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Comprehensive Toxicity Study of Nonylphenol and Short-Chain Nonylphenol Polyethoxylates on *Daphnia magna*

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Alkylphenol polyethoxylates (APnEOs) are among the most widely used nonionic surfactants in the world. The most significant commercial APnEOs are nonylphenol polyethoxylates (NPnEOs), accounting for approximately 80% of the total APnEOs produced. It has been reported that NPnEOs degrade starting at the hydrophilic ethoxylate (EO) chain in environmental media. Such a biotransformation pathway results in formation of a number of relatively stable metabolic products including nonylphenol (NP) and short-chain NPnEOs, such as NP1EO and NP2EO. These small metabolites, notably NP, are more lipophilic and toxic than the parent surfactants (Ahel et al. 1995). The 24h- and 48h-LC_{50} values for NP with *Daphnia magna* were reported to be 0.30 and 0.13 mg/L (Comber et al. 1993). Acute toxicity of NP for fish, as measured by 48h- to 96h-LC_{50} values, typically ranged from 0.1 to 1.0 mg/L, while these values for NPnEOs ranged from 1 to 1000 mg/L, with toxicity decreasing as the length of the EO chain increased (Yoshimura 1986). In addition to the direct toxicity, these metabolites have also been reported to have endocrine disrupting effects. These compounds compete with natural estrogen for binding to the estrogen receptor and, as a result, effects on reproduction and development could occur (Burkhardt et al. 2000).

Although there have been many reports on the toxicity of NP and short-chain NPnEOs ($n<3$), further studies on these small metabolites of NPnEOs have rarely been reported, such as the influence of these degradation products on the physiological behavior of aquatic organisms. Furthermore, NP, NP1EO and NP2EO usually coexist in the aquatic environment as a result of the degradation of NPnEOs. However, studies on their joint toxicities are lacking. In this study, in addition to the study of its acute and chronic toxicity on *D. magna*, the influence of hardness, pH and humic acid on the acute toxicity of NP was observed, the alteration of feeding behavior of *D. magna* exposed to NP was studied, and we also investigated the joint toxicities of NP with NP1EO and NP with NP2EO to *D. magna* at an equitoxic ratio based on their single acute toxicities.

MATERIALS AND METHODS

D. magna from a continuous laboratory culture were reared under experimental

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conditions, at 20±1℃ under light of 2000Lux intensity with a 16h:8h light : dark cycle, and fed with green algae *Chlorella vulgaris*. NP was purchased from Tokyo Chemical Synthesis Ind. Co. Ltd, Japan. NP1EO and NP2EO were purchased from Hayashi Pure Chemical Ind. Co. Ltd, Japan. A stock solution of 100 mg/L in hexane was used, respectively. Humic acid was the product of Aldrich Company. A stock solution of 4 g/L was used. The purity of these chemicals was 99%.

The 24-h acute toxicity of NP, NP1EO and NP2EO on *D. magna* were investigated. All acute tests were performed in dilution water with the following composition: 0.294g $CaCl_2 \cdot 2H_2O$, 0.123g $MgSO_4 \cdot 7H_2O$, 0.065g $NaHCO_3$ and 0.006g KCl in 1L of deionized water (total Ca + Mg hardness 2.5 m*M* with the ratio of Ca^{2+}:Mg^{2+} at 4:1, pH adjusted to 7.5-7.8). Certain amounts of the stock solution were first added into separate vessels. 100 ml of the dilution water was added after volatilization of the solvent by blowing N_2. Therefore, hexane content in the test medium could be ignored. For each chemical, five concentrations and a solvent control were used and each concentration was replicated. For each test, 10 neonates of the daphnids were introduced into a 150-ml vessel containing 100 ml of test medium. There was no feeding during the acute tests. The mortality was recorded after 24 hours, and the 24h-LC_{50} with 95% confidence interval was calculated. Acute toxicity tests for NP were also conducted under different hardness, pH and humic acid levels in order to elucidate their influence on the acute toxicity of NP. Hardness levels were adjusted to required levels by regulating concentrations of Ca^{2+} and Mg^{2+} at Ca^{2+}:Mg^{2+} proportion of 4:1. Four Ca + Mg hardness levels (1.3, 2.5, 3.8, 5.0 m*M*) were used. pH values were adjusted to required values (6.1-9.2) with 0.5 *M* NaOH and 0.5 *M* HCl. Certain amounts of the humic acid stock solution were added to dilution water to make the humic acid concentration of 0, 5, 10, 15 and 20 mg/L.

