with a gas-phase sequencer (model 473A, Applied Biosystems, CA, USA), following the procedure described by Oba et al. (2007). The samples containing rTBT-bp1 were pooled and then concentrated to 38 μM in phosphate buffered saline (PBS; 137 mM NaCl, 8.1 mM $Na_2HPO_4$ 12 $H_2O$, 2.68 mM KCl, 1.47 mM $KH_2PO_4$) by ultrafiltration.

### 2.6. Purification of nTBT-bp1 from the blood of Japanese flounder

One cultured Japanese flounder was obtained from Heisei Suisan (Fukuoka Prefecture, Japan) in May 2009. The blood collected from the caudal vessel of the fish with a syringe, clotted at 4 °C overnight and centrifuged at 12,000 × *g* for 10 min. The serum was separated and stored at −20 °C. The serum of the flounder (5 ml) was used for ammonium sulfate fractionation. Solid ammonium sulfate was added to 5 ml serum (to 50% saturation), and the solution was stirred for 30 min and then centrifuged for 30 min at 17,000 × *g*. Additional ammonium sulfate was added to the supernatant to achieve 70% saturation, and the procedure described above was repeated. The supernatant was dialyzed against of PBS (pH 7.4) by using an Amicon Ultra-15 Ultracel 30,000 membrane (Millipore). The following procedures were carried out at 4 °C.

The supernatant obtained from ammonium sulfate fractionation at 70% saturation was subjected to native polyacrylamide gel electrophoresis (native-PAGE) as described previously (Shapiro et al., 1967). Negative staining of proteins in polyacrylamide gels with imidazole-zinc salts was performed as described previously (Fernandez-Patron et al., 1992). The visualized proteins were cut out and eluted from the gel with a Model 422 Electro-Eluter (Bio-Rad Laboratories, CA, USA). The protein concentrations were calculated by means of a bicinchoninic acid acid assay (BCA protein assay reagent; Thermo Fisher Scientific K.K., Tokyo, Japan).

N-terminal amino acid sequence of nTBT-bp1 separated by SDS–PAGE was determined by Edman degradation with a PSQ-1 protein sequencer (Shimadzu, Kyoto, Japan) as described above. The proteins on the gel were visualized by means of silver staining kit (Bexel Biotechnology, CA, USA) according to the manufacturer's instructions. The purified nTBT-bp1 was digested with a set of de-*N*-glycosylation (Takara, Tokyo, Japan), and the deglycosylated sample was analyzed by SDS–PAGE that was conducted under the same conditions used for purification of rTBT-bp1.

### 2.7. Binding assay

TBT (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in dimethyl sulfoxide (DMSO), and the resulting solution was diluted with PBS. Equal volumes of 38 μM rTBT-bp1 solution and 380 μM TBT (2600 μl total) were mixed in a glass test tube and incubated for 16 h at 15 °C. After filtration with a 0.5-μm filter, 100 μl of the mixture was removed, and the rest was subjected to gel filtration chromatography on a TSK gel G2000SWxl column equilibrated with the elution buffer (50 mM sodium phosphate, 300 mM NaCl; pH 7.0). The flow rate was maintained at 0.6 ml/min, and eluents were fractionated at 1-min intervals for 27 min. The column was washed with distilled water for 20 min, and the eluent was collected in a test tube. The column was also washed with 50 mM sodium phosphate, 0.1 M NaCl and 10% DMSO (pH7.0) for 4 h and eluents were fractionated for 30 min. Then the fractions (except for 100 ml) were subjected to gas chromatography–mass spectrometry (GC–MS, model 6890 gas chromatograph, model 5973 mass spectrometer; Hewlett-Packard, Avondale, PA, USA) for the detection of TBT. A 100 μl aliquot of each fraction was analyzed by SDS–PAGE as described above, and protein concentrations were measured by means of a bicinchoninic acid acid assay.

### 2.8. Effect of rTBT-bp1 on inhibition of osteoblastic activity by TBTCl in the cultured scales of goldfish

We detected alkaline phosphatase activity in scales as a valid marker for osteoblastic activity with the method described in our previous study (Suzuki et al., 2006). Eight scales were collected from one goldfish under anesthesia with ethyl 3-aminobenzoate and methane sulfonic acid salt (MS-222, Aldrich Chemical Company, Inc., WI, USA), and each scale was cut into half. One half of each scale was incubated for 6 h in 1 ml of minimum essential medium supplemented with TBTCl ($10^{-8}$ to $10^{-6}$ M) or TBT-bp1-containing TBTCl ($10^{-8}$ to $10^{-6}$ M) less than cytotoxicity level (Grzyba et al., 2003; Raffray et al., 1993). The other half of each scale was incubated for 6 h in 1 ml of TBTCl-free medium as a control. After incubation, each half-scale was placed in a well of a 96-well microplate. A 100 μl aliquot of an alkaline buffer (100 mM Tris-HCl, pH 9.5; 1 mM $MgCl_2$; 0.1 mM $ZnCl_2$) was added to each well. The plate was immediately frozen at −85 °C and then kept at −20 °C until analysis. The plate was allowed to thaw, and 100 ml of *para*-nitrophenol-phosphate in an alkaline buffer was then added to the melted solution in each well of the microplate. The plate was incubated at 20 °C for 30 min with shaking. After incubation, the reaction was stopped by the addition of 50 μl of 2 N NaOH. 150 μl of the resulting colored solution was transferred to a new plate, and the absorbance was measured at 405 nm. The absorbance was converted into the amount of produced *para*-nitrophenol (pNP) using a standard curve for pNP. After measurement of alkaline phosphatase activity, each scale was dried and weighed.

The results are shown as means ± standard error. Statistical analyses were performed with Student's t-test tool in Microsoft Excel 2004 (Microsoft Co. Ltd., Tokyo, Japan). Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Purification of rTBT-bp1 and nTBT-bp1

We succeeded in expressing and purifying TBT-free rTBT-bp1. A Gateway entry clone and transfer vector for TBT-bp1 expression were constructed as described in materials and methods. The transfer vector pFBTBT-bp1-His6 was transformed into *E. coli* with BmDH10Bac cells harboring genomic DNA of BmNPV to induce *in vivo* transposition mediated by Tn7 transposase (Motohashi et al., 2005). The recombinant baculovirus was generated by transfecting the bacmid DNA into silkworm Bme21 cells, and the amplified P3 virus stock was injected into the hemocoels of silkworm larvae. rTBT-bp1 was secreted into the hemolymph of the larvae according to a signal sequence from Japanese flounder. The hemolymph was rapidly collected and purified by means of histidine affinity chromatography (Fig. 1A). For removal of contaminating proteins originating from the hemolymph of the silkworms, fractions containing rTBT-bp1 were pooled and subjected to gel filtration chromatography under appropriate conditions. The final sample exhibited two bands, one at about 30 kDa and another at 35 kDa, on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1B). These bands were identified as rTBT-bp1 by analysis of the $NH_2$-terminal amino acid sequence (Table 2).

Purification of nTBT-bp1 on a native-PAGE gel gave a band at 44 kDa on SDS–PAGE with visualization by silver staining (Fig. 2). This band was confirmed to be nTBT-bp1 by the analysis of the $NH_2$-terminal amino acid sequence (Ala–Pro–Thr–Glu–Glu–Glu–Ser–Gln–Leu–Val–Ser–Pro–Val). A total of 226.2 μg nTBT-bp1 was purified from 1 ml of serum.

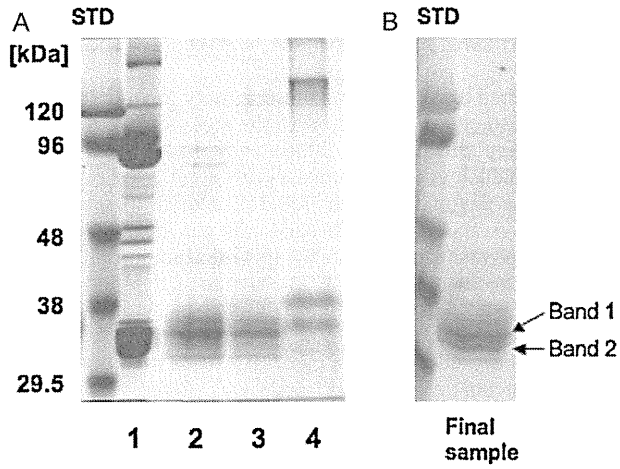


**Fig. 1.** Purification of rTBT-bp1 detected by SDS–PAGE. (A) Detection of His-tagged rTBT-bp1 fractionated by affinity chromatography: lane 1, flow-through material; lane 2, eluate at 50 mM imidazole; lane 3, eluate at 150 mM imidazole; lane 4, eluate at 450 mM imidazole. (B) Detection of final sample of His-tagged rTBT-bp1 fractionated by gelfiltration chromatography. STD is molecular weight standard.

**Table 2**
Results of N-terminal amino acid sequence analysis. Band 1 and 2 are upper and lower band of final sample, respectively.

| | N-terminal amino acid sequence |
|---|---|
| Band 1 (upper) | APTPEETSQLVSPVS |
| Band 2 (under) | APTPEEXXQLV |

### 3.2. TBT binding assay

A binding assay indicated that rTBT-bp1 bound TBT. After incubation of the rTBT-bp1 solution (38 μM) with TBT solution (380 μM) at 15 °C for 16 h, the mixture was fractionated by gel filtration chromatography. rTBT-bp1 was detected in fractions 18 and 23, and the peaks for TBT were corresponded to those for the protein (Fig. 3). This result indicated that rTBT-bp1 bound TBT because the molecular weight of rTBT-bp1 and TBT were 35 kDa and 289 Da, respectively. The molar ratios of rTBT-bp1 to TBT in fractions 18 and 23 were 6:1 and 4:1 (rTBT-bp1:TBT), respectively. The molecular weights of rTBT-bp1 estimated from the elution times were 27 kDa for fraction 18 and 3.2 kDa for fraction 23. These values did not agree with the values determined by means of SDS–PAGE analysis (*i.e.*, 30 and 35 kDa).

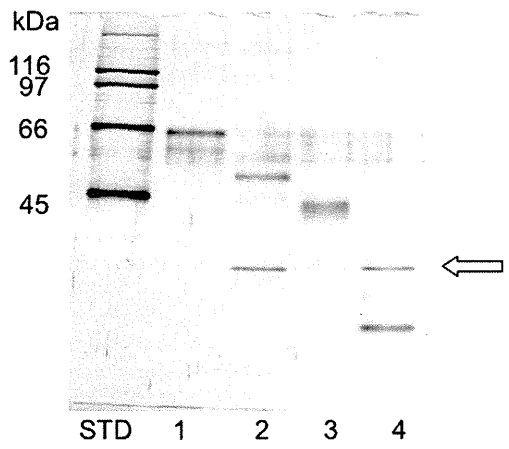


**Fig. 2.** SDS–PAGE of purified and deglycosylated TBT-bp1: lane 1, control glycoprotein; lane 2, deglycosylated control glycoprotein; lane 3, purified TBT-bp1; lane 4, deglycosylated TBT-bp1. The arrow indicates glycosidase F. STD is molecular weight standard.

