

Eco-toxicological evaluation of 4-nonylphenol diastereomers with delayed luminescence in the green alga *Pseudokirchneriella subcapitata*

Omagari R¹, Ishibashi Y¹, Katsumata M², Arizono K¹

¹ Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Kumamoto, Japan
862-8502

² Central Research Laboratory, Hamamatsu Photonics K. K. Hamamatsu, Shizuoka, Japan, 435-8558

Introduction

Nonylphenol (NP) is the raw material for production of nonylphenol ethoxylates (NPEO) which is widely utilized for industrial surfactant[®] and used as a plastic additive antioxidant^(1,2). NPEO in environment is mainly metabolized to 4-nonylphenol isomers (4-NP's). It is reported that 4-NP's have not only eco-toxicity but also endocrine disrupting effect to wild life animals. It is well known that the 4-NP's, which have the basic structure of branching of C-9 alkyl chain, were detected in water environment. Therefore, it was considered that the eco-toxicity of 4-NP's (both branched and linear compounds) should be carried out in isomer-specific manner. In this study, we have tried to evaluate eco-toxicity of six 4-NP diastereomers by algal test using DL. The stereo-structural relationship on eco-toxicity of 4-NP diastereomers as compared with the estrogenic activity and fish toxicity were also discussed.

Materials and methods

Test alga: The green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) was used for algal luminescence inhibition test. The test alga was provided as frozen algal kit preserved at -80°C (Hamamatsu Photonics, Japan). The frozen alga recovered to the exponential growth phase through a 1 h pre incubation (25°C, 50 µmol/m²/s) after thawing (Fig.1). At this time, the thawed alga was transferred to glass culture tube (Φ25mm×85mm), and diluted to ten times by the OECD medium.

Test chemicals: The six of 4-NP's (diastereomeric 4-nonyl phenol (NP) isomers [4-(3,4-dimethyl heptan-4-yl)phenol (NP-J, NP-L) and 4-(3,4-dimethyl heptan-3-yl)phenol (NP-K, NP-P), 4-(3,5-dimethyl heptan-3-yl)phenol (NP-E, NP-G), and 4n-NP were used as test chemicals (Fig.1). These 4-NP diastereomers were kindly supplied by Prof. Katase (College of Bioresource Sciences, Nihon University).

Exposure incubation: 0, 30, 100, 300, 1000, 3000 mg/L of 4-NP's were prepared as test solution. The behavior of DL from the alga in culture glass tube was measured at 0, 1, 6, 24 h exposure. The exposure solutions were incubated under continuous illumination from fluorescent lamps (50 µmol/m²/s) at a temperature of 25 ± 1°C in an orbital shaking culture during 24 h of exposure test.

Algal bioassay using delayed luminescence (DL): The DL from algae was measured by high sensitivity Lumino-meter (TYPE-7100, Hamamatsu Photonics, Japan). The relative DL intensity was expressed as a count.

EC₅₀ was determined on the basis of Ecotox-Statics analysis software. And, the both algal growth rate and inhibition ratio of algal growth were calculated by the same evaluating method that using in the TG-201 used the algal DFI instead of algal biomass.

Statistic: EC₅₀'s of 4-NP diastereomers were calculated by employing ANOVA4 on the Web and compared with those of branched chain 4-NP's.

Results and discussion

Calculation of prediction EC₅₀ values on alg: The EC₅₀ of DL values by 1, 6, 24 h NP exposure indicated with the estrogenic activity which related to E2 and fish toxicity. The EC₅₀ (µg/mL) of 24 h exposure each 4-NP diastereomer was revealed that NP-E (2.58), NP-G (1.38), NP-J (1.86), NP-L (0.84), NP-K (1.31), NP-P (0.64) and 4-n-NP (0.40), respectively. 4-n-NP which is 4-NP of linear chain type found stronger toxic influences to algae than other 4-NP diastereomers which are branched chain type.

Comparison of inhibition ratio to alga: The dose depended alteration of algal growth inhibition ratio(%) at 24 h after exposure revealed at a dose of 0-3.0 µg/mL. The results indicated that 4-NP diastereomers caused algal growth inhibition obviously. At 1.0 µg/mL, the inhibition ratio of β-carbon to the benzene ring isomers (NP-J:38.7%, NP-L:63.5%, NP-K:51.5%, NP-P:68.0%) were higher than that of γ-carbon to the benzene ring isomers (NP-E:28.5%, NP-G:36.7%). The linear 4-NP (4-n-NP:93.0%) indicated highest algal growth inhibition. NP-P and NP-L which are high field shift of the methyl proton signal having β-carbon to the benzene ring exhibited potent inhibition ratio than their diastereomers (NP-K and NP-J). On the other hand, the inhibition ratio at 0.03 to 0.3 µg/mL on 4-NP diastereomers indicates no significant difference.

