ISOMER-SPECIFIC ANALYSIS OF NONYLPHENOLS WITH ESTROGENIC ACTIVITY AND THEIR DISTRIBUTION IN AQUATIC ENVIRONMENT IN RELATION TO ENDOCRINE DISRUPTERS

Yun-Seok KIM[1], Takao Katase[1], Yuichi Horii[2], Nobuyoshi Yamashita[2], Mitsuko Makino[3], Taketo Uchiyama[3], Yasuo Fujimoto[3], Tadashi Inoue[1]

[1]College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa, Kanagawa, 252-8510, Japan
[2]National Institute of Advanced Industrial Science and Technology, 16-1 Onogawa, Tsukuba, Ibaraki 305-8569, Japan
[3]College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi, Chiba, 274-8555, Japan

Introduction

The effect of estrogen-exposure on levels of a larval storage protein of *Balanus amphitrite*, cypris major protein (CMP), which is related to barnacle vitellin, has been examined at low concentrations (0.01–1.0 μg/l) of 4-nonylphenol (NP) and 17β-estradiol (E2) (1.0 μg/l) from egg hatching until the nauplius cypris stage[1]. Eventually, the exposure to 0.01 μg/l of NP led to a ca. 50% increase in the optical density of the CMP. There are theoretically ca. 170 kinds of isomers of NP, based on the structure of the nonyl side chain in NP[2]. We fractionated a commercial NP by high performance liquid chromatography (HPLC) to give six fractions (Fr. 1- Fr. 6). Fr. 3 - Fr. 5 were further separated to afford 14 fractions by using gas chromatograph equipped with a preparative fraction collector (GC-PFC) and 11 NP isomers were identified by gas chromatograph equipped with mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR). The chemical structures of 11 isomers (NP1 to NP14) were characterized and estrogenicities of the selected isomers were tested in recombinant yeast screen system. The 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol (NP7) was found to exhibit the highest estrogenic activity corresponding to 1.9×10^{-3} that of E2[3,4]. The NP4 and 6 were structurally in diastereomer. The individual isomer of NP in aquatic samples taken from Ariake Sea and Tokyo, Japan was analyzed by steam distillation extraction in the present study.

Methods and Materials

Extraction of NP from aquatic samples: The water samples from sea, river and lake were collected at 3 locations in Ariake Sea and 16 locations in river and lake in Tokyo area between 2003 and 2004. The sea water samples were taken from river mouth (N33° 11' 28" and E130° 13' 57", at

0 point distance) toward offshore (N33˚ 04' 19" and E130˚ 14' 38", 12km far from the mouth) in Ariake Sea, Japan. Isomer specific analysis of 13 individual NP based on relative response factor (RRF) quantification by GC-MS in combination with steam distillation extraction (SDE) from water samples was carried out[5]. Suitable ions to quantify; m/z 107, 121, 135, 149, 163, 177, 191 and 220 was used for the identification and quantification of 13 isomers of NP. One litter of individual water sample was distillated for 5 hours by simultaneous extraction with 20 ml of cyclohexane. Supplementary clean-up of the extracts was performed by a glass silica solid-phase extraction cartridge with 10 ml of diethylether and hexane (3:7 v/v) to elute the NP. The elutes were identified and quantified by a large volume injection GC-MS using selected ion monitoring. Quantification of the individual NP isomers in the samples was carried out by the internal standard method using RRF. A portion of NP-$^{13}C_6$ of 15 ng was spiked into samples as internal standard. Recovery of NP-$^{13}C_6$ in spiked water samples was 50% to 100% for samples tested. The detection limit (ND) for total NP was 6 ng/l.

Method of Identification: Some of the fractions of NP by HPLC were further fractionated by GC-PFC. Structures of the major components in the each fraction were determined by analysis of their mass spectra and ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra[3,4].

Estrogenic activity: Each fraction by GC-PFC was tested for estrogenic activity by the recombinant yeast screen assay[3,6]. The yeast was kindly supplied by Dr. Sumpter, Brunel University, UK. The amounts of color development were plotted against the molar concentrations of sample to give a dose-response curve. From this curve, the minimal effective concentration was calculated as that gives the half of the maximum effect. Under our conditions, the minimal effective concentrations varied 20 to 50 % depending on samples. We therefore carried out 4 to 6 independent experiments and calculated the mean value. For comparison of the activities between the fractionated NP samples, the minimal effective concentration of each sample was compared with that of E2, which was included in all the assay plates as the standard.

