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Cytotoxicity, genotoxicity and ecotoxicity assay using human cell and environmental species for the screening of the risk from pollutant exposure

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Abstract

For the screening of the risk from environmental contamination, the cytotoxic/genotoxic effects of various model pollutants were determined using an *in vivo* system comprised of human HeLa cells; the ecotoxicity was also determined using the acute and genotoxicity tests on two aquatic sentinel species widely used in biomonitoring, namely, freshwater crustacean, *Daphnia magna* and larva of aquatic midge, *Chironomus tentans*. Nonylphenol (NP), bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEPH) and paraquat dichloride (PQ) were used as the model pollutants. The results showed that exposure of HeLa cells to NP, BPA and DEHP was sufficient for the expression of noticeable genotoxic and cytotoxic effects. Ecotoxicity results showed that, as expected, *D. magna* was more sensitive than *C. tentans* to chemical exposure. BPA may exert a genotoxic effect on *D. magna* and *C. tentans*, given that DNA strand breaks increased in both species exposed to this compound, whereas NP-induced DNA damage occurred only in *C. tentans*. *In vivo* genotoxic data obtained in aquatic sentinel species could provide valuable information for freshwater quality monitoring. From the results of the present study, the use of cytotoxic, genotoxic and ecotoxic tests using human cell system, as well as, biomonitoring species, seems to be relevant for preliminary evaluation of the human health and ecological effects of pollutants and thus, a promising screening tool for environmental monitoring and risk assessment.
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Keywords: Screening system; Cytotoxicity; Genotoxicity; Ecotoxicity; Environmental monitoring

1. Introduction

The reduction in point source pollution and the ban of some persistent chemicals have had positive effects on the level of environmental pollution over the last few decades. However, non-point source pollution by organic (e.g. pesticides, dioxins) and inorganic (e.g. heavy metals) compounds is still a global matter of concern. Moreover, numerous new industrial compounds have been synthesized for commercial and industrial purposes, which have generated environmental concerns, due to their high production and widespread use. Despite of the dramatic increase in the use of these chemicals, little information is available on their potential toxic effects on human health and living organisms. The potential deleterious effects on human and ecological health should be identified for the safe use of these chemicals. However, pollutions induced by these chemicals are caused by a complex mixture of compounds, making the exhaustive analyses of the contaminants present in polluted environments impossible, which limit the possibility of intensive toxicological studies (Risso-de-Faverney et al., 2001). Therefore, rapid and sensitive tools are needed for screening hazardous properties of such chemicals prior to intensive toxicological investigation and risk assessment. Short-term bioassay systems would appear to be relevant for the preliminary screening of the potential effects of environmental chemicals on human and ecological health.

Cytotoxicity and genotoxicity tests using mammalian cell lines are appropriate for screening of the potential hazardous effects of a large number of chemicals on human health. DNA strand breaks are potential pre-mutagenic lesions and are sensitive markers of genotoxic damage. 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. Since the protocol

