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# Genotoxic Effects of Nonylphenol and Bisphenol A Exposure in Aquatic Biomonitoring Species: Freshwater Crustacean, *Daphnia magna*, and Aquatic Midge, *Chironomus riparius*

**Sun-Young Park · Jinhee Choi**

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**Abstract** The geno-, and eco-toxicity of nonlyphenol (NP) and bisphenol A (BPA) were investigated in *Daphnia magna*, and *Chironomus riparius*. BPA may exert a genotoxicity on both species, whereas NP-induced DNA damage occurred only in *C. riparius*. In NP-exposed *D. magna*, increased mortality, without effect on DNA integrity was observed, an example of a false-negative result from the biomarkers perspective. False-positive results from the genotoxicity were observed in BPA-exposed *D. magna* and in NP-exposed *C. riparius*. Considering the importance of genotoxic biomarkers in eco-toxicity monitoring, DNA damage in these species could provide useful information.

**Keywords** *Daphnia magna* · *Chironomus riparius* · Nonlyphenol · Bisphenol A · Genetic toxicity · Environmental risk assessment

Among the available genotoxicity indicator tests, the Comet assay has recently attracted much attention. The Comet assay, also called the single-cell gel electrophoresis (SCGE) assay, primarily measures DNA strand breakage in single cells. DNA strand breaks are potential pre-mutagenic lesions and are sensitive markers of genotoxic damage. The Comet assay has been shown to respond quickly and accurately, and its findings are easy to measure. Thus, since the protocol was published by Singh et al. (1988), it has been increasingly used in different fields of study: clinical applications, human monitoring, radiation biology, and genetic toxicology. Application of Comet assay, using many different species, has been conducted in ecotoxicology (Clement et al. 2004; Cotelle and Ferard 1999; Palmqvist et al. 2003). Evaluation of genetic toxicity using Comet assay was performed on numerous wildlife organisms, including plants (Koppen and Verschaeve 1996; Navarrete et al. 1997), worms (Rajaguru et al. 2003), mollusks (Clement et al. 2004), fish (Mitchelmore and Chipman 1998; Schnurstein and Braunbeck 2001), amphibians (Ralph and Petras 1997) and mammalians (Tice et al. 2000). Only few genotoxic studies have been conducted on the aquatic invertebrates, such as, daphnid or chironomid (den Besten and Tuk 2000).

In this study, chemical-induced DNA damages were investigated by measuring DNA strand breaks in two biomonitoring species, the freshwater crustacean, *Daphnia magna*, and the larva of the aquatic midge, *Chironomus riparius*, in order to identify genotoxic biomarkers for risk assessment. They hold an important position in the aquatic food chain and are sensitive to many pollutants, easy to culture and have a short life cycle, and thus they are considered as suitable species for aquatic biomonitoring (Giesy et al. 1988; Cranston 1995; Choi et al. 2000; Atienzar et al. 2001). Taken into account of the importance of *D. magna* and *C. riparius* in the aquatic ecosystem, information concerning genotoxicity on these species can be valuable for freshwater monitoring and environmental risk assessment. As chemical stressors, two most representative endocrine disrupting chemicals (EDCs), nonlyphenol (NP), which is used in the polymer industry (EU 2002) and bisphenol A (BPA), which is an intermediary in the production of polycarbonate and epoxyresins (EU 2003), were selected. Despite the importance of EDC in aquatic ecosystems, few studies have been conducted on the genotoxic

S.-Y. Park · J. Choi (✉)
Faculty of Environmental Engineering, College of Urban Science, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Korea
e-mail: jinhchoi@uos.ac.kr

effect of these compounds on the aquatic ecosystem components. DNA damage was measured upon sublethal exposure condition in NP and BPA exposed *D. magna* and *C. riparius* using Comet assay and its involvement in response to oxidative stress was also investigated by measuring typical oxidative stress indicators, such as, lipid peroxidation and catalase activity. Conventional ecotoxicity tests, using growth and survival as toxic endpoints, were conducted, in order to validate ecotoxicological relevance of genotoxic biomarkers in these species as potential biomarker for environmental contamination.

## Materials and Methods

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), we obtained *D. magna* and *C. riparius* larvae from adults reared in our laboratory. *D. magna* were individually placed in glass beakers containing a culture medium, aerated M4 media (OECD 202 2004), for 2 days. Cultured daphnids were fed daily on the green alga *Chlorella* sp. at concentrations of $1 \times 10^6$–$10^9$ cells/mL; the larvae of *C. riparius*, which were fed with fish flake food (Tetramin, Tetrawerke, Melle, Germany), were reared in a 2 L glass chamber containing dechlorinated tap water and acid-washed and aerated sand. Culture of *D. magna* and *C. riparius* were maintained at 20 ± 1°C, 16 h light and 8 h dark cycle photoperiod regime.