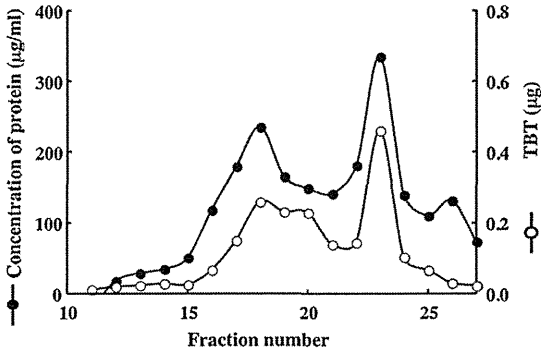


**Fig. 3.** Elution profile of mixture containing rTBT-bp1 and TBT obtained by gel filtration chromatography on a TSK gel G2000SWxl column.

### 3.3. In vitro assay of restoration of osteoblastic activity inhibited by TBT in cultured scales of goldfish

The results of the assay for the ability of rTBT-bp1 ($10^{-7}$ M) to restore osteoblastic activity inhibited by TBT are shown in Fig. 4A. The osteoblastic activities in the scales of the TBT treatment group significantly lower than those of the control ($10^{-8}$ M: $P=0.032$; $10^{-7}$ M: $P=0.006$; $10^{-6}$ M: $P=0.0006$). In contrast, osteoblastic activities were recovered in the group of co-treated with rTBT-bp1 ($10^{-7}$ M) and $10^{-8}$ or $10^{-6}$ M TBT, although at $10^{-6}$ M TBT osteoblastic activity remained significantly inhibited (Fig. 4B, $P=0.036$). These results indicate that rTBT-bp1 restored osteoblastic activity inhibited by TBT.

The results of a similar assay conducted with nTBT-bp1 ($10^{-7}$ M) are shown in Fig. 4C. Osteoblastic activity was restored in all the groups co-treated with nTBT-bp1 ($10^{-7}$ M) and TBT ($10^{-8}$, $10^{-7}$, or $10^{-6}$ M). The nTBT-bp1 restored osteoblastic activity as well or better than rTBT-bp1.

## 4. Discussion

We obtained histidine-tagged rTBT-bp1 by means of baculovirus gene expression system in silkworm larvae (Fig. 1). The predicted molecular weight of rTBT-bp1, which consists of 280 amino acid residues, is approximately 30 kDa. This value corresponds to the molecular weight calculated for the lower band (band 2) observed on the SDS–PAGE. However, the molecular weight of the upper band (band 1) was estimated to be 35 kDa. The difference between the molecular weight of the two bands might be due to glycosylation modification in the endoplasmic reticulum of the silkworm cells. nTBT-bp1 purified from blood of Japanese flounder is reported to be 42% glycosylated with N-linked sugar chains (Shimasaki et al., 2002). In the expression system using silkworm larvae, some recombinant glycoprotein is expressed in various glycoforms (Wu et al., 2002; Kato and Park, 2007). Kato and Park reported that the signal peptide might relate to glycosylation of two types of fusion proteins produced in Bm5 cells. In our expression system, the signal peptide of TBT-bp1 originating from Japanese flounder would have contributed to these two types of rTBT-bp1.

The TBT binding assay strongly suggests that rTBT-bp1 retains a binding ability by folding into the same structure as nTBT-bp1. Shimasaki et al. (2002) reported that the binding ratio of nTBT-bp1 to TBT is 30:1.

In gel filtration analysis, rTBT-bp1 and TBT co-eluted in the same two fractions 18 and 23. SDS–PAGE analysis indicated that molecular weights of proteins eluted during the gel filtration analysis were

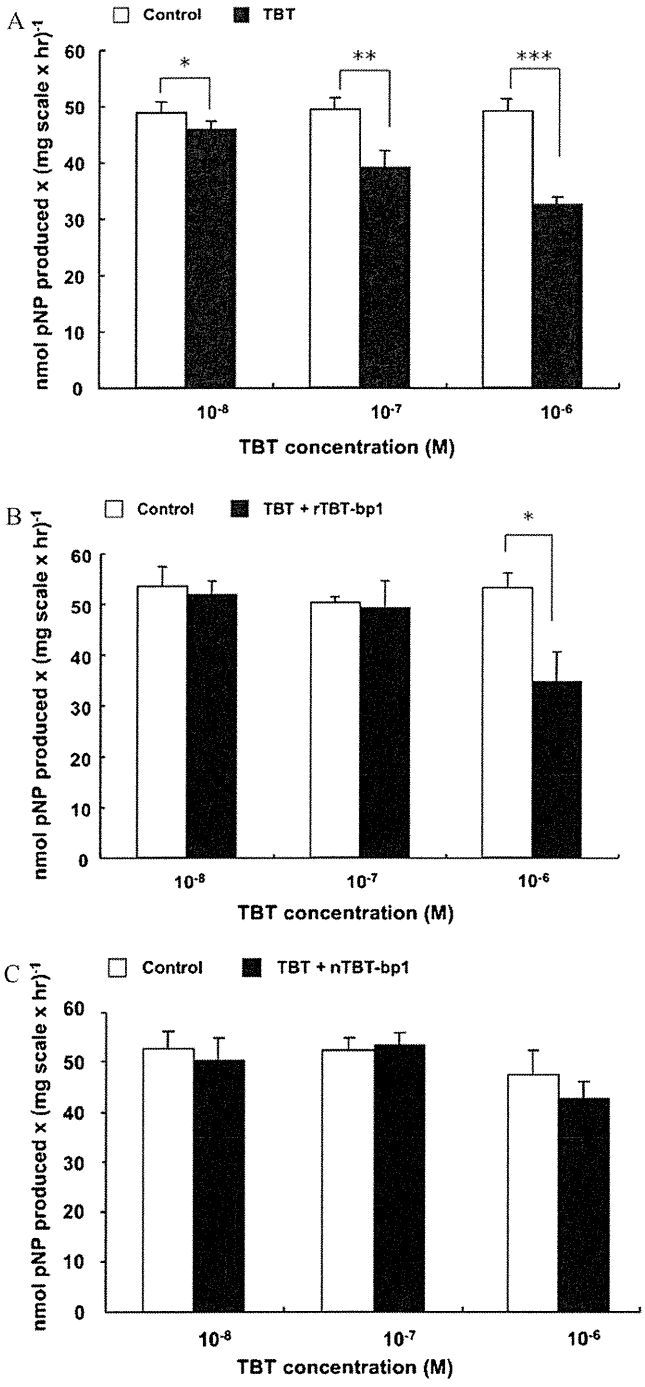


**Fig. 4.** Effects of (A) TBT, (B) TBT puls rTBT-bp1, and (C) TBT plus nTBT-bp1 on osteoblastic activity in cultured goldfish scales after 6 h of incubation. The alkaline phosphatase (ALP) activity was determined as a mesure of osteoblastic activity. *, **, and *** indicate statistically significant difference to the level of activity in the control scales, at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

30 and 35 kDa, respectively. These were confirmed to be rTBT-bp1 by N-terminal amino acid analysis. However, molecular weights of 27 kDa and 3.2 kDa were calculated for fractions 18 and 23, respectively. The result of gel filtration of glycoprotein tends to change according to its carbohydrate content, shape, adsorption and so on (Andrews, 1965). The rTBT-bp1 proteins in both fraction bound TBT, despite the proposed difference in glycosylation. This result suggests that glycosylation of TBT-bp1 did not contribute to the protein's ability to bind TBT.

The addition of rTBT-bp1 or nTBT-bp1 with TBT exerted the recovery of osteoblastic activity inhibited by a single exposure to TBT at $10^{-7}$ M (Fig. 4). It is suggested that TBT-bp1 binds to TBT and reduces its toxic effects on scale calcification or on bone tissues that results in malformation. In medaka larvae, it was demonstrated that the malformation of the spinal marrow and tail fin might be caused by TBT that has been maternally transferred to the embryo (Hano et al., 2007). In adult fish or mammals, there have been no reports of TBT induction of abnormal bone formation. Malformation of bone during developmental stage induced by TBT might arise from low levels of endogenous TBT-bp1 resulting in the inhibition of calcification. Further experiments are needed to clarify the expression of the TBT-bp1 gene during development.

The ability of rTBT-bp1 to restore the osteoblastic activity was lower than that of nTBT-bp1, even though it was demonstrated that rTBT-bp1 retained a higher binding ability than nTBT-bp1. The reason of this difference between recombinant and native protein is not clear. However, we expect that TBT-bp1 has another mechanism for TBT binding to protect the scales using for example sugar chain recognition.

TBT-bps in fish may generally respond to exposure of chemicals. Thirteen putative TBT-bp-like proteins have been found in eight species of teleost including medaka, zebrafish (*Danio rerio*), and spotted green pufferfish (*Tetraodon nigroviridis*) (Satone et al., 2008). In the brain of Japanese medaka, the expression levels of two types of genes encoding TBT-bp-like proteins are altered by polychlorinated biphenyls (Volz et al., 2005). The expression levels of mRNA for TBT-bp1 were up regulated in medaka liver exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Nakayama et al., 2008). Further experiments are required to reveal the binding ability of TBT-bps to chemicals.

In conclusion, we purified rTBT-bp1 and confirmed that it bind to TBT. We found that TBT-bp1 bind to TBT in the body of fish and restored osteoblastic activity inhibited by TBT. This report related to protective function of TBT-bp1 on fish body surface will be valuable to future experiments investigating physiological functions of TBT-bps and their homologs.

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—Rapid Communicatio

# Mutagenicities and Endocrine-disrupting Activities of 1-Hydroxy-2-nitropyrene and 1-Hydroxy-5-nitropyrene

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**The mutagenicities and endocrine-disrupting activities of two isomers of mononitrated 1-hydroxypyrene [1-hydroxy-*x*-nitropyrenes (1-OH-*x*-NPs); *x* = 2 and 5], which are not only photoreaction products of 1-nitropyrene (1-NP) but also constituent of ambient airborne particles, were evaluated for the first time using the Ames plate incorporation assay and the yeast two-hybrid assay, respectively. The mutagenicity of 1-OH-5-NP was weakly positive in the absence of rat liver S9, but was enhanced up to 3-fold with the metabolic activation by S9. On the contrary, 1-OH-2-NP did not exhibit significant mutagenicity in the presence or absence of S9. 1-OH-5-NP showed weak estrogenic activity, but 1-OH-2-NP did not show any estrogenic activity. The concentration of 1-OH-5-NP that gave 10% of activity of $1.0 \times 10^{-6}$ M 17$\beta$-estradiol ($E_2$) was $5.4 \times 10^{-7}$ M. 1-OH-5-NP exhibited stronger antiestrogenic and antiandrogenic activities than 1-OH-2-NP. 1-OH-5-NP at a concentration of $1.0 \times 10^{-6}$ M inhibited 71 and 90% of $\beta$-galactosidase activity induced by $1.0 \times 10^{-9}$ M of $E_2$ and $1.0 \times 10^{-8}$ M of 5$\alpha$-dihydrotestosterone (DHT), respectively. On the other hand, $1.0 \times 10^{-6}$ M of 1-OH-2-NP inhibited 16 and 43% of $\beta$-galactosidase activity induced by $1.0 \times 10^{-9}$ M of $E_2$ and $1.0 \times 10^{-8}$ M of DHT, respectively. These findings point out the need for determining the environmental sources and distribution of 1-OH-2-NP and 1-OH-5-NP as well as the other hydroxynitropyrene isomers.**