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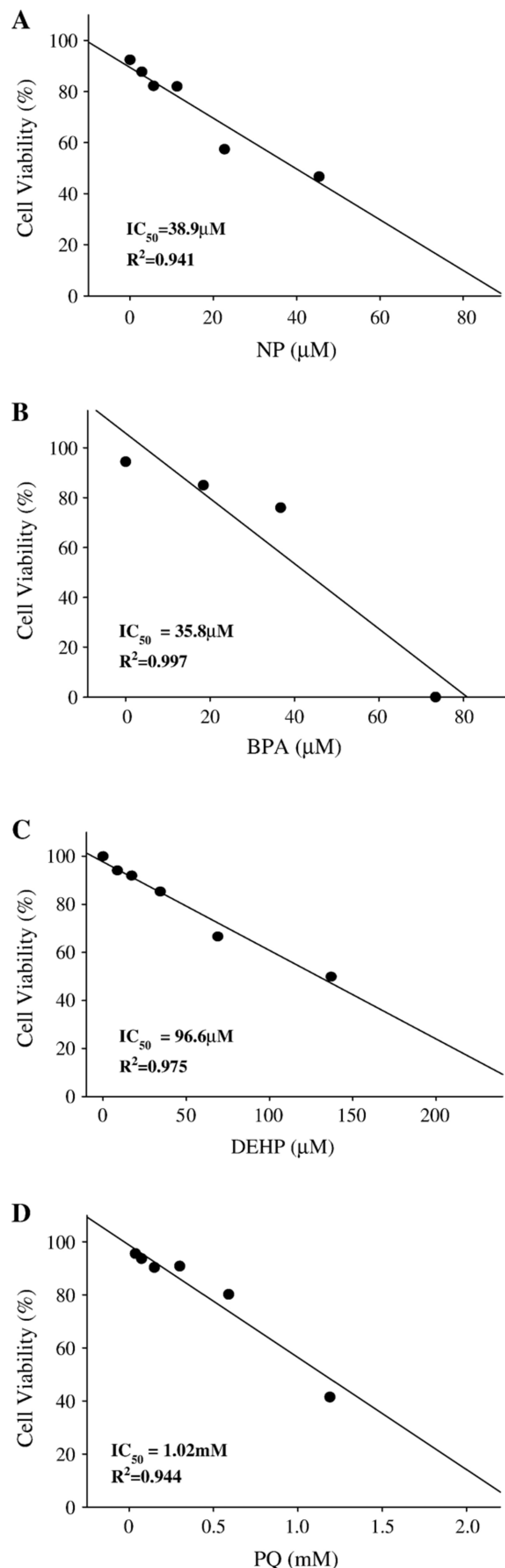

Fig. 1. Cell viability measured in HeLa cells exposed for 24 h to nonylphenol (NP), (A), bisphenol A (BPA), (B), bis(2-ethylhexyl) phthalate (DEHP), (C) and paraquat dichloride (PQ), (D) using trypan blue assay.

was published by Singh et al. (1988), it has been increasingly used in different fields of study, and it is considered a sensitive and rapid technique for the detection of DNA strand breaking, and is ideally suited as a non-specific biomarker of genotoxicity for environmental monitoring (Collins et al., 1997; Cotelle and Ferard, 1999; Tice et al., 2000; Brendler-Schwaab et al., 2005; Møller, 2006).

As for the screening of ecological health, the small-sized freshwater crustacean, *Daphnia magna* and the aquatic larvae of non-biting midges, *Chironomus tentans* were selected as biological model system. 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). The ecotoxicity tests using *Daphnia* and *Chironomus* system may provide an insight into the potential toxic effects of hazardous chemicals in aquatic environments. Moreover, information concerning genotoxicity, as well as, classical acute toxicity, on these species can be valuable for freshwater monitoring, taken into account of the importance of *D. magna* and *C. tentans* in the aquatic ecosystem.

In the present study, to identify a suitable tool for the development of a screening system prior to environmental monitoring, effects of various environmental contaminants on human and ecological health were evaluated using cyto-/genotoxicity (in human cell, HeLa) and acute-/genotoxicity tests (in two sentinel species, *D. magna* and *C. tentans*), respectively. Nonylphenol (NP), bisphenol A (BPA), bis(2-ethylhexyl) phthalate (DEPH) and paraquat dichloride (PQ) were studied as model pollutants, due to their widespread use and importance to human and environmental health. NP is used in the polymer industry (EU, 2002), BPA is an intermediate in the production of polycarbonate and epoxy resins (Staples et al., 1998), DEPH is a plasticizer in polymer products (EU, 2001) and PQ is an oxygen radical generating herbicide.

2. Materials and methods

2.1. Cell culture and cell treatment

The human HeLa cells were maintained in RPMI1640 (GIBCO BRL Life Technologies), supplemented with 10% (v/v) fetal bovine serum and 1% antibiotics, at 37 °C in a CO_2 atmosphere. Treated and control cells were incubated for 24 h, and then harvested for analysis.

2.2. Cell viability assay

The cell viability was measured using trypan blue (GIBCO BRL life technologies, MD, USA) reagent. After the treatment with 4 chemicals, treated and control cells were stained with 0.4% trypan blue and the total numbers of stained and unstained cells were counted using a hemocytometer. All experiments were performed in triplicate.