Joint toxicities of NP and NP1EO (or NP2EO) using an equitoxic mixture was measured as in single acute tests. Equitoxic mixtures are solutions in which each chemical is present at the same fraction of its individual 24h-LC_{50} (Warne and Hawker 1995). After 24 h, the mortality was recorded and the 24h-LC_{50} values of the mixtures were calculated. The Additive Index (AI) (Hagopian et al. 2001) was employed to analyze the data of joint toxicity tests. First, a sum toxic unit (TU) was calculated by summing the concentration of each chemical in the equitoxic mixture's 24h-LC_{50} value divided by its 24h-LC_{50} when present alone. The TU formula is:

$$TU=(LC_{50}/LC_{50,i})_A+(LC_{50}/LC_{50,i})_B$$

where A is chemical A, B is chemical B, LC_{50} is the concentration of each chemical in the mixture's 24h-LC_{50} value, $LC_{50,i}$ is the individual 24h-LC_{50}. The Additive Index (AI) was calculated using the following formula:

$$AI=1/TU-1, \text{ for } TU \leqslant 1, \qquad AI=1-TU, \text{ for } TU>1$$

AI=0 describes additive action; AI<0 describes antagonism; and AI>0 describes

synergism. Another model, the concentration-addition model (Otitoloju 2003), was used to classify the interactions between NP and NP1EO (or NP2EO). In order to evaluate the joint toxicity, a predicted 24h-LC_{50} value is derived by summing up the 24h-LC_{50} values of the separate toxicants according to the proportion of their contribution in the mixture. The predicted 24h-LC_{50} value is then compared to the observed 24h-LC_{50} value of the mixture so as to classify the interactions. The relationship between the observed 24h-LC_{50} value and the predicted 24h-LC_{50} value (RTU) is estimated as follows:

$$RTU = \frac{\text{predicted } 24h - EC_{50} \text{ value}}{\text{experimentally observed } 24h - EC_{50} \text{ value}}$$

RTU=1 describes concentration additive action; RTU<1 describes antagonism; RTU>1 describes synergism.

The effect of NP on feeding behavior of *D. magna* was investigated using 2-d old daphnids. Four concentrations (1/4, 1/2, 3/4 and 1/1 of the 24h-LC_{50} value) and a control were used. Three replicates of each concentration were prepared with 100 ml of test medium in 150-ml vessels. *Chlorella* was added to each vessel at a density of about 8×10^5 cells • ml^{-1} and the initial cell density was measured accurately. 10 daphnids were introduced into each vessel and were allowed to feed in the dark for 3 hours, and then removed from the vessels. After removal of the animals, cell density of the algae in each vessel was calculated. Filtration rate (F) and ingestion rate (I) were evaluated according to the equations (Villarroel et al. 2003) below:

$$F = \frac{V \times (\ln C_0 - \ln C_t)}{n \times t} \qquad I = F\sqrt{C_0 \times C_t}$$

where F is the filtration rate (μl • $animal^{-1}$ • h^{-1}), I is the ingestion rate (cell • $animal^{-1}$ • h^{-1}), V is the volume of medium in the test vessel (μl), C_0 is the initial density of *Chlorella* (cell • μl^{-1}), C_t is the final density of *Chlorella* (cell • μl^{-1}), n is the number of animals in each vessel, t is the duration of the feeding test (h).

Five concentrations (1/32, 1/16, 1/8, 1/4 and 1/2 of the 24h-LC_{50} value) and a control were used in the chronic tests. 100 ml of test medium was poured into a 150-ml vessel and 3 daphnids (6-24h old) were added. For each concentration, three replicates were prepared. The chronic test lasted for 21 days. Daphnids were fed after daily medium renewal. Survival, reproduction and the interval of molting were assessed whenever the test medium was renewed. Offspring were discarded after counting.

Data from feeding tests and chronic tests were both analyzed using analysis of variance (ANOVA) to detect significant differences between the control and exposed daphnids, followed by Dunnett's Procedure. The significance level (P) was set at < 0.05, while for molting, P was set at < 0.01.