**Key words**——nitropyrene, nitropyrenol, polycyclic aromatic hydrocarbon, mutagen, endocrine disruptor

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## INTRODUCTION

Numerous polycyclic aromatic hydrocarb (PAHs) have been detected in organic extract airborne particles, and concerns are rising they may affect human health through their tagenic and carcinogenic effects. Nitrated p cyclic aromatic hydrocarbons (NPAHs) are al class of mutagens/carcinogens found in the at sphere, and some of them exhibit stronger m genicity/carcinogenicity than their parent PAI 1-Nitropyrene (1-NP) is believed to be e ted into the atmosphere from combustion cesses of fossil fuel such as diesel fuel[2] an one of the most abundant NPAHs in the at sphere.[3] 1-NP taken up by humans and anin is metabolized to hydroxynitropyrenes (OHN Fig. 1), such as 1-hydroxy-3-nitropyrene (1-OH NP), 1-hydroxy-6-nitropyrene (1-OH-6-NP), an hydroxy-8-nitropyrene (1-OH-8-NP) in the p ence of cytochrome P450 enzymes.[4,5] We rece found that these OHNP isomers were also produ from a photoreaction of 1-NP in the atmospher well as the other isomers, 1-hydroxy-2-nitropyr (1-OH-2-NP) and 1-hydroxy-5-nitropyrene (1-( 5-NP).[6] Several groups reported that 1-OH-3- 1-OH-6-NP, and 1-OH-8-NP are weakly m

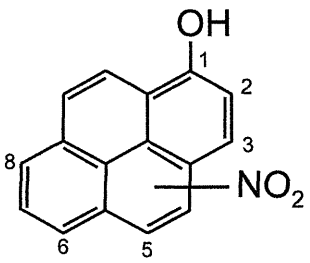


**Fig. 1.** Structure of OHNP

genic,[7–10] but the mutagenicities of 1-OH-2-NP and 1-OH-5-NP are unknown.

Recently several kinds of PAH derivatives have been found to act as endocrine disruptors which may cause the dysfunction of human and wildlife endocrine systems, abnormal development of reproductive systems, and immunodeficiencies. For example, several monohydroxylated derivatives of PAHs (OHPAHs) have significant estrogenic/antiestrogenic activities, as shown by a reporter gene assay[11] and by a yeast two-hybrid assay system based on the ligand-dependent interaction of the estrogen receptor (ER) and its co-activator.[12] Furthermore, mono- and dihydroxy metabolites of PAHs appear to act as antiandrogenic chemicals, as shown by a reporter gene assay based on Chinese hamster ovary (CHO) cells transiently cotransfected with a human androgen receptor (hAR) vector and an MMTV-LUC vector.[13] We have also found that 1-OH-3-NP, 1-OH-6-NP, and 1-OH-8-NP show significant estrogenic, antiestrogenic, and antiandrogenic activities in the yeast two-hybrid assay system.[14] These results imply that 1-OH-2-NP and 1-OH-5-NP, whose structures are similar to those of OHPAHs and other OHNP isomers, also exhibit endocrine-disrupting activities.

In this study, theirfore, we first examined the mutagenicities and endocrine-disrupting activities of 1-OH-2-NP and 1-OH-5-NP. For these analyses, we used the Ames plate incorporation assay and the yeast two-hybrid assay, respectively.

## MATERIALS AND METHODS

**Synthesis of OHNPs** —— 1-OH-2-NP was synthesized by nitration of 1-hydroxypyrene (OHPy) by 4-nitro-4-methyl-2,3,5,6-tetrabromo-2,5-cyclohexadien-1-one in diethyl ether at room temperature for 2 hr according to the literatures.[6, 15, 16] 1-OH-5-NP was obtained by a photoreaction of 1-NP according to the previous report.[6] Each OHNP isomer was purified by preparative normal phase HPLC (SUPELCO, St. Louis, MO, U.S.A.; Supelcosil PLC-SI, 21.2 mm ID × 25 mm, eluted with $CH_2Cl_2$ containing 0.5 mM $CH_3COOH$ at 10 ml/min). To identify the synthetic compounds, their EI-MS and $^1H$ NMR analyses were performed.

**Chemicals** —— 4-Nitro-4-methyl-2,3,5,6-tetrabromo-2,5-cyclohexadien-1-one was purchased from Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). 1-NP and OHPy were obtained from Sigma-Ald (St. Louis, MO, U.S.A.). 17$\beta$-Estradiol ($E_2$) 5$\alpha$-dihydrotestosterone (DHT) were purcha from Wako Pure Chemicals (Osaka, Japan). Hydroxytamoxifen (4-OHT) and hydroxyflutan (OHFl) were obtained from Sigma-Aldrich Toronto Research Chemical Inc. (North Y Canada), respectively. Test compounds were solved in ethanol and stored at −20°C until All other chemicals were of the highest qua available from commercial sources.

**Mutagenicity Assay** —— Mutagenic activities OHNPs were assayed with *Salmonella typhimur* strains TA98 and TA100 according to the met developed by Maron and Ames[17] including a sl modification of preincubation[18] in the presenc absence of S9 mix.

**Yeast Two-hybrid Assay** —— Estrogenic, an strogenic, androgenic, and antiandrogenic activi of OHNPs were evaluated with the yeast two-hyl assay following Nishikawa's method with s modifications.[12, 19, 20] Briefly, yeast cells (*Sacc romyces cerevisiae* Y190) expressing human trogen receptor (hER$\alpha$) and hAR or two-hyl system control yeast cells (*Saccharomyces c visiae* Y190 transfected with the pGBK7-53 pGADT7-T) were grown overnight at 30°C shaking in synthetic defined medium free from t tophan and leucine, and treated with each test c pound at 30°C for 4 hr. After the incubation, treated cells were collected and enzymatically gested with 1 mg/ml Zymolyase 20T at 37°C 30 min. 2-Nitrophenyl-$\beta$-D-galactoside was ad to the lysate to a final concentration of 4 mg/ml. ter incubation at 30°C for 45 min, the reaction terminated by the addition of 1 M $Na_2CO_3$. yeast debris was removed by centrifugation and absorbance of supernatant was measured at 415 Estrogenic activity was evaluated by the 10% r tive effective concentration ($REC_{10}$), which is fined as the concentration of the test compou showing 10% of the highest $\beta$-galactosidase ac ity of $E_2$. Antiestrogenic and antiandrogenic ac ities were evaluated by $IC_{20}$, which is the conc tration of the test compounds that inhibit 20% o galactosidase activity induced by $1.0 \times 10^{-9}$ M and $1.0 \times 10^{-8}$ M DHT, respectively.

## RESULTS AND DISCUSSION

Table 1 shows the mutagenicities of OHNPs

**Table 1.** Specific Mutagenicities of OHNPs and 1-NP, and Relative Mutagenicities of OHNPs to 1-NP Evaluated by Ames Assay

| Compound | TA98 | | TA100 | |
|---|---|---|---|---|
| | −S9 | +S9 | −S9 | +S9 |
| Specific mutagenicity [a] | | | | |
| 1-OH-2-NP | 28 | 46 | 118 | 164 |
| 1-OH-5-NP | 73 | 255 | 209 | 589 |
| 1-NP | 1605 | 165 | 539 | 236 |
| DMSO | 22 | 29 | 122 | 127 |
| Relative mutagenicity to 1-NP [b] | | | | |
| 1-OH-2-NP [a] | 0.02 | 0.3 | 0.2 | 0.7 |
| 1-OH-5-NP [a] | 0.05 | 1.5 | 0.4 | 2.5 |
| 1-OH-3-NP | 0.3 [c] | 0.6 [c] | 1.4 [d] | 0.8 [d] |
| 1-OH-6-NP | 0.06 [c] | 1.5 [c] | 2.5 [d] | 2.4 [d] |
| 1-OH-8-NP | 0.09 [c] | 0.3 [c] | — [e] | — [e] |

DMSO: used as a negative control. *a*) This study. Specific mutagenicity, expressed as revertants/40 nmol-test compound, was calculated by least squares linear regression from linear portion of dose-response curve. *b*) Calculated based on the specific mutagenicities. *c*) Obtained from reference 9. *d*) Obtained from reference 10. *e*) Not available in reference 10.

**Table 2.** $REC_{10}$ and $IC_{20}$ Values for OHNPs and Reference Chemicals in Yeast Two-hybrid Assay

| Compound | $REC_{10}$ [c] | $IC_{20}$ [e] | |
|---|---|---|---|
| | Estrogenic activity | Antiestrogenic activity | Antiandrogenic activity |
| 1-OH-2-NP [a] | — [d] | $1.3\times10^{-6}$ | $3.7\times10^{-7}$ |
| 1-OH-5-NP [a] | $5.4\times10^{-7}$ | $2.5\times10^{-7}$ | $3.2\times10^{-8}$ |
| 1-OH-3-NP [b] | $6.0\times10^{-7}$ | $1.1\times10^{-6}$ | $2.3\times10^{-7}$ |
| 1-OH-6-NP [b] | $6.0\times10^{-8}$ | $1.0\times10^{-6}$ | $3.1\times10^{-7}$ |
| 1-OH-8-NP [b] | $9.0\times10^{-7}$ | $7.0\times10^{-7}$ | $5.1\times10^{-8}$ |
| $E_2$ [a] | $6.0\times10^{-11}$ | | |
| 4-OHT [a] | | $5.3\times10^{-6}$ | |
| OHFl [a] | | | $5.3\times10^{-6}$ |

*a*) This study. *b*) Taken from reference 14. *c*) Concentration of the test compounds showing 10% of the highest $\beta$-galactosidase activity of $E_2$. *d*) Significant induction of $\beta$-galactosidase activity was not observed at concentrations between $1.0\times10^{-8}$ and $1.0\times10^{-6}$ M. *e*) Concentration of the test compounds that inhibit 20% of $\beta$-galactosidase activity induced by $10^{-9}$ M $E_2$ or $10^{-8}$ M DHT.

tained in this study. 1-OH-2-NP was not mutagenically active in either TA98 or TA100 with or without metabolic activation by S9. The mutagenicity of 1-OH-5-NP in the TA98 and TA100 strains was weakly positive in the absence of S9, *i.e.*, the numbers of revertants were 2–3-fold greater than the number of spontaneous revertants in the negative control [dimethyl sulfoxide (DMSO)]. On the other hand, the mutagenic activities of 1-OH-5-NP in both the strains were enhanced approximately 3-fold by the addition of S9. The mutagenicities of 1-OH-5-NP relative to the mutagenicity of 1-NP are given in Table 1 together with those of the other OHNP isomers. With S9 activation, they were 1.5 and 2.5 in TA98 and TA100, respectively, *i.e.*, the mutagenicity of 1-OH-5-NP was higher than that of 1-NI was the case of 1-OH-6-NP in the presence of These results are consistent with previous findi that some OHNP isomers need metabolic activa to exhibit mutagenicity.[8, 9, 21]

Figure 2 shows the estrogenic activities of and the two isomers of OHNP. $\beta$-Galactosidase tivity increased with increasing $E_2$ concentrat reaching a plateau at $1.0\times10^{-6}$ M. A signifi induction of $\beta$-galactosidase activity was also served for 1-OH-5-NP ($REC_{10} = 5.4\times10^{-7}$ M), no activity was observed for 1-OH-2-NP at conc trations between $1.0\times10^{-8}$ and $1.0\times10^{-6}$ M. $REC_{10}$ and $IC_{20}$ values for the OHNP isomers tained in this study and for the reference chemi

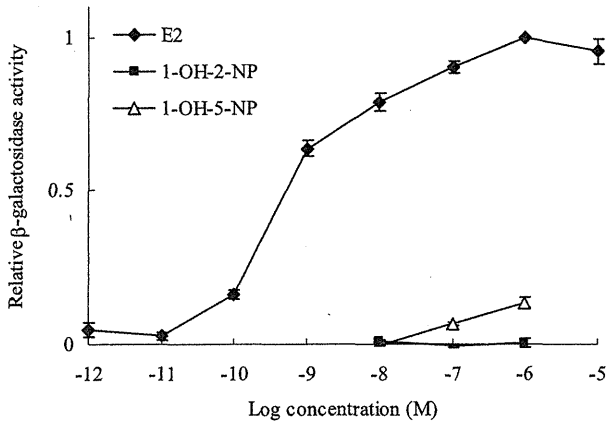


**Fig. 2.** Dose Response Curves of Estrogenic Activity of $E_2$ and 1-OH-*x*-NPs ($x$ = 2 and 5) in a Yeast Two-hybrid Assay System

Each data point is the mean ± S.D. ($n$ = 3).