2.3. Daphnia and Chironomus culture

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), we obtained *D. magna* and *C. tentans* larvae from adults reared in our laboratory. *D. magna* were individually placed in glass beakers containing a culture medium, aerated M4 media, for 2 days. Cultured daphnids

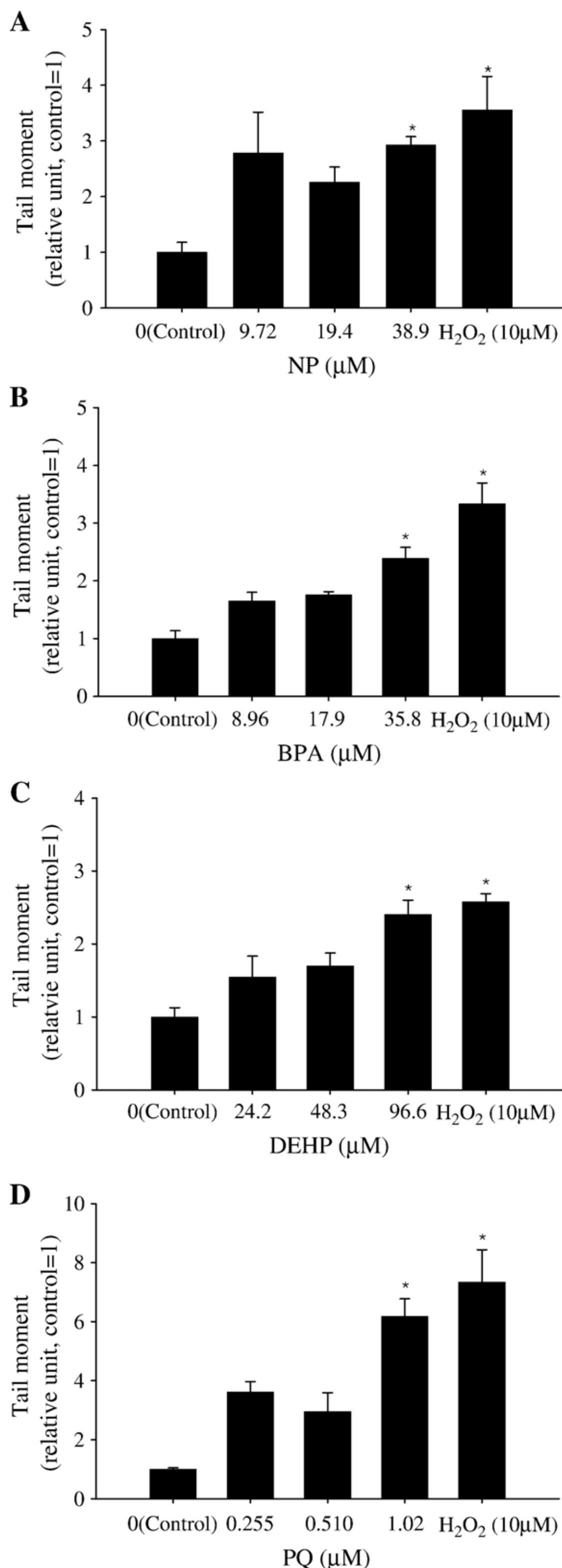

Fig. 2. DNA damage measured in HeLa cells exposed for 24 h to of nonylphenol (NP; A), bisphenol A (BPA; B), bis(2-ethylhexyl) phthalate (DEHP; C) and paraquat dichloride (PQ; D) using single-cell gel electrophoresis. The results were expressed as tail moment (number=3, mean±standard error of mean, *$p<0.05$).

were fed on the green alga *Chlorella* sp. at concentrations of 1×10^6–10^9 cells/mL every 2 days; the larvae of *C. tentans*, 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. tentans* were maintained at 20 °C±1 °C, 16 h light and 8 h dark cycle photoperiod regime.