Results and Discussion

Isomer-specific analysis of NP in aquatic environmental samples: Total concentrations of NP isomers in Ariake sea water samples are shown in Fig. 1. The NP in the water samples in Ariake Sea was diminishing toward offshore (St.C) from river mouth (St.A); St.A; 55 ng/l, St.B; 32 ng/l, St.C; 17 ng/l. The detection limit (ND) was 6 ng/l. Bester *et al*. (2001)[7] reported that concentration of NP in North Sea between coast and 100 km-offshore were from 0.7 to 4.4 ng/l. The NP concentrations in Ariake Sea were 10 time higher than in North Sea. The NP-concentration of 10 ng/l effected on barnacles according to a report by Billinghurst *et al*. (2000) [1], which is one fifth of the concentration observed at St.A in Ariake Sea, and is just slightly lower than that at St.C. The NP in Ariake Sea might effect on the marine livings. Total concentrations of NP isomers in river water varied from ND (0.038 μg/l) to 5.4 μg/l (Fig. 2). The waters at the locations, St.2, St.11, St.4, St.12 and St.13 might be polluted by industrial wastewater. The concentration of NP isomers in Kasumigaura lake water (St.15; 0.14 μg/l) was observed higher than that in river water (St.16; 0.05 μg/l). The concentrations were lower than those in Europe rivers (up to 180 μg/l) [8,9] but similar to those in rivers in the US (ND-0.64 μg/l)[10]. Isobe *et al*. (2001) reported that chromatogram of NP consists of 10 isomer peaks and the NP concentration in river water of Tokyo area ranged from

0.051 to 1.08 μg/l samples with higher concentrations in summer and spring than in winter[11]. The NP in lake might be input from the rivers and accumulated here. The Japanese Ministry of the environment reported that the predicted no-effect concentration of NP for Japanese medaka (*Oryzias latipes*) was 0.6 μg/l[2]. Concentrations of NP in some locations were higher than this level. Concentration of individual NP isomer in aquatic environments by development is shown in Fig. 3. Method of isomer specific quantification for thirteen individual NP isomers was based on relative response factor quantification by GC-MS. The compositions of individual NP isomer in aquatic environment were shown in Fig. 4. In this study on coefficient of variation value, the significant variation of the isomer composition of NP varied between 4%~75% in sea water and between 11%~38% in river water. This suggests that NP isomers might be independently degraded in aquatic environmental samples. However, Isobe *et al*. (2004) pointed out that isomer compositions between source material and those detected in various environmental samples was uniform and not varied[12].

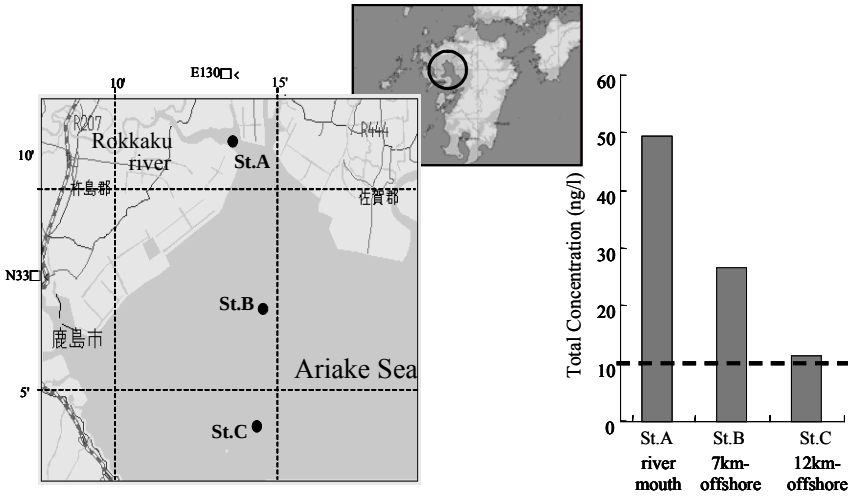

Fig. 1 Concentration of NP in sea water taken from Ariake Sea, Japan. ND ; < 6 ng/l. ---- ; effect level (10 ng/l) of NP for barnacles *B. amphitrite*.