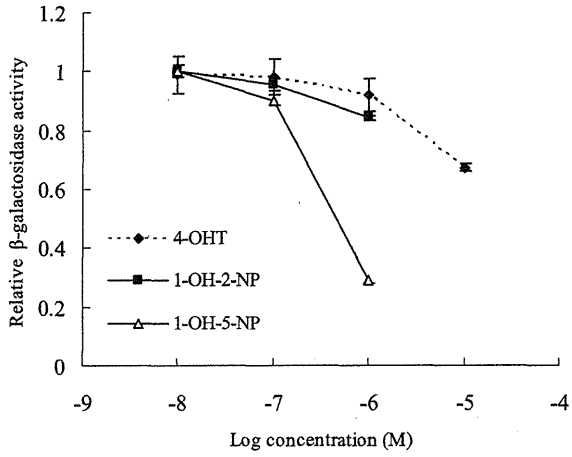


**Fig. 3.** Antiestrogenic Activity of 4-OHT and 1-OH-*x*-NPs ($x$ = 2 and 5) against the Estrogenic Activity of $E_2$ in a Yeast Two-hybrid Assay System

Antiestrogenic activities of 4-OHT and 1-OH-*x*-NPs were expressed as β-galactosidase activity relative to the level induced by $1.0 \times 10^{-9}$ M $E_2$. Each data point is the mean ± S.D. ($n$ = 3).

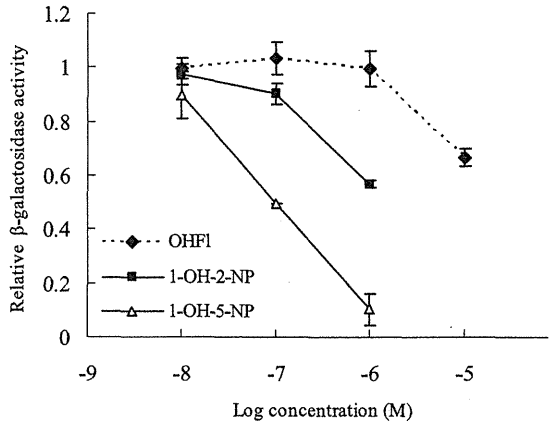


**Fig. 4.** Antiandrogenic Activity of OHFl and 1-OH-*x*-($x$ = 2 and 5) against the Androgenic Activity of I in a Yeast Two-hybrid Assay System

Antiandrogenic activities of OHFl and 1-OH-*x*-NPs were pressed as β-galactosidase activity relative to the level induce $1.0 \times 10^{-8}$ M DHT. Each data point is the mean ± S.D. ($n$ = 3).

are summarized in Table 2. The estrogenic activity of 1-OH-5-NP was lower than that of 1-OH-6-NP, but higher than the estrogenic activities of the other OHNP isomers previously reported[14] or bisphenol A ($REC_{10}$ = $3 \times 10^{-6}$ M),[12] a known estrogenic compound. Figure 3 shows the antiestrogenic activities of the tested OHNPs in the concentration range from $1.0 \times 10^{-8}$ to $1.0 \times 10^{-6}$ M. To obtain these data, we used an $E_2$ concentration of $1.0 \times 10^{-9}$ M, which induced about 50% of the maximum β-galactosidase activity. At a concentration of $1.0 \times 10^{-6}$ M, each of the OHNP isomers decreased the induction of β-galactosidase activity by $E_2$. 1-OH-2-NP and 1-OH-5-NP showed 4 and 21 times higher antiestrogenic activity, respectively, than 4-OHT, a typical ER antagonist (Table 2). Figure 4 shows the results of the antiandrogenic activities for tested OHNPs. In the presence of OHNPs at c centrations between $1.0 \times 10^{-8}$ and $1.0 \times 10^{-6}$ the activity of $1.0 \times 10^{-8}$ M DHT, which indu about 50% of the highest β-galactosidase activit DHT, was inhibited concentration-dependently. highest inhibitory effect among the five OHNP mers was observed with 1-OH-5-NP as was the c with antiestrogenic activity. The antiandrogenic tivities of 1-OH-2-NP and 1-OH-5-NP were 14 166 times higher than the antiandrogenic activit OHFl (Table 2). At concentrations less than 1. $10^{-6}$ M, neither OHNP isomer was cytotoxic to control yeast cells, which supports the idea that decreases of β-galactosidase induction observe this study were due to antiestrogenic/antiandroge effects rather than cytotoxic effects. Neither 1-( 2-NP nor 1-OH-5-NP showed androgenic acti at concentrations between $1.0 \times 10^{-8}$ and 1.( $10^{-6}$ M. OHPAHs having four aromatic rings, s as hydroxybenz[*a*]anthracenes, hydroxychrysei and hydroxybenzo[*c*]phenanthrenes, were show have strong endocrine-disrupting activities.[20] In dition, it was reported that the four rings an phenolic hydroxyl group needed to be in a r angular plane in order for OHPAHs to bind to site of the receptor.[20] The OHNP isomers have same planar structure, which could account for t endocrine-disrupting activities.

Because of the significant biological eff of OHNPs, further studies of their environme sources, sinks, and distributions are needed to be assess their risks.

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Short communication

# Hydrogen peroxide–sodium hydrosulfite chemiluminescence system combined with high-performance liquid chromatography for determination of 1-hydroxypyrene in airborne particulates

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ABSTRACT

In this research, a highly sensitive chemiluminescence method based on a sodium hydrosulfite ($NaHSO_3$)–hydrogen peroxide ($H_2O_2$) reaction for the determination of 1-hydroxypyrene (1-OHP) was developed. The response of this system was linear in the range from 0.5 to 50 pmol ($R^2$ = 0.9983). The limit of detection for 1-OHP was 100 fmol ($S/N$ = 3). 1-OHP in airborne particulates was well separated from interfering compounds using an ODS column with 75% methanol as the mobile phase in isocratic mode. The proposed method was successfully applied to determine the 1-OHP in airborne particulates collected in Kanazawa, Japan. The average concentration of 1-OHP in the atmosphere was 2.0 pg/m$^3$ (9.2 fmol/m$^3$).



## 1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants [1,2] that are generated from incomplete combustion of organic matters such as fuel oil and coal [3,4]. In the atmosphere, PAHs react with the co-pollutants, such as ozone and nitrogen oxides ($NO_x$) to produce substituted PAHs [5,6]. One type of substituted PAHs is hydroxylated PAHs (OH-PAHs). They may be generated from photochemical reactions of PAHs or combustion of coal [7–9]. OH-PAHs have endocrine disrupting activities such as estrogenic activities or antiestrogenic activities [10,11]. Atmospheric concentrations of OH-PAHs were reported to be in the pg/m$^3$ range, which are 1–3 orders of magnitude lower than that of PAHs [8,9]. Therefore, it is necessary to establish a highly sensitive and reliable method for determination of OH-PAHs in atmosphere [12].

Gas chromatography/mass spectrometry (GC–MS) is one of the most commonly used methods in detecting OH-PAHs in the atmosphere [7–9,13,14]. A disadvantage of GC–MS is that it requires a derivatization step before analysis, which is time-consuming, and increases the risk of contamination or loss of OH-PAHs [15]. Another method for determining OH-PAHs is high-performance liquid chromatography (HPLC) with electrochemical detection [16], but the sensitivity was too low to detect OH-PAHs in the atmosphere. Kishikawa et al. determined six kinds of OH-PAHs by HPLC with a fluorescence detector using the internal standard method [15]. However, interference peaks were found in the chromatogram, which may affect the accuracy of the method.

In this study, we developed a highly sensitive and simple chemiluminescence (CL) method to determine 1-OHP in the airborne particulates for the first time. Airborne particulate sample might contain many interfering compounds. Therefore, an ODS separation column was connected to the system to separate 1-hydroxypyrene from interfering compounds. The method avoids the time-consuming derivatization step required by GC–MS method [14]. Moreover, the method was simple and convenient. The possible mechanism of this CL reaction was proposed. We used the method to determine 1-OHP in airborne particulates in Kanazawa city in Japan.

## 2. Experimental

### 2.1. Reagents and chemicals

Sodium hydrosulfite ($NaHSO_3$) and hydrogen peroxide ($H_2O_2$) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan).

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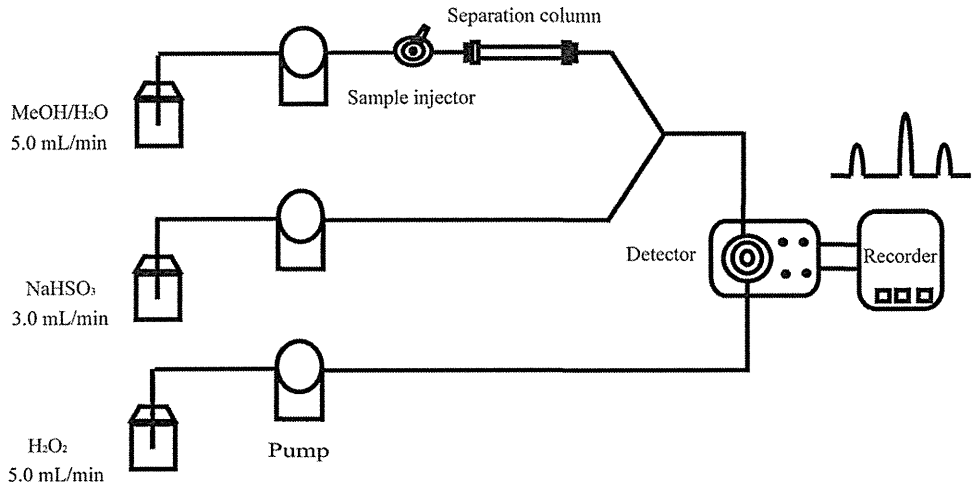


**Fig. 1.** Schematic diagram of the $NaHSO_3$–$H_2O_2$ CL system to detect 1-OHP.

$NaHSO_3$ solution was freshly prepared by dissolving appropriate amounts of $NaHSO_3$ powder in ultrapure water. $H_2O_2$ solution was freshly prepared by volumetric dilution of commercial 30% $H_2O_2$ solution. 1-OHP was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Solution of 1-OHP was prepared by dissolving powder with methanol. Standard solution of 1-OHP was diluted with 75% methanol. All the organic solvents (methanol, hexane, dichloromethane, diethyl ether, benzene, and ethanol) were HPLC grade and from Wako Pure Chemical Industries, Ltd. Ultrapure water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

### 2.2. Sample collection

Airborne particulates were collected on the roof of a seven-storey building of Kanazawa University (Kakuma-machi) in Kanazawa, Japan. Sampling was conducted in August, 2010 using a high-volume air sampler (Model 120, Kimoto Electrics, Osaka, Japan) with a 2500 QAT-UP quartz fiber filter (8″ × 10″, Pallflex Products, Putnam, CT, USA) for 24 h at a flow rate of 1000 L/min (total volume 1434.9 $m^3$). The filters with airborne particulates were stored in a freezer at −20 °C until analysis.