2.4. Acute toxicity test

Acute toxicity test for *D. magna* was performed using immobilization as an endpoint. Ten neonates aged less than 24 h were individually transferred into 100 mL glass beakers filled with 50 mL of test solution and incubated at 20±1 °C for 24 h. The 24 h 10%, 50% and 90% effective concentration (EC10, EC50 and EC90) values were determined for swimming inhibition of daphnids by the probit method. Acute toxicity for *C. tentans* was determined after 24 h of exposure, using death of individuals as an endpoint. To evaluate 24 h 10%, 50% and 90% lethal concentration (LC10, LC50 and LC90) of *C. tentans* to 4 chemicals, groups of 10 larvae were exposed to four concentrations of each chemical, whereas other groups were kept as control.

2.5. Comet assay

For the preparation of the HeLa cell, about 4×10^5 cells/well were seeded in 6 well plates and incubated for 24 h prior to treatment. After the treatment with the 4 chemicals, the cells were washed with PBS, the cell suspension precipitated by vortexing. For the preparation of *Daphnia* and *Chironomus*, a total of 15 *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. Treated organisms were placed in 1 mL of phosphate-buffered saline (PBS) containing 20 mM ethylene diamine tetra-acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO) and disintegrated mechanically by mincing. Treatment of hydrogen peroxide (H_2O_2, 10 μM) was used as the positive control.

Table 1
Estimation of 24 h L(E)C10, L(E)50 and L(E)90 to nonylphenol bisphenol-A, bis(2-ethylhexyl) phthalate and paraquat dichloride in *D. magna* and *C. tentans*

Species	Chemicals		24 h L(E)C (μM)	Interval of confidence (95%)
D. magna	Nonylphenol	EC10	0.91	0.44<EC10<1.16
		EC50	1.38	1.04<EC50<1.80
		EC90	2.08	1.64<EC90<4.21
	Bisphenol A	EC10	0.47	0.03<EC10<0.74
		EC50	1.04	0.49<EC50<1.46
		EC90	2.37	1.63<EC90<13.03
	Bis(2-ethylhexyl) phthalate	EC10	0.97	0.08<EC10<1.30
		EC50	1.82	1.38<EC50<7.11
		EC90	3.40	2.21<EC90<432
	Paraquat dichloride	EC10	1.36	0.002<EC10<3.09
		EC50	4.38	0.53<EC50<7.13
		EC90	14.1	8.46<EC90<257
C. tentans	Nonylphenol	LC10	3.34	0.68<LC10<4.75
		LC50	5.76	3.63<LC50<9.62
		LC90	9.96	6.94<LC90<55.0
	Bisphenol A	LC10	8.98	2.77<LC10<12.3
		LC50	14.3	9.08<LC50<19.1
		LC90	22.7	17.3<LC90<50.9
	Bis(2-ethylhexyl) phthalate	LC10	104	0<LC10<329
		LC50	1124	387<LC50<3503
		LC90	12,078	2512<LC90
	Paraquat dichloride	LC10	770	175<LC10<1203
		LC50	1586	929<LC50<2663
		LC90	3280	2112<LC90<13,717

LC: lethal concentration, EC: effective concentration.