We conducted the experiment at a constant temperature of 20 ± 1°C under light conditions of 16–8 h of light and darkness using 7-day-old *Daphnia* and the fourth instar larvae of *Chironomus*. For the chemical treatment, based on the results of the acute toxicity test (Lee and Choi 2006, 2007; Park and Choi 2007), three concentrations corresponding to 1/1,000, 1/100 and 1/10 of the 24-h L(E)C50 were selected for sublethal exposure conditions. *Daphnia magna* were exposed to 0.3, 3, and 30 μg/L for NP and BPA, whereas, *C. riparius* were exposed to 1, 10 and 100 μg/L for NP and 5, 50 and 500 μg/L for BPA. For each experiment, we added 0.1 mL of the test solution into the experimental beakers before introducing the larvae. Acetone was used as solvent. Three concentrations of each test chemical, solvent control (acetone) was prepared for each experiment. Three replicates were prepared for each concentration.

Twenty juveniles of *Daphnia* and 10 larvae of *Chironomus* were collected 24 h after treatment from the control and experimental tanks and were pooled for a Comet assay, as described previously (Park and Choi 2007). Briefly, a suspension of cells is mixed with low melting point agarose and spread onto a microscope glass slide. Following lysis of cells with detergent at high salt concentration, DNA unwinding and electrophoresis is carried out at a pH 13 above. Before analysis, the slides were stained with ethidium bromide, then analyzed at 400× magnification using a fluorescence microscope (Nikon, Kanagawa, Japan). DNA damage was expressed as the olive tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK). Twenty juveniles of *Daphnia* and 10 larvae of *Chironomus* were collected 24 h after treatment from the control and experimental tanks and pooled for enzyme activity measurements. Catalase (CAT) activity and malonyldialdehyde (MDA) measurement were conducted, as described previously (Lee et al. 2008). Survival and growth were investigated using 20 *Daphnia* and 10 larvae of *Chironomus*, as described previously (Lee et al. 2008).

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

## Results and Discussion

Genotoxicity testing in vivo is performed for hazard identification and is part of the risk assessment process. Results from in vivo DNA damage detection assay, such as, Comet assay, contribute to hazard identification and to dose-response assessment. In this study, genetic toxicity of NP and BPA was investigated in aquatic sentinel species, *D. magna* and *C. riparius* by investigating DNA strand breaks using Comet assay (Table 1). Exposure concentration-dependant increases in Olive tail moment were observed in both NP and BPA exposed *Daphnia* and *Chironomus*. For *D. magna*, statistically significant increase in Olive tail moments was observed only at 3 and 30 μg/L of BPA exposure, whereas, for *C. riparius*, Olive tail moment increased significantly at all concentrations of both chemicals tested. BPA may exert a genotoxic effect on *D. magna* and *C. riparius*, given that DNA strand breaks increased in both species exposed to this compound, whereas NP-induced DNA damage occurred only in *C. riparius*. In aquatic environment, most of genotoxic tests using Comet assays have been performed in vitro system from aquatic species, mostly using fish-driven cell lines (Cotelle and Ferard 1999; Nehls and Segner 2005). In this study, however, *D. magna* and *C. riparius* were exposed to each chemical in vivo and DNA damage was assessed in cells subsequently isolated from them. In vivo genotoxic biomarker obtained in aquatic sentinel species, as in our study, could be a powerful tool in environmental monitoring. Indeed, according to Ohe et al. (2004) and Chen and

**Table 1** DNA strand break, malonyldialdehyde (MDA) formation and catalase (CAT) activities measured in *D. magna* and *C. riparius* exposed to nonylphenol (NP) and bisphenol A (BPA) for 24 h

| Species | Chemicals | Concentration (μg/L) | DNA damage Olive tail moment | Lipid peroxidation Malonyldialdehyde (MDA) | Antioxidant enzyme Catalase (CAT) |
|---|---|---|---|---|---|
| *D. magna* | NP | 0.3 | 1.14 ± 0.18 | 5.34 ± 1.71* | 0.75 ± 0.04* |
| | | 3 | 1.19 ± 0.06 | 4.09 ± 0.17* | 0.84 ± 0.02 |
| | | 30 | 1.36 ± 0.13 | 3.13 ± 0.81* | 0.86 ± 0.07 |
| | BPA | 0.3 | 1.15 ± 0.05 | 3.76 ± 1.28 | 0.59 ± 0.01* |
| | | 3 | 1.49 ± 0.05* | 0.46 ± 0.41 | 0.56 ± 0.02* |
| | | 30 | 1.56 ± 0.11* | 0.41 ± 0.38 | 0.42 ± 0.01* |
| *C. riparius* | NP | 1 | 1.34 ± 0.15 | 0.95 ± 0.41 | 1.18 ± 0.06* |
| | | 10 | 1.62 ± 0.15* | 1.96 ± 0.85 | 0.81 ± 0.05* |
| | | 100 | 2.10 ± 0.02* | 3.55 ± 0.02* | 0.90 ± 0.02* |
| | BPA | 5 | 0.31 ± 0.07 | 0.44 ± 0.31 | 0.61 ± 0.02* |
| | | 50 | 1.65 ± 0.05* | 0.49 ± 0.26 | 1.19 ± 0.22 |
| | | 500 | 1.57 ± 0.13* | 1.32 ± 0.32 | 0.72 ± 0.03* |