### 2.3. Sample preparation

The whole sample filter was cut into small pieces and extracted ultrasonically with 100 mL benzene/ethanol mixture solvent (1:1, v/v) for 15 min twice. Both extracts were combined and filtered with a cellulose acetate filter (No. 5C, Advantec MFS, Dublin, CA, USA) to remove solid residue. Then the solution was evaporated to near dryness at 30 °C in a rotary evaporator under reduced pressure. The residues were reconstituted in 10 mL hexane (under ultrasonication for 2 min), and filtered with a 0.45 μm membrane filter.

The residues were loaded onto a Sep-Pak®Plus Silica cartridge (Waters, Milford, MA, USA) which was preconditioned with 10 mL hexane. The target compound was eluted by 10 mL hexane/ethyl acetate (7/1, v/v). The eluate was gently evaporated to near dryness under the gentle nitrogen gas stream, and then re-dissolved in 1.0 mL methanol/water mixture solvent (75/25, v/v). An aliquot of 100 μL of the sample solution was injected into the HPLC system.

### 2.4. HPLC-CL system

The HPLC system with a CL detector is shown in Fig. 1. This HPLC-CL system consists of three HPLC pumps (LC-10A, LC-10A, and LC-6000, Shimadzu, Tokyo, Japan), a sample injector with a 100 μL loop, a separation column (Cosmosil 5C18-AR-II; 10.0 mm i.d. 250 mm, Nacalai Tesque), and a column oven (Sugai, U-620). The temperature of the column oven was set at 30 °C. The CL detector is S-3400 (Soma Optics, Tokyo, Japan). An aliquot of 100 μL of the standard mixture or the airborne particulate sample was injected into the HPLC system, and detected directly by the CL detector.

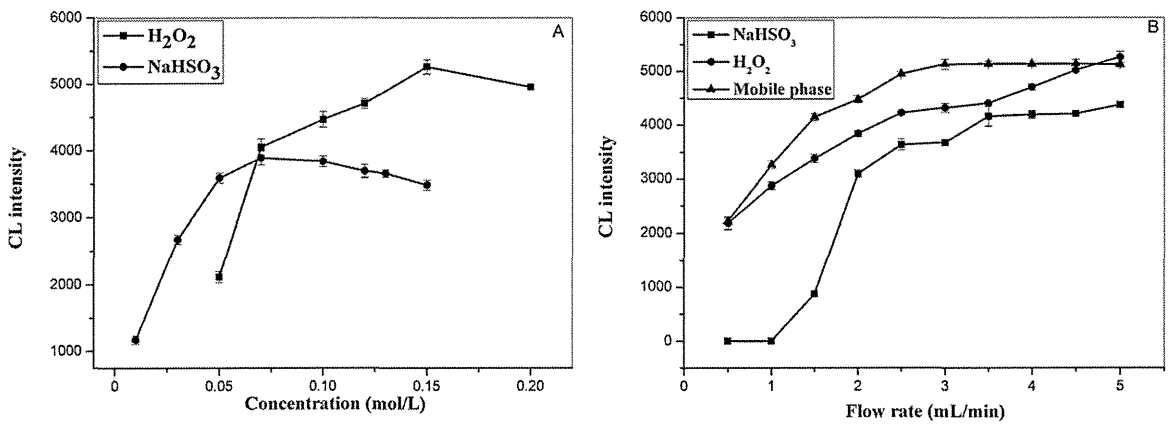


**Fig. 2.** Effects of concentrations and flow rates on CL intensity: (A) concentrations of $NaHSO_3$ and $H_2O_2$. Flow rates of $NaHSO_3$, $H_2O_2$ and mobile phase were 5.0, 3.0, and 5.0 mL/min, respectively; (B) flow rates of $NaHSO_3$, $H_2O_2$ and mobile phase. Concentrations of $NaHSO_3$ and $H_2O_2$ were 0.1 mol/L and 0.15 mol/L.

**Table 1**
Precision of the method for determining 1-OHP in airborne particulates and recovery of 1-OHP in airborne particulates.

| | Intra-day ($n$=3) | | | Inter-day ($n$=3) | | |
|---|---|---|---|---|---|---|
| Added (nmol/L) | 0 | 50 | 100 | 0 | 50 | 100 |
| Found (nmol/L) | 7.0 | 53 | 110 | 7.0 | 53 | 110 |
| Recovery (%)[a] | – | 92% | 103% | – | 92% | 103% |
| RSD (%) | 9.5% | 8.2% | 2.4% | 15.5% | 11.5% | 4.7% |

[a] Expressed as [(found concentration – non-spiked concentration)/added concentration] × 100.

Mobile phase was methanol/water mixture (75/25, v/v) in isocratic mode at a flow rate of 5.0 mL/min.

## 3. Results and discussion

### 3.1. Optimization of HPLC-CL system

In order to obtain a high sensitivity for determination of 1-OHP, factors influencing CL intensity, such as concentration and flow rate of CL reagents were investigated independently.

We investigated the concentration of $H_2O_2$ in the range from 0.05 to 0.2 mol/L. CL intensity increased with $H_2O_2$ concentration up to 0.15 mol/L and then leveled off (Fig. 2A). In addition, too high concentration of $H_2O_2$ caused gas bubble to form in the tube, which would affect the stability of the CL system. Therefore, a $H_2O_2$ concentration of 0.15 mol/L was used for the following experiments.

The effect of $NaHSO_3$ concentration on CL intensity was studied in the range 0.01–0.15 mol/L. The CL intensity increased linearly with the increasing of $NaHSO_3$ concentration, but at concentrations of $NaHSO_3$ higher than 0.10 mol/L, the CL intensity began to decrease (Fig. 2A). Therefore, the concentration of $NaHSO_3$ solution was selected to be 0.1 mol/L for further study.

The flow rate of the CL reagent solution strongly affected sensitivity of the CL method. We investigated the flow rates of $NaHSO_3$, $H_2O_2$, and mobile phase from 0.5 to 5.0 mL/min. The optimized flow rates of $NaHSO_3$ and $H_2O_2$ were 5.0 mL/min and 3.0 mL/min (Fig. 2B). When the flow rate of the mobile phase was higher than 3.0 mL/min, the CL intensity remained constant (Fig. 2B). But the separation efficiency of the column was the highest at a flow rate of 5.0 mL/min. Therefore, the flow rate of the mobile phase was set at 5.0 mL/min.

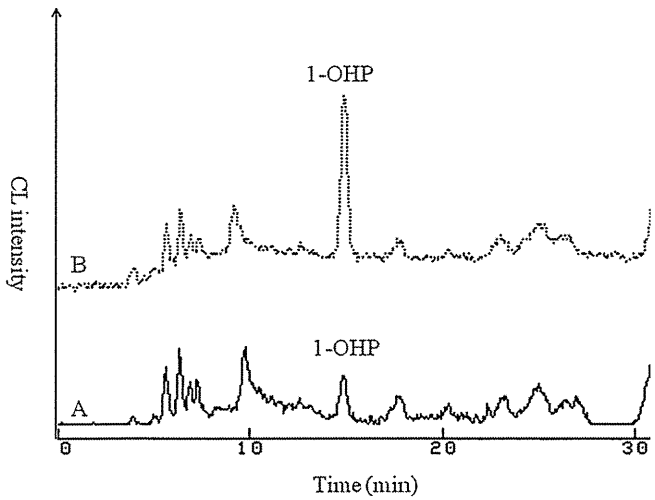


**Fig. 3.** Chromatograms of 1-OHP (A) in airborne particulates and (B) airborne particulates spiked with 1.0 pmol 1-OHP. Mobile phase was 75% methanol at 5.0 mL/min; concentrations of $NaHSO_3$ and $H_2O_2$ were 0.10 mol/L and 0.15 mol/L; their flow rates were 3.0 mL/min and 5.0 mL/min respectively.

Methanol and acetonitrile are organic solvents that are often used as the mobile phase in HPLC analyses. The effect of methanol and acetonitrile on the CL intensity was compared. Methanol gave higher sensitivity for 1-OHP and better separation. Therefore, methanol was selected for further study.

### 3.2. Possible mechanism of CL system

In the $NaHSO_3$–$H_2O_2$ CL reaction, $HSO_3^-$ was oxidized by $H_2O_2$ to produce sulfite radical ($^\bullet SO_3^-$) [17], which dimerized to dithionate ion ($S_2O_6^{2-}$) [18,19].

$$HSO_3^- + H_2O_2 \rightarrow {}^\bullet OH + SO_3^{\bullet -} + H_2O \quad (1)$$

The OH radical, a strong oxidant, rapidly reacted with $HSO_3^-$ to give $^\bullet SO_3^-$ radical [20,21].

$$^\bullet OH + HSO_3^- \rightarrow SO_3^{\bullet -} + H_2O \quad (2)$$

$$k = 1.5 \times 10^9\ M^{-1}\ s^{-1}$$

$$^\bullet SO_3^- + {}^\bullet SO_3^- \rightarrow S_2O_6^{2-} \quad (3)$$

Excited sulfur dioxide ($SO_2^*$) was generated by the decomposition of $S_2O_6^{2-}$ [18,19]. The emission wavelength of $SO_2^*$ is 260–480 nm [22,23], which overlaps the excitation wavelength of 1-OHP [15]. Therefore, when 1-OHP is present in the CL system, $SO_2^*$ will transfer its energy to 1-OHP. This greatly increased the CL emission. CL intensity was highly correlated with 1-OHP concentration. Based on this mechanism, 1-OHP could be detected sensitively through the following reactions.

$$S_2O_6^{2-} \rightarrow SO_4^{2-} + SO_2* \quad (4)$$

$$SO_2* \rightarrow SO_2 + h\nu \quad (5)$$

$$SO_2* + \text{1-OHP} \rightarrow SO_2 + \text{1-OHP}* \quad (6)$$

$$\text{1-OHP}* \rightarrow \text{1-OHP} + h\nu \quad (7)$$

### 3.3. Calibration curve, detection limit and reproducibility

Under the optimized conditions, standard 1-OHP was successfully determined by the $NaHSO_3$–$H_2O_2$ CL system without a separation column. The calibration curve was obtained with a good linear relationship between the concentrations of 1-OHP and peak heights. The linear range was from 0.005 to 10 pmol/injection (seven calibration points; $R^2$ = 0.9994). The slope and intercept of the regression equation of calibration curve (mean ± error) were $5.77 \times 10^{11} \pm 5.63 \times 10^9$ and 635 ± 220. The detection limit ($S/N$ = 3) was 1.0 fmol/injection. This CL detection method for detecting 1-OHP showed higher sensitivity than the previous methods reported [15,24]. However, 1-OHP cannot be detected exactly only with the proposed CL method due to the interference compounds in airborne particulates. An ODS column was incorporated to separate 1-OHP and the interference compounds in airborne particulates. A good linear relationship was observed between the peak height and concentrations of 1-OHP in the range from 0.5 to 50 pmol/injection (ten calibration points) with 100 μL per injection

($R^2 = 0.9983$). The slope and intercept of the regression equation (mean ± error) were $2.92 \times 10^{11} \pm 8.94 \times 10^9$ and $674 \pm 65.7$. The detection limit ($S/N = 3$) for 1-OHP detected by the proposed method was 100 fmol/injection.

