST showed high sensitivity toward chromium and fenitrothion exposure [7]. Acetylcholinesterase activity did not change by NP exposure, which suggests that neurotoxicity might not be important in NP toxicity of *C. riparius* larvae.

Emergence seems to be a more sensitive parameter than pupation with respect to NP exposure because NP did not affect pupation and emergence failure increased with NP exposure. Decrease of emergence rate, an indicator of animal development, at the highest concentration of NP suggests that alteration of this parameter might be considered a consequence of a serious progression of the toxic effect. Whereas much more variability in emergence and less success of female emergence compared with higher NP levels and the control was observed in the experiment with low level of exposure (1 μg/L; Fig. 5). Our data are not sufficient to provide a clear explanation for this phenomenon. If more concentration levels with longer

Table 4. Coefficients of correlation[a] between molecular-, biochemical-, physiological-, and ecophysiological-level parameters measured in nonylphenol (NP)-exposed *Chironomus riparius*

	Molecular		Biochemical				
	Tail moment (Comet assay)	Heat shock protein 70	Catalase	Peroxidase	Glutathione peroxidase	Selenium-dependent glutathione peroxidase	Glutathione-*S*-transferase
Nonylphenol	0.470 (0.530)	−0.113 (0.887)	0.951 (0.049)*	−0.979 (0.021)*	−0.741 (0.259)	0.864 (0.136)	0.198 (0.802)
Tail moment (Comet assay)		0.786 (0.214)	0.457 (0.543)	−0.459 (0.541)	−0.178 (0.822)	0.850 (0.150)	0.954 (0.046)*
Heat shock protein 70			−0.186 (0.814)	0.054 (0.946)	0.453 (0.547)	0.380 (0.620)	0.932 (0.068)
Catalase				−0.869 (0.131)	−0.901 (0.099)	0.829 (0.171)	0.171 (0.829)
Peroxidase					0.595 (0.405)	−0.845 (0.155)	−0.212 (0.788)
Glutathione peroxidase						−0.545 (0.455)	0.102 (0.898)
Selenium-dependent glutathione peroxidase							0.662 (0.338)
Glutathione-*S*-transferase							
Acetylcholine esterase							
Water content							
Body dry wt							
Pupation failure							
Emergence failure							
Time to the completion of emergence							
Male adult emergence							

[a] Pearson correlation test; p value in parentheses.
* Statistically significant ($p < 0.05$).
** Statistically significant ($p < 0.01$).

exposure periods had been tested, this could probably be evaluated and explained to a greater extent.

It is widely accepted that sex-specific effects, effects on sexual differentiation, alteration in sex ratios, or a combination of effects induced by a chemical compound hint at the potential of the substance to disturb hormonal processes [32]. Bogart [33] suggested that the sexual differentiation processes in animals, including arthropods, are based on the ration of male and female sex hormones. Because vertebrate sex steroids are either absent in insects or have not been found to exert any specific effects [34], a model is suggested in which ecdysone acts both as a direct precursor of the active molting hormone 20-hydroxyecdysone and as a male sex steroid [35]. Thus, possessing estrogenic potential in vertebrates, NP might not directly alter pathways related to invertebrate sex hormones.

Because chironomids are considered good biological models for the study of the chronic effects of environmental pollutants, the response of several biomarkers to toxicants has already been evaluated [7,36,37]. Results of this study suggest that population-level changes are less sensitive than molecular response. Statistical tests revealed significant correlations between the nominal concentrations of NP and CAT/Px activities, between DNA damage and GST activity, and between DNA damage/GPx and development descriptors. However, correlation study alone cannot provide any causal relationships between these parameters. Direct experimental demonstrations of the wider relationships between biochemical-level effects and their subsequent consequences at higher levels of biological organization are needed to establish causal relationships. Characterization of the causal relationships between biomarker responses and effects at higher biological levels would help to define the sublethal hazards of chemicals in this animal.