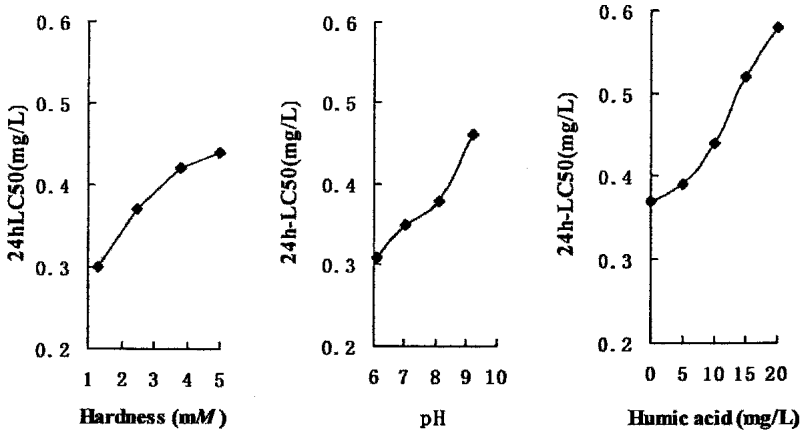

Figure 1. Influence of hardness, pH and humic acid on the toxicity of nonylphenol (NP) to *Daphnia magna*.

RESULTS AND DISCUSSION

Acute toxicities of NP, NP1EO and NP2EO were high, with 24h-LC_{50} values being 0.37 mg/L (0.33-0.42 mg/L), 0.39 mg/L (0.38-0.42 mg/L) and 0.56 mg/L (0.53-0.60 mg/L), respectively. The toxicity was NP > NP1EO > NP2EO. The influence of hardness, pH and humic acid on the toxicity of NP for *D. magna* are shown in Figure 1.

It can be seen that an increase in water hardness resulted in a decrease in the toxicity of NP. The reduction of toxicity was attributed to the competitive uptake between NP and divalent hardness cations (Ca^{2+} and Mg^{2+}), especially Ca^{2+}, for available binding sites, which has been documented for Cd^{2+} (Penttinen et al. 1995). Therefore, when Ca^{2+} was elevated, toxicity of NP decreased. As for pH, the toxicity of NP decreased as pH was elevated. The results can be interpreted in terms of pH-dependent ionization of NP. NP is an ionizable organic compound with a pKa of 10.7 reported by Nagasaki et al. (2004). When pH is lower than the pKa, the neutral molecule, NPOH, is the dominant species; whereas when pH approaches the pKa, the anionic molecule, NPO^-, begins to replace NPOH. It has been recognized that neutral molecules can penetrate cell membrane more easily than anionic molecules (Fent and Looser 1995). Hence, it is easy to understand that NP is less toxic under alkaline conditions. Humic substances are thought to play an important role in the fate of organic pollutants in the aquatic environment. The binding of the pollutant to humic substances depends on its chemical properties and its hydrophobicity (Fent and Looser 1995). In the presence of elevated concentration of humic acid, increasing portions of NP might combine with humic substances, thus the freely dissolved species is reduced. However, only freely dissolved NP species are bioavailable. Therefore a decrease in the toxicity of NP occurred in the presence of humic acid.

The 24h-LC_{50} values of NP, NP1EO and NP2EO singly and in two equitoxic mixtures are displayed in Table 1. Analysis of the data by the method of AI and the concentration-addition model revealed that the type of interactions between NP and NP1EO (or NP2EO) was in agreement with the model of antagonism (Table 1).

Table 1. Joint toxicity of nonylphenol (NP), nonylphenol monoethoxylate (NP1EO) and nonylphenol diethoxylate (NP2EO).

Chemicals	24h-LC_{50} with 95% CL (mg/L)	TU	AI	RTU
NP	0.37 (0.33-0.42)	----	----	----
NP1EO	0.39 (0.38-0.40)	----	----	----
NP2EO	0.56 (0.53-0.60)	----	----	----
NP-NP1EO	0.63 (0.57-0.70)	1.66	-0.66(<0)	0.63(<1)
NP-NP2EO	0.76 (0.71-0.81)	1.64	-0.64(<0)	0.64(<1)

The mechanism responsible for the antagonistic interaction between NP and NP1EO (or NP2EO) was attributed to the competition for uptake sites at the biological interface between various chemicals (Otitoloju 2003). When NP, NP1EO and NP2EO coexist in the aquatic environment, NP1EO and NP2EO compete with NP for binding to the uptake sites and hence NP's entrance into the cell is hindered. NP1EO and NP2EO are both less toxic than NP and hence antagonistic interactions occurred when they coexisted. Meanwhile, as a surfactant, the presence of NP1EO and NP2EO are thought to enhance the water phase solubility of NP, and therefore reduce the fugacity of NP. Therefore, the fraction of NP entering into the cell and, accordingly, its toxicity decreased. To our knowledge, antagonistic interactions between NP and NP1EO and NP2EO have not been previously reported. We think it is an advantage for environmental management because NP, NP1EO and NP2EO are often found to exist simultaneously. Antagonism implies that interactions between the constituents result in the lowering of the toxicity of NP, or NP1EO and NP2EO against the living species (Otitolojo 2003), and hence ecorisks caused by the use and degradation of NPnEO are reduced.