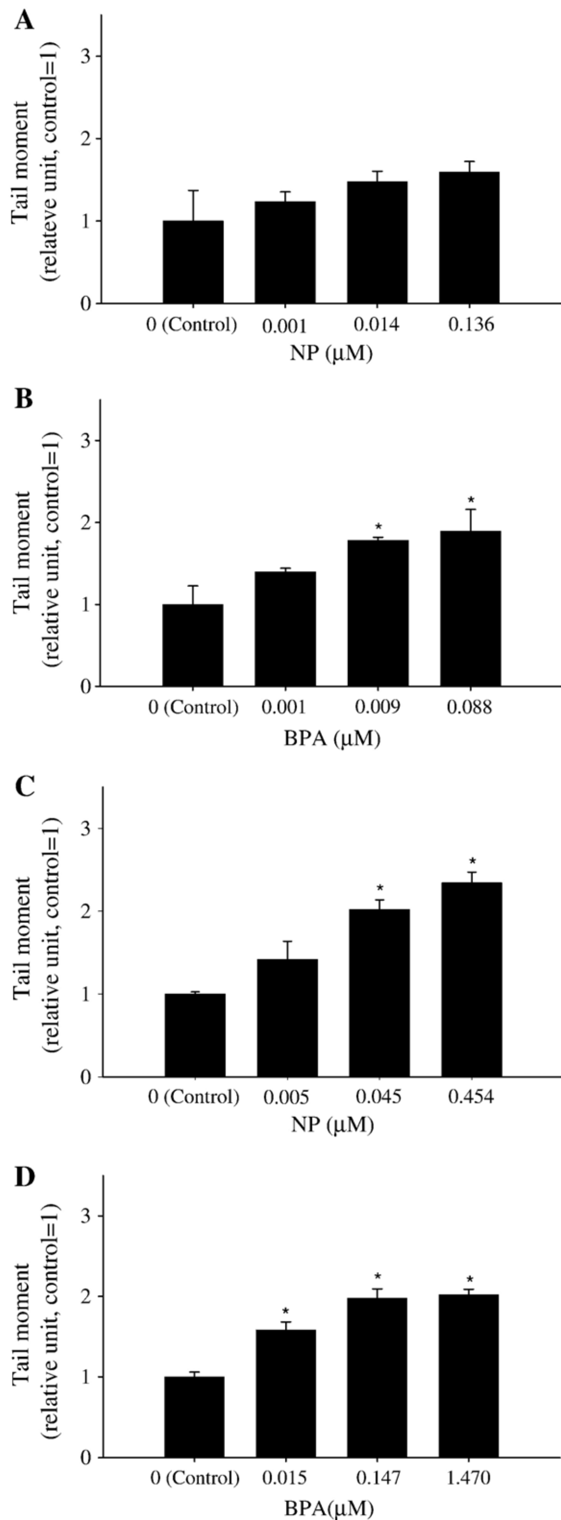

Fig. 3. DNA damage measured in *D. magna* and *C. tentans* exposed for 24 h to sublethal concentrations of NP and BPA using single-cell gel electrophoresis. The results were expressed as tail moment measured in nonylphenol (NP)-exposed *D. magna* (A), bisphenol A (BPA)-exposed *D. magna* (B), nonylphenol (NP)-exposed *C. tentans* (C) and bisphenol A (BPA)-exposed *C. tentans* (D; number=3, mean±standard error of mean, *$p<0.05$).

An alkaline comet assay was performed, as described by Singh et al. (1988). Briefly, 100 μL of 1% low melting point (LMP) agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 NaCl, 10% DMSO(only organisms), 10% Triton X-100, pH 10), and subsequently exposed to alkali conditions (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow the DNA to unwind and the alkali-liable sites to be expressed. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min, after which, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place prior to image analysis. Before their analyses, the slides were stained with 50 μL ethidium bromide (5 μg/mL), then analyzed using a fluorescence microscope (Nikon, Kanagawa, Japan) equipped with an excitation filter of BP 546/12 nm and a barrier filter of 590 nm at 400× magnification. Approximately, 50 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail moment using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.6. Data analysis

Log-probit transformation of the data was used in order to estimate 24 h L(E)C10, L(E)C50 and L(E)C90 values and the corresponding 95% confidence intervals. The Comet assay results were tested for significance using analysis of variance (ANOVA) test with Dunnett's multiple comparison test. All statistic tests were performed using SPSS® 12.0 KO (SPSS Incorporated, Chicago, IL, USA).

3. Results

Fig. 1 shows the effects of the 4 environmental pollutants on the cell viability. The order of cytotoxicity, expressed as median inhibitory concentration (IC50) observed on the HeLa cells was NP ≥ BPA > DEPH > PQ. Based on the results of cell viability assay, 3 concentrations – corresponding to IC50, 1/2 of IC50 and 1/4 of IC50 – were selected for the exposure conditions of the Comet assay. They were 9.72, 19.4 and 38.9 μM for NP; 8.96, 17.9 and 35.8 μM for BPA; 24.2, 48.3 and 96.6 μM for DEHP; and 0.26, 0.51 and 1.02 mM for PQ.

DNA damage, particularly DNA strand breaks, was measured by Comet assay to evaluate whether the studied chemicals induced any genetic toxicity. Fig. 2 shows that all 4 studied compounds seemed to have genotoxic potential at a high level of exposure (IC50), since the tail moment obtained from the Comet assay increased significantly for the cell exposed to the highest concentration; whereas, DNA strand breaks were not observed at lower concentrations.