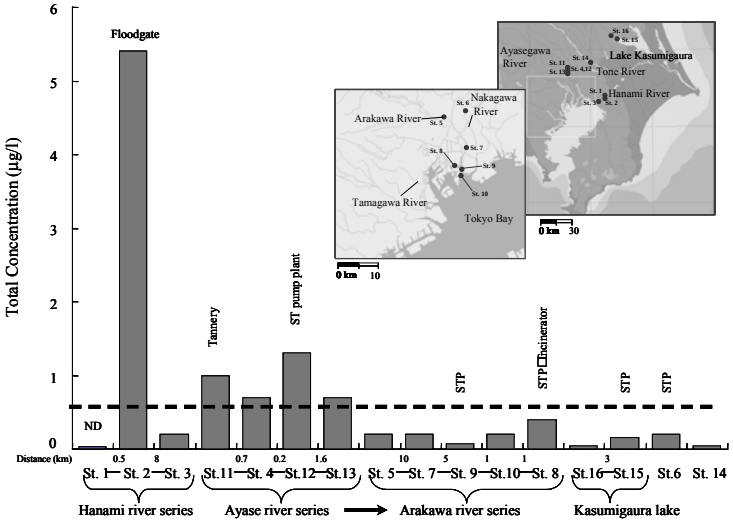

Fig. 2 Concentration of NP in river water taken from Tokyo area, Japan. STP; sewage treatment plant. ND ; < 0.038 μg/l. -----; no-effect level (0.6 μg/l) of NP for Japanese Medaka.

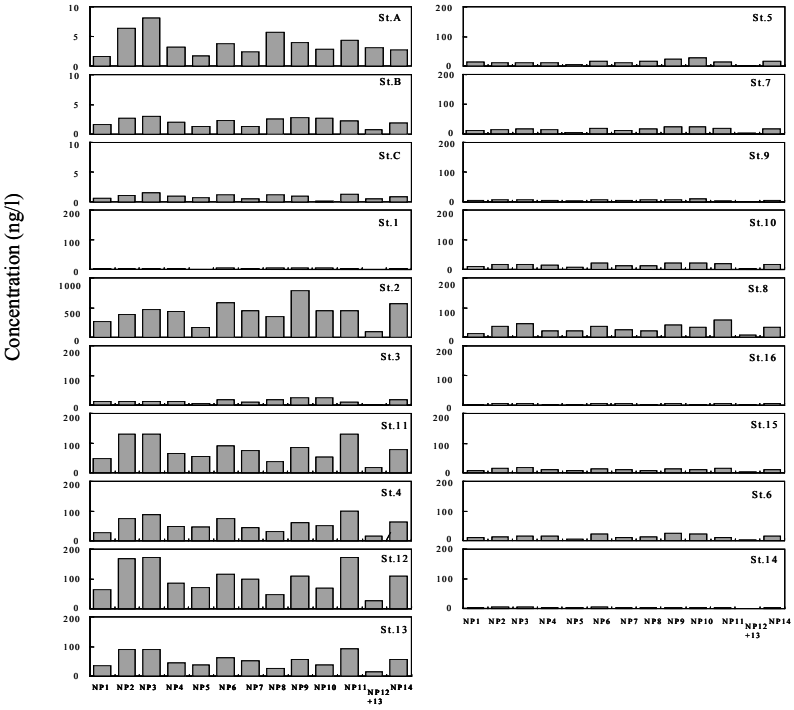

Fig. 3 Concentration of individual NP isomer in aquatic environment. (St.A~C for Ariake Sea, St.1~St.16 for river and lake water in Tokyo area)