In freshwater ecosystems, chemical pollution is frequently caused by a complex mixture of pollutants. This considerably increases the difficulty in predicting pollutant effects and emphasizes the need for studies on multiple biological endpoints to identify pertinent biomarkers. Simultaneous measurement of various biological parameters gives the opportunity to obtain data at different levels of biological organization, and it might help to fully understand the effects of a toxicant on organisms. In addition, determination of population-level parameters improves the interpretation of data collected at lower biological levels [38].

A series of descriptors at different levels of biological organization (molecular, biochemical, and physiological) were investigated in NP-exposed *C. riparius* larvae. This approach could be used in environmental biomonitoring programs. The data obtained from this study could be an important contribution to knowledge of the toxicology of NP in *C. riparius*, about which little data are available. The biomarkers found to be most sensitive to the treatment were HSP70 gene expression and DNA strand break, and they can be used as an early warning sign of exposure to low concentrations of chemical exposure.

CONCLUSIONS

This study, designed as a short-term experiment under controlled laboratory conditions with a nominal concentration of NP, demonstrated that *C. riparius* larvae are sensitive to this chemical and are of potential use as a biomonitoring species. The results of this laboratory experiment to evaluate biomarker responses to NP at different levels of biological organization in *C. riparius* will enable a simple suite of biomarker tests to be defined for risk assessment of NP exposure. However, further research is required to clarify dose–response relationships in *C. riparius* to NP over an extended period. Naturally, a study of the effects of NP in a natural environment should also follow this experiment. Moreover, quantification of both NP in water and in animal tissue will contribute to an understanding of the mechanistic aspects of the deleterious effects of NP on *Chironomus*.

Table 4. Extended.

Biochemical	Physiological		Ecophysiological				
Acetylcholine esterase	Water content	Body dry wt	Pupation failure	Emergence failure	Time to the completion of emergence	Male adult emergence	Female adult emergence
−0.443 (0.557)	0.232 (0.768)	0.380 (0.620)	−0.708 (0.292)	0.744 (0.256)	0.604 (0.396)	−0.740 (0.260)	−0.080 (0.920)
0.118 (0.882)	0.712 (0.288)	0.470 (0.530)	−0.950 (0.050)*	0.284 (0.716)	−0.404 (0.596)	0.211 (0.789)	−0.127 (0.873)
0.167 (0.833)	0.424 (0.576)	0.023 (0.977)	−0.613 (0.387)	−0.346 (0.654)	−0.762 (0.238)	0.621 (0.379)	0.198 (0.802)
−0.166 (0.834)	0.442 (0.558)	0.631 (0.369)	−0.652 (0.348)	0.912 (0.088)	0.515 (0.485)	−0.627 (0.373)	−0.383 (0.617)
0.601 (0.399)	−0.086 (0.914)	−0.196 (0.804)	0.712 (0.288)	−0.594 (0.406)	−0.626 (0.374)	0.772 (0.228)	−0.124 (0.876)
−0.124 (0.876)	−0.485 (0.515)	−0.773 (0.227)	0.328 (0.672)	−0.993 (0.007)**	−0.493 (0.507)	0.526 (0.474)	0.677 (0.323)
0.197 (0.803)	0.546 (0.454)	0.497 (0.503)	−0.964 (0.036)*	0.608 (0.392)	0.129 (0.871)	−0.320 (0.680)	−0.123 (0.877)
	0.648 (0.352)	0.318 (0.682)	−0.832 (0.168)	0.013 (0.987)	−0.626 (0.374)	0.450 (0.550)	−0.023 (0.977)
	0.715 (0.285)	0.659 (0.341)	0.133 (0.867)	0.184 (0.816)	−0.691 (0.309)	0.748 (0.252)	−0.812 (0.188)
		0.912 (0.088)	−0.576 (0.424)	0.581 (0.419)	−0.507 (0.493)	0.418 (0.582)	−0.775 (0.225)
			−0.418 (0.582)	0.831 (0.169)	−0.171 (0.829)	0.124 (0.876)	−0.921 (0.079)
				−0.412 (0.588)	0.102 (0.898)	0.103 (0.897)	0.033 (0.967)
					0.398 (0.602)	−0.448 (0.552)	−0.710 (0.290)
						−0.978 (0.022)*	0.195 (0.805)
							−0.230 (0.770)

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