The precision of the determination of 1-OHP and the recovery of 1-OHP were investigated by spiking standard 1-OHP solutions into the airborne particulates. The results are shown in Table 1. The relative standard deviation (RSD) of intra-day and inter-day ranged from 2.4% to 11.5%. The recoveries for the spiked airborne particulates of intra-day and inter-day were in the range 93–103%.

### 3.4. Determination of 1-OHP in airborne particulates

Pyrene is one of major PAHs in the atmosphere and exists in both gas and particulate phase. The vapor pressure of 1-OHP is much lower than that of pyrene because of the hydroxy group; 1-OHP may mainly exist in particulate phase. Therefore, the proposed method was applied to determine 1-OHP in airborne particulates collected in Kanazawa, Japan in August 2010. The chromatogram of real airborne particulates showed the peak at the retention time of 1-OHP and the peak height increased by the addition of 1-OHP standard solution (Fig. 3(A) and (B)). From the chromatogram, the average concentration ($n = 3$) of 1-OHP in airborne particulates in Kanazawa city was $2.0 \pm 0.2$ pg/m$^3$ ($9.2 \pm 0.9$ fmol/m$^3$). Although only a few reports have examined the concentrations of 1-OHP in airborne particulates, our results were in the range of previous values. For example, the concentration of 1-OHP in Nagasaki, Japan was 5.97–63.25 pg/m$^3$ [15].

## 4. Conclusions

In this research, a highly sensitive HPLC-CL method based on $NaHSO_3$–$H_2O_2$ coupled with an ODS separation column to detect 1-OHP in airborne particulates was proposed. This is the first time that the $NaHSO_3$–$H_2O_2$ CL system reaction was combined with an HPLC system to detect air pollutants. The proposed method was successfully applied to detect trace levels of atmospheric 1-OHP in Kanazawa city in summer.

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# Recent Changes in Atmospheric Polycyclic Aromatic Hydrocarbons (PAHs) and Nitropolycyclic Aromatic Hydrocarbons (NPAHs) in Shenyang, China

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Airborne particulates were collected in three size fractions by using Anderson low-volume air samplers in Shenyang, China, in winter and summer in 2007. Compared with data obtained in 2001 at the same sites, the total concentrations of nine polycyclic aromatic hydrocarbons (PAHs) in winter decreased by 67% at one site and decreased by 40% at the other site, while the total concentrations of four nitropolycyclic aromatic hydrocarbons (NPAHs) did not decrease. This suggests that environmental countermeasures begun in 2001 were effective in decreasing the concentration of PAHs. However, in summer, the concentrations of PAHs and NPAHs rose by the factors of 4 and 5, respectively, possibly because of an increase in the number of motor vehicles.

Keywords: polycyclic aromatic hydrocarbon (PAH), nitropolycyclic aromatic hydrocarbon (NPAH), 1-nitropyrene/pyrene ratio ([1-NP]/[Pyr]), air pollution, Shenyang government Blue Sky Project

Many polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs) are carcinogenic and/or mutagenic. The International Agency for Research on Cancer (IARC). has ranked benzo[*a*]pyrene (BaP) in Group 1 (carcinogenic to humans) and 1-nitropyrene (1-NP) in Group 2A (probably carcinogenic to humans) (IARC, 2005). Several PAHs also have estrogenic/antiestrogenic or antiandrogenic activities (Kizu et al., 2000). Atmospheric PAHs such as BaP and NPAHs such as 1-NP mainly originate from imperfect combustion and pyrolysis of organic matters (Hayakawa et al., 1995a; Rogge et al., 1993). In addition, some NPAHs, such as 2-NP, are formed in the atmosphere via reactions of their parent PAHs and $NO_2$ (Arey et al., 1986). We previously reported that the main contributors to atmospheric PAHs and NPAHs were automobiles in Japanese and South Korean commercial cities (Sapporo, Kanazawa, Toyama, Tokyo and Seoul) (Kakimoto et al., 2000; 2002; Hayakawa et al., 2000; 2002; Tang et al., 2002a; 2005), while they were coal combustion systems in Chinese and Far-eastern Russian cities (Beijing, Shenyang, Fushun, Tieling and Vladivostok) (Hattori et al., 2007; Hayakawa et al., 2007; Tang et al., 2002a; 2005; 2009).

During the past three decades, large increases in the consumption of petroleum and coal in China have led to air pollution. In Shenyang, a city that is the economic center of northeast China, the main sources of air pollution are exhausts from domestic heating, industry, and motor vehicles. In Shenyang in 2001 to 2002, the average concentrations of total PAHs (nine types of PAHs) and NPAHs (seven types of NPAHs) were 534 pmol $m^{-3}$ and 677 fmol $m^{-3}$, respectively (Hattori et al., 2007). These concentrations were significantly higher than those in Kanazawa, Japan in the same period. To reduce air pollution, the Shenyang government undertook the Blue Sky Project (Shenyang Environmental Protection Bureau of China [SYEPB]), in which 100 factories were transferred to the outer city, and 5,000 inefficient boilers for domestic heating were removed from 2001 to 2007. These changes helped to reduce the annual average concentrations of $PM_{10}$ from 190 $\mu$g $m^{-3}$ in 2001 to 120 $\mu$g $m^{-3}$ in 2009 (SYEPB).

In this study, airborne particulates (APs) were collected by using the same method and at the same sites as our previous investigation in 2007 (Hattori et al., 2007). Our objectives were to clarify the effect of the above countermeasures and to understand the present pollution status of PAHs and NPAHs in Shenyang, China.

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## Materials and Methods

*Sampling Sites*

Sampling sites in this study were the same as our previous study (Hattori et al., 2007). SY-1 (Heping Elementary School) was in a commercial area and SY-2 (Taishan Elementary School) was in a mixed residential and commercial area. Airborne particulates were collected simultaneously at the two sites using Andersen low-volume air samplers (AN-200, Shibata Sci. Tech., Tokyo, Japan) at a flow rate of 28.3 L $min^{-1}$. The height of the intake varied from 1 to 3 m above ground level. APs were separately collected only in three fractions according to their aerodynamic size: >7 $\mu$m, 2.1–7 $\mu$m, and <2.1 $\mu$m onto quartz fiber filters (2500QAT-UP, Pallflex Products, Putnam, CT,USA), which differed from our previous study (Hattori et al., 2007; APs were collected in nine fractions and separated in same three granulometric groups for PAHs and NPAHs analysis). Air samples were collected at two sites on March 10–17 (winter) and September 17–24, 2007 (summer). Filters were replaced daily. At each site, seven three-layer filters were used for each of the above sampling periods. The filters were dried in a desiccator in the dark, weighed and stored at −20°C until use.

*Chemicals*

USEPA 610 PAHs mix, a mixture of 16 PAHs including fluoranthene (FR), pyrene (Pyr), benz[*a*]anthracene (BaA), chrysene (Chr), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), BaP, benzo[*ghi*]perylene (BgPe) and indeno[1,2,3-*cd*]pyrene (IDP) were purchased from Supelco Park (Bellefonte, PA, USA). Two internal standards for PAHs (pyrene-$d_{10}$ (Pyr-$d_{10}$) and benzo[*a*]pyrene-$d_{12}$ (BaP-$d_{12}$)) were purchased from Wako Pure Chemicals (Osaka, Japan). 1-NP, 6-nitrochrysene (6-NChr), 6-nitrobenzo[*a*]pyrene (6-NBaP) and an internal standard for NPAHs (2-fluoro-7-nitrofluorene (FNF)) were purchased from Chiron AS (Trondheim, Norway). All other chemicals used were of analytical reagent grade.

*Sample Treatment and Analytical Procedures*

Three-layer filters (>7 $\mu$m, 2.1–7 $\mu$m, <2.1 $\mu$m) of each sample were cut into small pieces and placed in a flask, respectively. By this treatment, the concentrations of PAHs and NPAHs in the three groups were obtained for each sample. Both PAHs and NPAHs were extracted ultrasonically twice with benzene/ethanol (3:1, v/v) and then the solution was filtered with a 0.45 $\mu$m membrane filter (HLC-DISK13, Kanto Chemical Co., Inc., Tokyo, Japan). Internal standards, Pyr-$d_{10}$, BaP-$d_{12}$ and FNF, were added to the flask prior to the ultrasonic extraction. In the case of PAHs, the filtrate was evaporated to dryness. The residue was dissolved in 0.5 mL of acetonitrile, and then injected into the HPLC system for PAHs. In the case of NPAHs, the filtrate was washed once with 5% (w/v) sodium hydroxide solution, once with 20% (v/v) sulfuric acid solution, and twice with water for removing acid and base substance. Then the solution was evaporated to dryness with rotation evaporator. The residue was dissolved in 1 mL of 75% ethanol-0.02 M acetic acid-sodium acetate buffer (pH 5.5). The solution was filtered with a 0.45 $\mu$m HLC-Disk membrane filter (Kanto Chemical Co., Inc., Tokyo, Japan), and an aliquot of this solution was injected into the HPLC system for NPAHs. Other conditions were the same as in our previous reports (Hayakawa et al., 1991; Tang et al., 2002b).

The nine PAH species were determined by using HPLC with fluorescence detection. The PAH HPLC system consisted of a reversed-phase column (Inertsil ODS-P, 4.6 i.d. × 250 mm, GL Sciences Inc., Tokyo, Japan) with an acetonitrile/water gradient and fluorescence detection. The flow rate was 1 mL/$min^{-1}$. The time program of the fluorescence detector was set to detect at the optimum excitation and emission wavelengths for each PAH. Other conditions were the same as in our previous report (Tang et al., 2002b).

The four NPAH species were determined by using HPLC with chemiluminescence detection. The HPLC system consisted of two reversed-phase columns (Cosmosil 5C18-MS, 4.6 i.d. × (250 + 150) mm, Nacalai Tesque, Tokyo, Japan) connected in series with chemiluminescence detection. The mobile phase was 10 mM imidazole buffer (pH 7.6)-acetonitrile (1:1, v/v), and the chemiluminescence reagent solution was an acetonitrile solution containing 0.02 mM bis(2,4,6-trichlorophenly)oxalate and 15 mM hydrogen peroxide. The flow rate was 1 mL $min^{-1}$ for each solution. Other conditions were the same as in our previous report (Hayakawa et al., 1991; Tang et al., 2003).