Results were expressed as the mean value compared to control (solvent control = 1; number = 3; mean ± standard error of mean)

* Significantly different from the control value $p < 0.05$

White (2004), DNA damage in wildlife species measured by Comet assay could provide a sensitive and rapid genotoxic biomarker in environmental monitoring.

Aquatic organisms can provide model systems for investigation of how genotoxicants damage cellular components, how cells respond, and how repair mechanisms ameliorate this damage (Di Giulio et al. 1989; Livingstone et al. 1994). Moreover, aquatic organisms are more sensitive to exposure and toxicity compared to terrestrial organisms including mammals and in this respect they may provide experimental data for evaluation of subtle effects of genotoxicity, oxidative stress, and other adverse effects of pollutants (Lackner 1998). Involvement of DNA damage in response to oxidative stress was investigated by measuring typical oxidative stress indicators, such as, lipid peroxidation and catalase activity, in NP and BPA exposed *D. magna* and *C. riparius* (Table 1). Statistically significant increase in MDA was observed in NP-exposed *Daphnia* and *Chironomus*, whereas, CAT activity rather decreased in both chemicals exposed animals. The result suggests that oxidative stress related response may be involved in NP and BPA toxicity, however, the exact physiological meaning of increased lipid peroxidation and decreased catalase activity is difficult to explain. To fully understand the involvement of oxidative stress in NP- and BPA-toxicity, experimental evidence provided in this study was not sufficient. Broad range of oxidative stress-related parameters and their physiological meanings are needed to be investigated. Until recently few studies have addressed the production of reactive oxygen species (ROS) for in vivo experiments, either in the presence or in the absence of toxic chemicals, in aquatic organisms, because of technical difficulties of appropriate measurements. But evidence was provided by indirect measurements of free radical formation (spin trapped) in digestive gland cell mixture (mussels) and DNA strand breaks (comet assay; Livingstone et al. 1997; Mitchelmore et al. 1998). Also, despite the numerous studies (laboratory and field) on the antioxidant defenses found widely in aquatic organisms, our knowledge of the regulation of antioxidant systems in aquatic organisms in relation to either endogenous or exogenous (pollutants) sources of ROS is limited (Livingstone 2001). The resulting oxidative damage to lipids, DNA, and proteins and the adverse effects on the antioxidant, enzymatic and nonenzymatic, defense mechanisms of aerobic organisms have been used in recent years as biomarkers for monitoring environmental pollution. The current knowledge that such processes of oxidative damage occur in aquatic organisms gave the impetus to extend environmental and ecotoxicological studies to aquatic organisms as sentinels of environmental contamination by toxic chemicals. All these studies indicate that oxidative biomarkers in combination with other types of biomarkers, such as genotoxic biomarker, in aquatic organisms can be useful in large-scale environmental monitoring programs (Almeida et al. 2003; Monserrat et al. 2003).

Biomarkers can be used to assess changes at individual and/or population levels. However, it has been widely recognized that the implementation of biomarkers, including genotoxic biomarkers in environmental monitoring is hampered by the lack of knowledge of how biomarker responses are related to population dynamics of the species in which the biomarker is applied (den Besten 1998; Hyne and Maher 2003). Indeed, although pollutants

**Table 2** Body fresh weight, body dry weight and survival rate measured in nonylphenol (NP) and bisphenol A (BPA) exposed *D.magna* and *C.riparius*

| Species | Chemicals | Concentration (μg/L) | Body fresh weight | Body dry weight | Survival rate |
|---|---|---|---|---|---|
| *D. magna* | NP | 0.3 | 1.02 ± 0.08 | 1.01 ± 0.18 | 0.79 ± 0.21 |
| | | 3 | 1.13 ± 0.08 | 1.09 ± 0.24 | 0.71 ± 0.23 |
| | | 30 | 1.00 ± 0.08 | 1.18 ± 0.14 | 0.86 ± 0.03* |
| | BPA | 0.3 | 1.03 ± 0.20 | 0.95 ± 0.20 | 0.96 ± 0.04 |
| | | 3 | 1.10 ± 0.20 | 1.09 ± 0.14 | 1.00 ± 0.00 |
| | | 30 | 1.08 ± 0.16 | 0.96 ± 0.19 | 1.00 ± 0.00 |
| *C. riparius* | NP | 1 | 1.13 ± 0.04 | 1.31 ± 0.09 | 1.00 ± 0.06 |
| | | 10 | 1.12 ± 0.03 | 1.17 ± 0.03 | 0.88 ± 0.00 |
| | | 100 | 1.10 ± 0.05 | 1.17 ± 0.07 | 0.82 ± 0.06 |
| | BPA | 5 | 1.16 ± 0.06 | 1.27 ± 0.11 | 1.06 ± 0.00 |
| | | 50 | 1.12 ± 0.03 | 1.17 ± 0.03 | 1.00 ± 0.06 |
| | | 500 | 1.08 ± 0.03 | 1.13 ± 0.06 | 0.99 ± 0.07 |