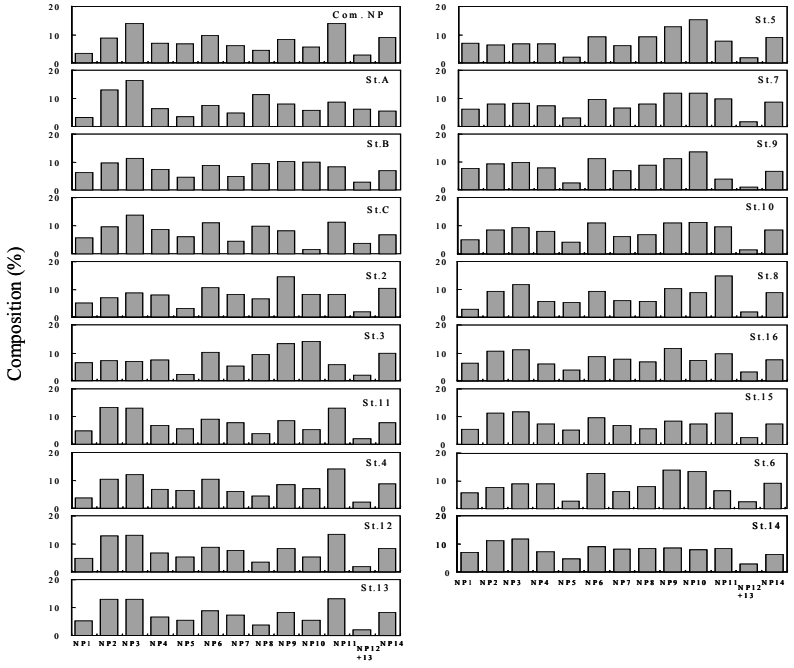

Fig. 4 Composition of individual NP isomer in aquatic environment. (St.A~C for Ariake Sea, St.2~St.16 for river and lake water in Tokyo area)

Estrogenic activity of NP isomers and their identification by NMR: The relative estrogenic activity of 12 isomers of NP by recombinant yeast screen assay is shown in Fig. 5. The NP7 exhibited the highest estrogenic activity corresponding to 1.9×10^{-3} that of E2. The second highest estrogenic isomer was the NP3 as well as NP12. The activity of NP11 was the least and NP9 and NP10 were not attempted. The extent of estrogenicities of the fractions was calculated as relative values to that of E2. The tertiary NP, 4-(1,1-dimethyl-heptyl)-phenol was synthesized in our preliminary study and this synthetic NP also exhibited the estrogenic activity[3]. The structural elucidation and the estimation of the purity of separated fractions were performed by analyses of their NMR spectra. Their isomers, NP1 to NP14 were attempted to be identified structurally as shown in Fig. 5. The three isomers, NP8, 13 and 14 still remain to be identified. The following results by the present study were obtained; the chemical structures of 11 isomers of NP1 to NP14 were confirmed as 4-(1,3-dimethyl-1-propyl-butyl)-phenol for NP1 (63%), 4-(1,1,3-trimethyl-hexyl)-phenol for NP2 (74%), 4-(1,4-dimethyl-1-ethyl-pentyl)-phenol for NP3 (61%), 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol for NP4 (80%), 4-(1,1,4-trimethyl-hexyl)-phenol for NP5 (68%), 4-(1,3-dimethyl-1-ethyl-pentyl)-phenol for NP6 (87%), 4-(1,1-dimethyl-2-ethyl-pentyl)-phenol for NP7 (68%), 4-(1,2-dimethyl-1-ethyl-pentyl)-phenol for NP9 (90%), 4-(1,2-dimethyl-1-propyl-butyl)-phenol for NP10 (53%), 4-(1,1,2-trimethyl-hexyl)-phenol for NP11 (75%) and 4-(1-ethyl-1-methyl-hexyl)-phenol for NP12 (63%) using GC-MS and NMR spectra. NP4 and 6 were structurally in diastereomer. A structure-activity relationship will be discussed on our further work.

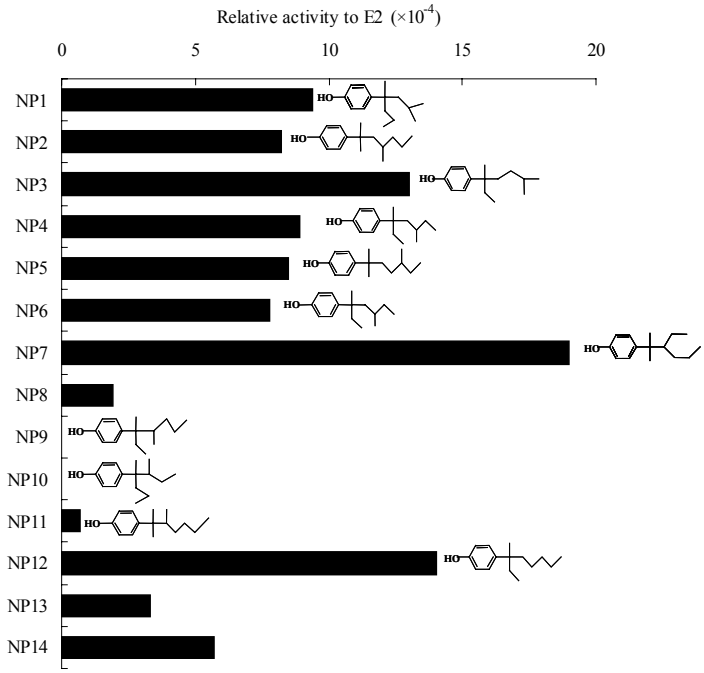

Fig. 5 Relative estrogenic activity of NP isomers, NP1 to NP14. The activities of NP9 and 10 were not measured yet. The structures of NP8, 13 and 14 are not determined yet.