*Data Analysis*

The major sources of PAHs and NPAHs were identified by a cluster analysis with Ward's method and standardized squared Euclidean distance. The statistical analysis software program used in this study was kindly provided by Dr. Susumu Hayakari of Aomori Prefectural Institute of Public Health and Environment (Aomori, Japan). For the cluster analysis, we used the concentration ratios of 1-NP to Pyr, 6-NBaP to BaP and individual PAH to total PAHs in the airborne particulates collected at two sites in 2001 – 2002 (Hattori et al., 2007) and 2007. The data of Tokyo in summer in 2004 and in winter in 2005 were used grouping (Tang et al., 2005). The data of average temperature, dew point and wind speed in Shenyang during our sampling periods were obtained from USA National Climatic Data Center (2009) and average humidity were calculated by using the data of average temperature and dew point.

## Results and Discussion

*Concentrations*

The PAH and NPAH concentrations were higher in winter than in summer at each site and they were both higher at SY-1 than

***Table 1.*** *Atmospheric concentrations of nine polycyclic aromatic hydrocarbons (PAHs) and four nitropolycyclic aromatic hydrocarbons (NPAHs) in 2007 at two sites in Shenyang, China*

| | | Summer | | Winter | |
|---|---|---|---|---|---|
| | | SY-1 | SY-2 | SY-1 | SY-2 |
| PAH | FR | 7.97 ± 3.55 | 7.22 ± 3.33 | 67.9 ± 36.0 | 51.6 ± 26.7 |
| (pmol $m^{-3}$) | Pyr | 6.99 ± 2.60 | 6.41 ± 2.63 | 70.1 ± 34.8 | 60.3 ± 31.8 |
| | BaA | 7.11 ± 3.63 | 5.41 ± 2.94 | 63.1 ± 28.2 | 57.9 ± 35.2 |
| | Chr | 13.4 ± 7.52 | 9.91 ± 6.19 | 79.8 ± 34.3 | 67.5 ± 34.0 |
| | BbF | 34.3 ± 28.5 | 28.7 ± 27.9 | 69.9 ± 32.9 | 61.8 ± 32.4 |
| | BkF | 11.2 ± 8.66 | 9.71 ± 8.62 | 26.5 ± 11.9 | 24.9 ± 12.6 |
| | BaP | 11.4 ± 6.45 | 10.2 ± 6.70 | 44.1 ± 19.3 | 42.9 ± 22.1 |
| | BgPe | 24.6 ± 13.8 | 23.1 ± 14.9 | 46.0 ± 17.1 | 56.2 ± 26.5 |
| | IDP | 14.4 ± 8.57 | 14.6 ± 10.0 | 22.2 ± 9.24 | 27.5 ± 13.1 |
| | Total PAHs | 131 ± 80.2 | 115 ± 79.6 | 491 ± 215 | 451 ± 226 |
| NPAH | 9-NA | 718 ± 400 | 600 ± 369 | 5400 ± 3280 | 5430 ± 3210 |
| (fmol $m^{-3}$) | 1-NP | 159 ± 49.8 | 147 ± 54.7 | 857 ± 459 | 555 ± 184 |
| | 6-NC | 205 ± 100 | 191 ± 86.0 | 544 ± 260 | 343 ± 157 |
| | 6-NBaP | 31.9 ± 15.6 | 32.4 ± 14.6 | 116 ± 58.5 | 93.4 ± 64.1 |
| | Total NPAHs | 1110 ± 526 | 972 ± 493 | 6910 ± 3450 | 6420 ± 3410 |

All data represent mean±standard deviation; $n = 7$.

at SY-2 (Table 1). Measurements made in 2001 to 2002 showed similar increases in winter (Hattori et al., 2007).

Figure 1 compares the total concentrations of nine PAHs and three NPAHs measured in the two studies (One of NPAHs measured in the present study was not measured in the earlier study). In winter, the concentrations of PAHs decreased by 40% at SY-1, which is in a commercial area, and decreased by 67% at SY-2, which is in a more residential area (Figure 1A). However, the concentration of NPAHs did not change markedly at either site, which suggests that Shenyang's Blue Sky Project was more effective against PAHs than NPAHs in winter. In summer, the concentrations of PAHs and NPAHs rose by a factor of at least 4 at both sites. A likely contributing factor was an increase in the number of motor vehicles from 360,000 in 2000 to 560,000 in 2007 ((SYEPB).

### *Distribution of PAHs, NPAHs and APs in Different Size Particulate Fractions*

The concentrations and compositions of air pollution matters are often affected by meteorological conditions (Yamasaki et al., 1982). PAH, NPAH and AP concentrations were found to be highly correlated with humidity at the two sites in summer (Table 2). However, no such correlations were found with temperature or wind speed, possibly because of their smaller ranges (15–22°C and 1.3–2.4 m $s^{-1}$) and because rainfall was only one-third the normal amount during the summer sampling period. However, in winter, AP (>7 $\mu$m) was negatively correlated with humidity and positively correlated with wind speed at both sites. Temperature also seemed to be positively correlated with PAH and NPAH levels in all size fractions. These observations suggest that cold and dry air, which blows predominantly from the inner Asian continent in winter, increased the concentrations of coarse particulates (>2.1 $\mu$m) and decreased the concentrations of PAHs, NPAHs and fine particulates (<2.1 $\mu$m) in the atmosphere (Table 2).

The main sources of atmospheric PAHs and/or NPAHs in Shenyang in summer are automobiles, while in winter they seem to be coal stoves and coal boilers used for domestic heating (Tang et al., 2005; Hattori et al., 2007; Kong et al., 2010). In 2007, the percentages of total PAHs and NPAHs in the fine particulate fraction, which is the fraction that most adversely

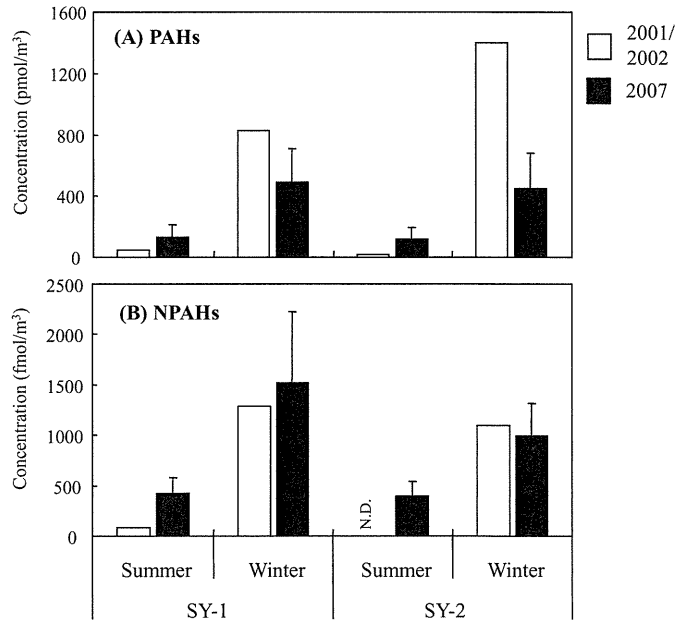


**Figure 1.** Graph for comparison of atmospheric polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs) at two sites in Shenyang in 2001–2002 and in 2007. Each bar means the average concentration of total PAHs or NPAHs; PAHs includes FR, Pyr, BaA, Chr, BbF, BkF, BaP, BgPe and IDP; NPAHs includes 1-NP, 6-NC and 6-NBaP. The data of Shenyang in 2001 –2002 are from Hattori et al. (2007).

*Table 2. Correlation coefficients between polycyclic aromatic hydrocarbons (PAHs), nitropolycyclic aromatic hydrocarbons (NPAHs), and airborne particulates (APs) in different particulate fractions and several meteorological conditions*

| | | Winter | | | Summer | | |
|---|---|---|---|---|---|---|---|
| | | Temperature (°C) | Wind speed (m $s^{-1}$) | Humidity (%) | Temperature (°C) | Wind speed (m $s^{-1}$) | Humidity (%) |
| Meteorological Condition | Wind speed (m $s^{-1}$) | −0.58 | | | −0.71 | | |
| | Humidity (%) | 0.27 | −0.81* | | −0.63 | 0.19 | |
| SY-1 | PAHs (>7 μm) | 0.24 | −0.64 | 0.79* | *0.20 | 0.12 | −0.83** |
| | PAHs (7 − 2.1 μm) | 0.54 | −0.74 | 0.82** | 0.04 | 0.31 | −0.75* |
| | PAHs (<2.1 μm) | 0.43 | −0.81* | 0.92** | −0.11 | 0.56 | −0.63 |
| | NPAHs (>7 μm) | 0.41 | −0.47 | 0.37 | 0.18 | −0.18 | −0.73 |
| | NPAHs (7 − 2.1 μm) | 0.61 | −0.65 | 0.50 | 0.03 | −0.02 | −0.67 |
| | NPAHs (<2.1 μm) | 0.27 | −0.63 | 0.70 | 0.13 | 0.18 | −0.82** |
| | APs (>7 μm) | −0.49 | 0.90** | −0.84** | 0.04 | −0.04 | −0.63 |
| | APs (7− 2.1 μm) | −0.11 | 0.70 | −0.58 | −0.05 | −0.04 | −0.55 |
| | APs (<2.1 μm) | 0.76 | −0.64 | 0.57 | −0.20 | 0.14 | −0.49 |
| SY-2 | PAHs (>7 μm) | 0.48 | −0.81* | 0.83** | 0.21 | 0.07 | −0.84** |
| | PAHs (7 − 2.1 μm) | 0.62 | −0.77* | 0.61 | 0.09 | 0.17 | −0.76* |
| | PAHs (<2.1 μm) | 0.34 | −0.77* | 0.79* | −0.14 | 0.57 | −0.52 |
| | NPAHs (>7 μm) | 0.42 | −0.75* | 0.73 | 0.34 | 0.05 | −0.88** |
| | NPAHs (7 − 2.1 μm) | 0.66 | −0.92** | 0.76* | 0.16 | 0.21 | −0.79* |
| | NPAHs (<2.1 μm) | 0.63 | −0.87** | 0.79* | 0.18 | 0.10 | −0.86** |
| | APs (>7 μm) | −0.39 | 0.90** | −0.90** | 0.47 | −0.21 | −0.90** |
| | APs (7 − 2.1 μm) | −0.00 | 0.50 | −0.62 | 0.18 | 0.01 | −0.79* |
| | APs (<2.1 μm) | 0.68 | −0.66 | 0.53 | −0.04 | 0.19 | −0.61 |

$n = 7$. The data of average temperature, dew point and wind speed in Shenyang during the sampling periods were obtained from National Climatic Data Center (2009) and average humidity were calculated by using the data of average temperature and dew point.

affects human health, were about 92% and 81%, respectively at both sites (Figure 2), in agreement with previous reports. (Hayakawa et al., 1995b; Kawanaka et al., 2004). However, in 2002, the percentages of total PAHs and total NPAHs in the fine fraction were 70% and 60% of the total, respectively. In winter 2002, the coarse size particulates had high PAH and NPAH levels (Figure 2). In general, inefficient boilers contributed greatly to the large size particulates in the atmosphere because of their imperfect combustion. Therefore, the increase in the ratio of the total PAHs and total NPAHs in the fine fraction from 2002 to 2007 was probably due to the removal of approximately 5000 boilers by the Shenyang government.