Results were expressed as the mean value compared to control (solvent control = 1; number = 3; mean ± standard error of mean)

* Significantly different from the control value $p < 0.05$

may influence the genetic constitution of populations by causing direct damage to DNA molecules within the individual cell nucleus, the ecological relevance of changes in single cells within some tissues of some individual organisms is extremely difficult to assess (Depledge 1998). Nonetheless, sensitive detection of DNA damage in wildlife species is necessary, as pollutant-induced DNA damage might influence the genetic constitution of populations. Therefore, in this study, to provide insight into the relative sensitivity and higher biological level consequences of DNA damage observed in Table 1, conventional ecotoxicity tests, using growth and survival as toxic endpoints, were conducted (Table 2). Studied concentrations of NP and BPA exposure do not seem to affect the general physiological status of *C.riparius* and *D.magna*, as any statistically significant change was not observed on body fresh and dry weights in NP- and BPA-exposed *D. magna* and *C.riparius*. The survival rate was evaluated at the end of the growth experiments in *D. magna* and *C. riparius*. In both species no clear trend among treatments was apparent, except at the highest level of NP exposure for both *Daphnia* and *Chironomus*, (30 and 100 μg/L, respectively), where statistically significant decrease in survival rate was observed.

The experiments with NP-exposed *Daphnia* show that 96 h effects on survival, while no 24 h effect on DNA integrity were found. Moreover, NP exposure seems to provoke long-term (21-day) reproduction failure in *D. magna* (Ha and Choi 2007). This may 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 that mechanism other than genetic alteration might be involved in NP-induced mortality and reproduction failure in *Daphnia*. Indeed, it would not be ruled out that membrane damage may be related with higher level consequences, as increase in lipid peroxidation was observed in NP-exposed *D. magna* (Table 1). As for *C. riparius*, increased mortality by NP exposure was also observed (Table 2), and our previous study revealed 100 μg/L of NP exposure induced long-term development impairment (Lee and Choi 2006). The fact that DNA damage occurred concomitantly with decrease in organism and population level toxicity indicators (survival, development) suggests DNA alteration by this compound might provoke higher level consequences. Impairment of survival and development might be considered as a consequence of a serious progression of the sub-organisms level toxicities, such as increased DNA and lipid damage and decreased antioxidant activity in *Chironomus*. However, our data are not sufficient to provide a clear explanation for this phenomenon. If more sub-cellular parameters had been tested with longer exposure period, involvement of observed DNA damage in physiological pathway could probably be better evaluated and explained. On the other hand, effects on DNA integrity in BPA-exposed *D. magna* and *C. riparius* were not related to a degree of impairment of growth or survival of both organisms. Moreover, our previous studies revealed that BPA exposure did not seem to lead alteration on reproduction (Ha and Choi 2007) or on development (Lee and Choi 2007) for *D. magna* and *C. riparius*, respectively. False-positive results from genotoxic biomarker obtained in BPA-exposed *D. magna* and *C. riparius* make it more difficult to use DNA damage as an early warning biomarker.

The relationships between genotoxic biomarker responses and physiological/individual/population effects

are complicated because of compensatory mechanisms that regulate physiological/individual fitness and population dynamics in a natural system. Some biomarkers do not appear to have a direct relationship to a higher level of biological organization. In this case, the use of biomarker will not give a reliable prediction of toxic effects upon organisms and is, therefore, only ever likely to indicate exposure to chemicals. In using such biomarkers of exposure, it is difficult to predict effects at the population level from biomarker changes measured in a sample of individuals (Depledge and Fossi 1994; Hyne and Maher 2003). However, as the mere presence of genotoxic compounds, which are potentially carcinogenic, is a major concern in human and ecosystem health, sensitive and rapid detection of genotoxic property in aquatic system itself is considered important, although it does not necessarily include alteration at a higher level of biological organization. Considering the potential of *D. magna* and *C. riparius* as bioindicator species, and the importance of genotoxic biomarkers in ecotoxicity monitoring, measurement of DNA damage in these species could provide useful information for freshwater monitoring and risk assessment.

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