We have already found that EC₅₀ values of 4-NP's (12 branched 4-NP's and the linear 4-NP) range 0.3µg/mL ~3.0µg/mL using DL as the toxic influence on algae⁸⁾. Although the toxicity of linear 4-NP to the alga was stronger than that of the other branched 4-NP's, the toxicity of NP's differs according to the isomers. Makino et al⁹⁾ reported that the 3 paired of 4-NP diastereomers were found in commercial NP mixture such as 4-(3,4-dimethyl heptan-4-yl)phenol (NP-J, NP-L), 4-(3,4-dimethyl heptan-3-yl)phenol (NP-K, NP-P) and [4-(3,5- dimethyl heptan-3-yl)phenol (NP-E, NP-G)]. In this study, we have tried to evaluate eco-toxicity of six 4-NP diastereomers by algal test using DL. The stereo-structure relationship on DL by 4-NP diastereomers are compared with both the estrogenic activity and fish toxicity.

The structure-relationship on eco-toxicity of 4-NP diastereomers were evaluated both EC₅₀ values of DL to alga at 1h, 6 h and 24h and the dose dependent alternation patterns of inhibition ratio during 24h by DL to alga. NP-P and NP-L having the methyl proton signal at the β-carbon in a higher field, showed more toxic effect to both EC₅₀ at 24h exposure and the alternation patterns of inhibition ratio of 4-NP diastereomers as compared with the corresponding diastereomers (NP-K and NP-J). From the set of the diastereomers having a methyl group to the γ-carbon, NP-G appeared higher than NP-E. It showed that The isomers (NP-P, NP-L and NP-G) of the higher field methyl proton signal appeared more toxic effect to algae than the corresponding diastereomers (NP-K, NP-J and NP-E), and the toxicity of γ-carbon to benzene ring (NP-G and NP-E) is not so high as compared to that of β-carbon (NP-P, NP-K, NP-L and NP-J).

NP-P (14.6) and NP-L (8.8) were also 2 and 4 times more potent estrogenic activity than their diastereomers (NP-K (6.5) and NP-J (2.0)), respectively. And NP-E (3.8) and NP-G (5.4) were showed lower estrogenic activity. The toxicity of alga and the estrogenic activity indicated almost same tendency related to their stereo-structure. The toxicity of 4-NP diastereomers to *Oryzias latipes* is relatively higher than linear chain 4-NP (4-n-NP). But stereo-structural effect to fish toxicity did not find clearly. Because the DL of alga is significantly affected by linear chain 4-NP (4-n-NP), 4-n-NP has severe action to algae more than fish.

Table 1 EC₅₀ of alga and LC₅₀ (*Oryzias latipes*) and the estrogenic activity exposed to 4-NP's, in vitro estrogenic activity which indicated related E2 and its chemical shift condition.

Isomers	EC50 ¹⁾ 1h	EC50 6h	EC50 24h	IR(% ²⁾ 1µg/mL	LC ₅₀ ³⁾ 96h	NOEL ⁴⁾ E2	
NP-E	0.45	0.84	2.58	28.6	347	3.8	0.76 ⁵⁾
N-G	1.25	0.82	1.39	36.7	360	5.4	0.49 ⁵⁾
NP-J	0.95	0.68	1.86	38.7	287	2.0	0.88 ⁶⁾
NP-L	ND	0.51	0.85	51.5	297	8.8	0.58 ⁶⁾
NP-K	1.37	0.90	1.31	63.5	314	6.5	0.87 ⁶⁾
NP-P	0.92	0.66	0.64	68.0	347	14.6	0.58 ⁵⁾
4-n-NP	0.21	0.34	0.38	93.0	439	-	-
4-NP			0.40			7.2	-

- 1) EC₅₀ of alga: DL after 24hr NP exposure(1µg/mL).
- 2) The inhibition ratio to alga after 24hr NP at 1µg/mL exposure (%).
- 3) LC₅₀ (*Oryzias latipes*) after 96hr NP exposure(1µg/mL). (Kobayashi et al 2013).
- 4) The estrogenic activity which indicated related E2(Murase et al 2008).
- 5) γ-carbon, the δ-methyl proton signal,
- 6) β-carbon, the γ-methyl proton signal

References

- 1 Naylor, C. G. ((1995) *Textile Chemist and Colorist*, **27**, 29-33.
- 2 Ishibashi H, Hirano M, Matsumura N, Watanabe N, Takao Y and Arizono K (2006) *Chemosphere*: **65**, 1019-1026.
- 3 Tatarazako N (2012) *Journal of Printing Science and Technology*: **49**, 42-44.
- 4 Katsumata M, Koike T, Nishikawa M and Tsuchiya H (2005) *Journal of Japan Society on Water Environment*, **28**, 23-28.
- 5 Katsumata M, Koike T, Nishikawa M, Kimiko Kazumur K and Tsuchiya H. (2006) *Water Research*: **40**, 3393-4000.