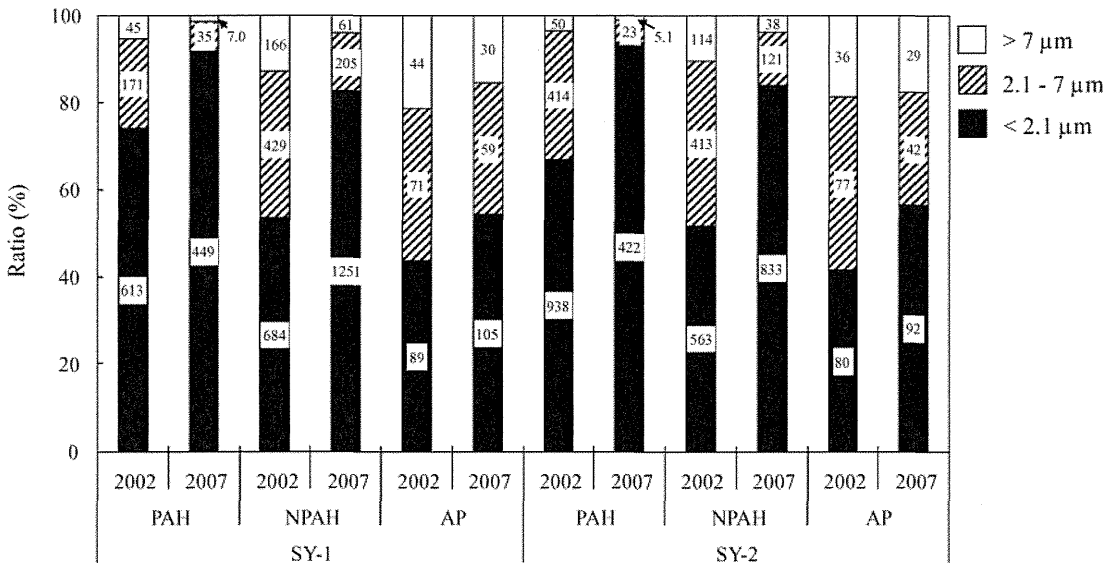


**Figure 2.** Graph of particulate partitions of polycyclic aromatic hydrocarbons (PAHs), nitropolycyclic aromatic hydrocarbons (NPAHs), and airborne particulates (APs) in three size fractions at two sites in Shenyang in 2001–2002 and in 2007. Three size fractions mean larger 7 μm, 7 − 2.1 μm and smaller than 2.1 μm. The data of Shenyang in 2001–2002 are referenced from Hattori et al. (2007). The concentrations of PAHs, NPAHs and APs in each particulate size fraction are shown within each box.

However, the difference of atmospheric concentrations of NPAHs between 2002 and 2007 was not significant. According to our previous study, the concentrations of NPAHs in the particulates collected in automobiles were significantly higher than those in coal combustion systems. By contrast, the concentrations of PAHs were lower in automobiles (Hayakawa et al., 2000; Tang et al., 2005). In addition, the particulate sizes originating from automobiles were very small (Ho et al., 2006). These observations suggest that the increase in the number of motor vehicles also contributed to the fine particulate fractions of PAHs and NPAHs, especially the latter.

*Compositions and Main Sources*

Multivariate statistical analysis methods and several diagnostic ratios have been used to identify possible emission sources of PAHs and NPAHs and to compare their compositions in the atmosphere (Bourotte et al., 2005; Kakimoto et al., 2002; Rogge et al., 1993). The compositions of PAHs and NPAHs were compared by a cluster analysis using Ward's method. As parameters, we used the concentration ratios of 1-NP to Pyr, 6-NBaP to BaP and individual PAHs to total PAHs in the airborne particulates collected at the two sites in 2001–2002 and 2007. The same compound pairs were investigated in Tokyo in summer in 2004 and in winter in 2005 (Tang et al., 2005) and are used here as a reference. As shown in Figure 3, two large clusters were observed according to the compositions of PAHs and NPAHs at all sites. Cluster 1 includes all winter samples collected at the two sites in Shenyang. All summer samples collected at the two sites in Shenyang and summer and winter samples in Tokyo were grouped into Cluster 2. This result indicates that the compositions of PAHs and NPAHs in winter were different from the compositions in summer at the two sites in Shenyang and that the compositions of PAHs and NPAHs in summer at these sites were similar to those in Tokyo in both summer and winter. Previously, we reported that the main sources of atmospheric PAHs and NPAHs were motor vehicles in all seasons in Tokyo, while they were coal combustion systems in winter and motor vehicles and coal combustion systems in summer in Shenyang (Tang et al., 2005; Hattori et al., 2007). These results suggest that the major contributors of atmospheric PAHs and NPAHs in Shenyang did not change significantly during 2001–2002 to 2007. However, the data in Cluster 1 in Figure 3 indicate that the compositions of PAHs and NPAHs in winter at the two sites in Shenyang slightly differed between 2002 and 2007. Possible causes of the differences in 2007 include the air pollution countermeasures and the increase in the number of vehicles.

The [1-NP]/[Pyr] ratio is a useful indicator for estimating the contributions of motor vehicles and coal combustion systems to atmospheric PAHs and NPAHs (Tang et al., 2005). Although 1-NP can form during the sampling and a part of Pyr is distributed in the gas phase, the yields of 1-NP that formed in the filter were smaller than 5% and the partition ratios in the gas and particulate phases of Pyr depend on temperature (Nielsen, 1983; Yamasaki et al., 1982). Figure 4 shows the [1-NP]/[Pyr]

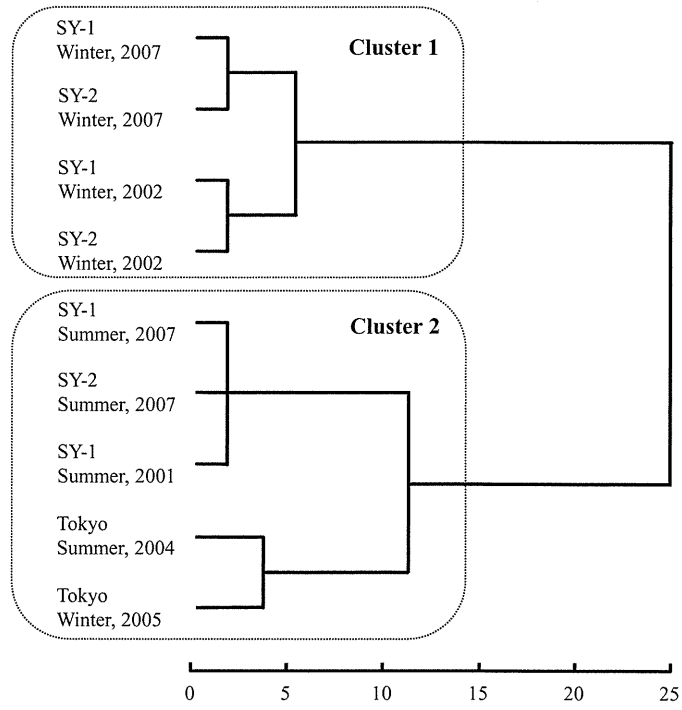


**Figure 3.** Cluster analysis dendrogram of atmospheric polycyclic aromatic hydrocarbons (PAHs) and nitropolycyclic aromatic hydrocarbons (NPAHs)at two sites in Shenyang and in Tokyo by using Ward's method and standardized squared Euclidean distance. The parameters used for the cluster analysis were the concentration ratios of 1-NP to Pyr, 6-NBaP to BaP and individual PAH to total PAHs in the airborne particulates collected at two sites in 2001–2002 and 2007.

ratios in the atmospheric particulates collected at the two sites of Shenyang in 2001–2002 and 2007. During the intervening 5 years, the [1-NP]/[Pyr] ratios in winter increased from 0.003 to 0.014 at SY-1 and from 0.002 to 0.010 at SY-2. In summer, the [1-NP]/[Pyr] ratios also increased from 0.008 (2001) to 0.025 (2007) at SY-1. Although no data was obtained at SY-2 in

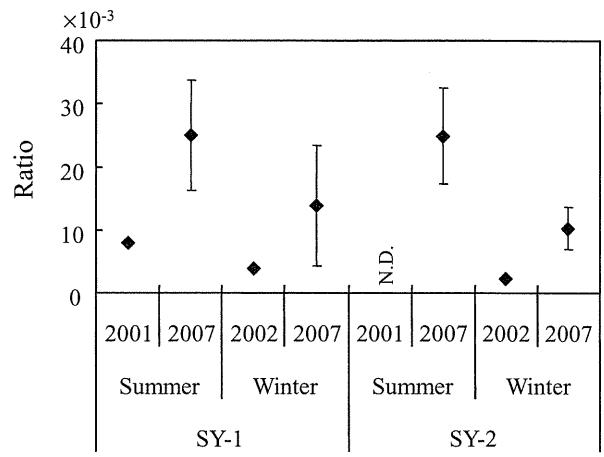


**Figure 4.** Graph for comparison of [1-NP]/[Pyr] ratios at two sites in Shenyang. The data of Shenyang in 2001–2002 are from Hattori et al. (2007).

summer 2001, the [1-NP]/[Pyr] ratio was very high (0.025) as well. Furthermore, the [1-NP]/[Pyr] ratio variance at SY-1 was larger in summer than in winter. The [1-NP]/[Pyr] ratio of particulates emitted by coal combustion systems (0.001) was much smaller than the ratio of particulates emitted by diesel-engine automobiles (0.36) (Tang et al., 2005). These results suggest that motor vehicles became one of the major contributors of atmospheric PAHs and NPAHs in Shenyang in 2007 not only in summer but also in winter.

## Conclusions

The concentrations and distribution of PAHs and NPAHs in different particulate size fractions changed between 2001–2002 and 2007. In winter, the concentrations of coarse size particulates (>2.1 $\mu$m) in the atmosphere decreased from 57.5% to 44.6% over the 5-year period. Concurrently, the concentrations of atmospheric PAHs and NPAHs in winter in the coarse size particulate fractions, which might have originated from inefficient boilers, decreased from 0.35 pmol $\mu g^{-1}$ to 0.15 pmol $\mu g^{-1}$, and 0.55 fmol $\mu g^{-1}$ to 0.32 fmol $\mu g^{-1}$, respectively, during the 5 years. Especially, the concentrations of PAHs and NPAHs in winter 2007 decreased significantly from the concentrations in winter 2002, probably largely as a result of Shenyang's Blue Sky Project. However, both PAH and NPAH concentrations in summer increased from 2001 to 2007, and the concentrations of NPAHs in winter did not change markedly between 2002 and 2007. Our comparative data on the particulate distributions, seasonal variations and compositions of PAHs and NPAHs in the atmosphere show that motor vehicles have become one of the major contributors of atmospheric PAHs and NPAHs in Shenyang not only in summer but also in winter.

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