Filtration rates (F) decreased from 1512 (the control) to 1202, 870, 737, 449 $\mu l \cdot animal^{-1} \cdot h^{-1}$ at 0.09, 0.19, 0.28, 0.37 mg/L of NP, respectively, in the feeding tests. Significant differences ($P<0.05$) between exposed groups and the control were found at NP concentrations of 0.19 mg/L and higher. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) were 0.09 and 0.19 mg/L, respectively. Ingestion rates (I) declined from 1.22×10^6 (the control) to 1.04×10^6, 8.24×10^5, 6.21×10^5, 3.43×10^5 cell $\cdot$ animal^{-1} $\cdot$ h^{-1} at 0.09, 0.19, 0.28, 0.37 mg/L of NP, respectively. Significant differences ($P<0.05$) between exposed groups and the control were at NP concentrations of 0.19 mg/L and higher. The NOEC and the LOEC were 0.09 and 0.19 mg/L, respectively. The results showed that NP had already influenced the feeding behavior of *D. magna* at sublethal concentrations. Constant exposure of *D. magna* to NP over their entire life cycle may reduce

their ability to obtain adequate nutrition and result in a decrease in survival and reproduction. On the other hand, *D. magna* as aquatic filter feeders tend to be primary consumers, playing a key role at the base of food chain in aquatic ecosystems. Therefore, influence of pollutants on their feeding behavior could have significant consequences on ecosystem structure and function (Villarroel et al. 2003).

The results of chronic tests are shown in Table 2. On the whole, survival, reproduction and interval of molting of *D. magna* were affected by NP at sublethal concentrations. Survival of *D. magna* did not significantly decrease with increasing concentrations of NP until the concentration of NP was higher than 0.050 mg/L ($P<0.05$). The NOEC and the LOEC for survival were 0.025 and 0.050 mg/L, respectively. The reproduction of *D. magna* was significantly reduced by NP at concentrations higher than 0.025 mg/L ($P<0.05$). The NOEC and the LOEC for reproduction are 0.013 and 0.025 mg/L, respectively. NP did not significantly affect the molting process of *D. magna* until 0.050 mg/L ($P<0.01$), with the NOEC and the LOEC being 0.025 and 0.050 mg/L, respectively. *D. magna* exposed to NP took less time to complete a molting process than the control did. The results showed that NP could hasten the molting process of *D. magna*.

Table 2. Survival, reproduction and interval of molting of *Daphnia magna* exposed to various concentrations of nonylphenol (NP).

NP (mg/L)	Survival (%)	Number of offspring per adult	Interval of molting (d)
Control	100	131	2.47
0.013	100	118	2.42
0.025	88.9	*109	2.41
0.050	*77.8	*94	**2.32
0.100	*55.6	*77	**2.25
0.200	*33.3	*55	**2.12

* $P < 0.05$, ** $P < 0.01$

Villarroel et al. (2003) have reported that reproduction is a more sensitive index in chronic toxicity tests, which was true in our study. Compared with other pollutants in the aquatic environment, NP has greater effects on the reproduction of *D. magna*. This is because of its endocrine disrupting activity. NP competes with natural estrogens in binding to the estrogen receptor after entering the organism. As a consequence, effects on reproduction occurred.

Molting is an important physiological process for *D. magna* because it allows for growth and development of organisms with a rigid exoskeleton. The molting process is regulated by a multihormonal system, but is under immediate control by molt-promoting steroid hormones, called ecdysteroids (Lachaise et al. 1993). NP, acting as an estrogenic substance, has a strong affinity to ecdysteroid receptors. When entering the organism, NP binds to and activates ecdysteroid receptors, preventing the endogenous ecdysteroids from binding to and

activating their receptors. NP has a stronger activation to ecdysteroid receptors in comparison with ecdysteroids, thus interfering with the molting process of *D. magna* (Zhou and Fingerman 1997). As a consequence, the interval of molting was shortened.

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