Twenty-four-hour L(E)C10, L(E)C50 and L(E)C90 of NP, BPA, DEHP and PQ were estimated in neonates of *D. magna* and in fourth instar larvae of *C. tentans* (Table 1). The order of acute toxicity (EC50) from the results of the *D. magna* immobilization tests was BPA ≥ NP > DEHP > PQ, which was very similar to the cytotoxicity results and as for *C. tentans*, the order acute toxicity, expressed as LC50 was NP > BPA >> DEHP > PQ. As for the cytotoxicity data (Fig. 1), again, PQ exhibited a tolerability compared to the other chemicals tested. As for the response toward exposure to DEHP, compared to *D. magna*, strikingly high tolerance of *C. tentans* was observed. Based on the results of acute toxicity, genotoxicity test was performed on two chemicals, which have two lowest L(E)C50 values, and they were NP and BPA for both species (Table 1). Three concentrations – corresponding to 1/10, 1/100 and 1/1000 of L(E)C50 – were selected for the sublethal exposure conditions of the Comet assay. They were 0.001, 0.014 and 0.136 μM for NP and 0.001, 0.009 and 0.088 μM for BPA in *D. magna*; and 0.005, 0.045 and 0.454 μM for NP and 0.015, 0.147 and 1.470 μM for BPA in *C. tentans*.

DNA strand breaks were measured using Comet assay in order to evaluate whether NP and BPA induce any genetic toxicity in *D. magna* and in *C. tentans* (Fig. 3). As shown in Fig. 3, tail moment increased significantly in cells isolated from *D. magna* that have been exposed to BPA (0.009 and 0.088 μM), whereas, NP did not seem to

induce DNA strand breaks in this species at the studied exposure levels. As for *C. tentans*, tail moment increased significantly by both chemicals tested. Increased tail moment by chemical exposure occurred at all concentrations tested by BPA exposure (0.015, 0.147 and 1.470 μM), whereas at two highest concentrations by NP exposure (0.045 and 0.454 μM).

4. Discussion

The cytotoxicity and ecotoxicity, as well as, genotoxicity, tests were performed on human cell (HeLa cell) and on two aquatic sentinel species (*D. magna* and *C. tentans*), for the toxicity screening of environmental pollutants (e.g. NP, BPA, DEPH and PQ). The order of cytotoxicity observed on the HeLa cells was NP ≥ BPA > DEPH > PQ, as shown in Fig. 1, and the IC50 of PQ was more than 20-fold higher than that of NP or BPA, and almost 10-fold higher than that of DEHP. PQ exhibited such a high tolerability compared to the other chemicals tested (Fig. 1) and it was also observed in ecotoxicity data on *D. magna* and *C. tentans* (Table 1), which may imply that the high hydrophilicity of this chemical impedes its membrane penetration. The cytotoxic effects of NP and BPA may indicate early cellular changes, with possible biological consequences, which should be considered in the preliminary evaluation of the risk of populations exposed to these chemicals *in vivo*. Despite the constraints in the extrapolation of *in vitro* to *in vivo* data in humans, the cytotoxicity of the studied compounds requires special attention in view of the major damage they cause to cell function, which result in the inability of cells to proliferate. These disturbances frequently appear long before genotoxic effects manifest, or even in the absence of the latter. Thus, the cytotoxic effect may be considered an earlier indication of cellular damage, with possible biological consequences; therefore, this should be taken into account in the preliminary evaluation of the risk to populations exposed *in vivo*, as has already been suggested (Ekwall, 1983).

The overall results shown in Figs. 1 and 2 suggest that exposure of HeLa cells to NP, BPA and DEHP is sufficient for the expression of noticeable genotoxic and cytotoxic effects. However, caution should be taken if using the present results to estimate the risk to populations exposed to low doses of these compounds, as the appropriate assessment of the genotoxic potential of an agent requires the use of different assays that will permit the evaluation of different genetic events in different cell types.