Environmental estrogen-effective concentration of NP: In our present study, environmental composition of NP was found to vary. An estrogen-effective concentration of NP in environment might be needed to calculate because individual isomer has different activity as shown in Fig. 5. The estrogen-effective concentration (EEC) of NP is defined as follows:

$$EEC = C(NP_n) \times E(NP_n)$$

where $E(NP_n)$ =estrogenic activity of individual isomer, NP1 to NP14.
$C(NP_n)$ =concentration of individual isomer.

For example, EEC of NP in Ariake Sea is shown in Table 1. The concentration of NP (A) in Ariake Sea supposed to be 2.7-to- 3.0-times overestimated estrogen-effectively.

Table 1 Estrogen-effective concentration (EEC) of NP in Ariake Sea.

	Concentration (A) (ng/l)			Estrogenic activity (B)*	Estrogen-effective concentration (EEC) (C=A×B) (ng/l)		
	St.A	St.B	St.C		St.A	St.B	St.C
NP1	1.6	1.6	0.6	0.49	0.8	0.8	0.3
NP2	6.4	2.6	1.1	0.43	2.8	1.1	0.5
NP3	8.0	3.0	1.6	0.68	5.5	2.0	1.1
NP4	3.2	2.0	1.0	0.47	1.5	0.9	0.5
NP5	1.7	1.2	0.7	0.45	0.8	0.6	0.3
NP6	3.7	2.3	1.2	0.41	1.5	1.0	0.5
NP7	2.4	1.3	0.5	1.00	2.4	1.3	0.5
NP8	5.7	2.5	1.1	0.10	0.6	0.3	0.1
NP9	3.9	2.7	0.9	0.00	0.0	0.0	0.0
NP10	2.8	2.6	0.2	0.00	0.0	0.0	0.0
NP11	4.3	2.2	1.3	0.04	0.2	0.1	0.0
NP12+13	3.1	0.7	0.4	0.46**	1.4	0.3	0.2
NP14	2.7	1.8	0.8	0.30	0.8	0.6	0.2
Total	49	27	11	-	18	8.9	4.2
Ratio (A / C)	2.7	3.0	2.7		-	-	-

* Relative activity to NP7

** Estrogenic activity for NP12+13 was calculated as follows : {E (NP12)+ E (NP13)}/2

Acknowledgments

The authors are thankful to Dr. E. Sumi and Dr. N. Hanari, Advanced Industrial Science and Technology (AIST) for their cooperation in collecting environmental samples and Dr. S. Ueda, Nihon University for his useful discussion and valuable comments of this report.

References

1. Billinghurst Z., Clare A.S., Matsumura K., Depledge M.H. (2000) Aquat. Toxicol. 47, 203.
2. Ministry of the Environment (2001) Report on the test of results of endocrine disrupting effects of nonylphenol on fish (draft). Government of Japan, Tokyo.
3. Kim Y.S., Katase T., Sekine S., Inoue T., Makino M., Uchiyama T., Fujimoto Y. and Yamashita N. (2004a) Chemoshphere 54, 1127.
4. Kim Y.S., Katase T., Makino M., Uchiyama T., Fujimoto Y., Inoue T. and Yamashita N. (2004b) Australasian J. Ecotoxicology. in submission.
5. Horii Y., Katase T., Kim Y.S. and Yamashita N. (2004) Bunsekikagaku. in submission.
6. Routledge E.J. and Sumpter J.P. (1996) Environ. Toxicol. Chem. 15, 241.

7. Bester K., Theobald N. and Schroder H.Fr. (2001) Chemosphere 45, 817.
8. Ahel M., Giger W. and Schaffner C. (1994) Wat. Res. 28, 1143.
9. Blackburn M.A. and Waldock M.J. (1995) Wat. Res. 29, 1623.
10. Naylor C.G., Mieure J.P., Adams W.J., Weeks J.A. and Castaldi F.J. (1992) JAOCS. 69, 695.
11. Isobe T., Nishiyama H., Nakashima A. and Takada H. (2001) Environ. Sci. Technol. 35, 1041.
12. Isobe T. and Takada H. (2004) Environ. Toxicol. Chem. 23, 599.