As well as studying the effects of chemicals on human health using cytotoxic and genotoxic tests (Figs. 1 and 2), it was our intention to obtain additional ecotoxicological information, which could be used in the preliminary evaluation of the effects to environmental health, especially on aquatic ecosystems. The most widely-used aquatic ecotoxicity tests, the *Daphnia* immobilization bioassay and *Chironomus* acute toxicity tests (Table 1) may provide a degree of insight into the relative sensitivity of these animals to pollutants, which may also provide information on the impact of chemicals on water systems, as these species hold an important position in aquatic ecosystems (OECD, 1984; Okamura et al., 1999; Kikuchi et al., 2000). As expected, short-term response to each chemical was more sensitive in *Daphnia* than in *Chironomus*.

The continued presence of genotoxic and potentially carcinogenic compounds in the aquatic environment is of major concern with respect to the health of aquatic media biota and humans (Houk and Waters, 1996, Ohe et al., 2004, Nehl and Segner, 2005). To efficiently assess the presence of mutagens in the water, aside from conventional physio-chemical analysis, genotoxicity assays should be included as additional parameters in water quality monitoring programs. There are many reports on the studies linking the DNA damage to subsequent molecular, cellular and tissue level alteration of aquatic organisms (Ohe et al., 2004). Genotoxic parameters, therefore, have been proven to be sensitive and reliable tools in the detection of mutagenic activity in the aquatic environment and thus currently the most valuable biomarkers for ecological risk assessment. Application of Comet assay, using many different species, has widely been conducted in ecotoxicology (Cotelle and Ferard, 1999, Guecheva et al., 2001, Palmqvist et al., 2003, Clement et al., 2004, Gravato et al., 2005).

As shown in Fig. 3, BPA may exert a genotoxic effect on *D. magna* and *C. tentans*, given that DNA strand breaks increased in both species exposed to this compound, whereas NP-induced DNA damage occurred only in *C. tentans* at the studied exposure levels. For screening of genotoxic activities in aquatic environment, most genotoxic tests using Comet assays have been performed *in vitro* system from aquatic species, mostly using fish-driven cell lines (Cortelle and Ferard, 1999; Nehl and Segner, 2005); in this study, however, *D. magna* and *C. tentans* were exposed to each pollutant *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 White (2004), DNA damage in wildlife species measured by Comet assay could provide a sensitive and rapid genotoxic biomarker in environmental monitoring. Although pollutants 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. 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. tentans* 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.

However, genotoxic biomarker alone is not sufficient for evaluating the toxicological response of pollutants in organisms. The multi-parametric approach, wherein different biological responses ranging from molecular/cellular to physiological/ecological are evaluated, is essential to perform a better

prospective assessment of the risks engendered by the presence of pollutants in the ecosystems. Simultaneous measurement of various toxicological/ecological parameters gives the opportunity to obtain data at different levels of biological organization and it may help to fully understand the effects of pollutants on organisms. In addition, the determination of population-level parameters improves the interpretation of data collected at lower biological levels (Lee and Choi, 2006).

Because of complex nature of chemical pollution, diagnosis of general environmental conditions requires screening with a minimum set of assays prior to intensive toxicological investigation and risk assessment and the appropriate remedial actions. Identification of suitable tools for the development of a screening system is therefore, required for environmental monitoring. Short-term bioassay, using cells or environmental species, systems would appear to be relevant for the preliminary screening of the potential effects of chemicals on human and environmental health. Short-term bioassays have the advantage of integrating the toxic potency of all compounds in a sample and of being more rapid and inexpensive than analytical chemistry techniques (Schirmer et al., 2004). This is particularly important if many samples need to be assessed for large scale field monitoring, such as, *in situ* investigations in large contaminated areas or in the long-term monitoring of specific areas.

From the results of the present study, the use of cytotoxic, genotoxic and ecotoxic test systems would appear to be relevant for the preliminary screening of human health, as well as the ecological effects of environmental chemicals, and seems to be a promising tool for environmental monitoring and risk assessment. If necessary, simultaneous measurements of various toxicological parameters, giving the opportunity of obtaining a full set of data on the effects of pollutants, which may help to more fully understand the effects of a toxicant on human and ecological health, could follow on from this screening test system.

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