Determination of 3,6-dinitrobenzo[*e*]pyrene in Surface Soil and Airborne Particles, and Its Possible Sources, Diesel Particles and Incinerator Dusts

Tomohiro Hasei,*, [a] Tetsushi Watanabe,[a] Osamu Endo,[b] Kazutoshi Sugita,[c] Masaharu Asanoma,[d] Sumio Goto,[b] and Teruhisa Hirayama[a]

[a]*Department of Public Health, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan,* [b]*School of Life and Environmental Science, Azabu University, 1–17–71 Fuchinobo, Sagamihara, Kanagawa 229–8501, Japan,* [c]*Analysis Division, Mitsubishi Chemical Analytech, 8–5–1 Chuo, Ami-machi, Inashiki-gun, Ibaraki 300–0332, Japan and* [d]*Nagoya City Public Health Research Institute, 1–11 Hagiyamacho, Mizuho-ku, Nagoya 467–8615, Japan*

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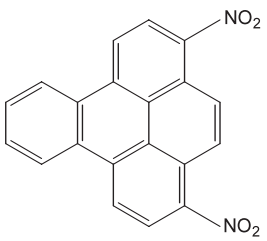
3,6-Dinitrobenzo[*e*]pyrene (3,6-DNBeP) is an extremely strong bacterial mutagen, and was recently identified in highly mutagenic surface soil samples. In a previous study, a sensitive analytical method was developed using high-performance liquid chromatography (HPLC) and fluorescence detection. In this study, we analyzed 3,6-DNBeP in surface soil, airborne particles, diesel particles, and incinerator dusts using this analytical method to reveal the distribution of 3,6-DNBeP in the environment. 3,6-DNBeP was detected in all surface soil samples, and the mutagenic contribution ratio of 3,6-DNBeP to the mutagenicity of the soil extracts toward *Salmonella* (*S.*) typhimurium TA98 was 17.3% on average. A positive correlation was observed between the mutagenicity of surface soil and the amount of 3,6-DNBeP (r = 0.8653). 3,6-DNBeP was detected in airborne particles in the range of 19–76 fg/m^3. The particle-size-distribution ratios of 3,6-DNBeP in <1.1, 1.1–2.0, 2.0–3.3, 3.3–7, and >7 μm of airborne particles were 13.1%, 13.8%, 37.0%, 19.1%, and 17.0%, respectively. 3,6-DNBeP was detected in diesel particles from general automobiles and industrial forklifts, and incinerator dusts. These results suggested that 3,6-DNBeP was a major mutagen in surface soil, and diesel engines and incinerators were possible sources of 3,6-DNBeP distributed in surface soil and air. This is the first report on the detection of 3,6-DNBeP in diesel particles and incinerator dusts.

Key words —— 3,6-dinitrobenzo[*e*]pyrene, particle size, diesel particle, incinerator dust, surface soil, airborne particle

INTRODUCTION

Nitrated polycyclic aromatic hydrocarbons (NPAHs) are emitted into the air by various anthropogenic sources, such as industrial power plants,[1,2] municipal incinerators,[3] and motor vehicles.[4–6] NPAHs are known as strong mutagen/carcinogens.[7] Many epidemiological studies have shown that outdoor air pollution tends to be associated with the incidence of lung cancer and cardiopulmonary mortality.[8–13] The previous study reported the detection of 3,6-dinitrobenzo[*e*]pyrene (DNBeP) (Fig. 1) as a novel mutagen in surface soil collected in Osaka and Aichi prefectures, Japan.[14] 3,6-DNBeP induced as many as 285000 revertants/nmol in *Salmonella* (*S.*) typhimurium TA98 without a mammalian metabolic system (S9 mix). The mutagenicity of 3,6-DNBeP was comparable to that of 1,8-



3,6-Dinitrobenzo[e]pyrene
(3,6-DNBeP)

Fig. 1. Structure of 3,6-DNBeP. 3,6-DNBeP is a Strong Bacterial Mutagen; Inducing 285000 Revertants/nmol in *S.* typhimurium TA98 in the Absence of S9 Mix

*To whom correspondence should be addressed: Department of Public Health, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607–8414, Japan. Tel.: +81-75-595-4650; Fax: +81-75-595-4769; E-mail: hasei@mb.kyoto-phu.ac.jp

dinitropyrene (DNP), which is known as the most potent bacterial mutagen identified so far in the literature.[15] 3,6-DNBeP showed genotoxicity *in vitro* to mammalian cells, such as mutagenicity in *hprt* gene and induction of sister chromatid exchange and micronucleus.[16] Furthermore, 3,6-DNBeP produced DNA damage in the cells of several organs in mice in the comet assay. Recently, a sensitive analytical method for 3,6-DNBeP was developed using high-performance liquid chromatography (HPLC) equipped with an on-line reduction apparatus and a fluorescence detector.[17] By this method, a few numbers of airborne particles and surface soil samples were analyzed, and the results suggested the possibility that 3,6-DNBeP is distributed widely in surface soil and ambient air. However, data on the distribution of 3,6-DNBeP in the environment is quite limited, and there are no reports on the sources of 3,6-DNBeP.

The purpose of this study was to reveal the distribution of 3,6-DNBeP in the surface soil and airborne particles, and sources of 3,6-DNBeP. Surface soil samples were collected in three metropolitan areas, the Kinki, Chukyo, and Kanto regions of Japan. Airborne particles were collected in Nagoya city, Aichi prefecture, the Chukyo region and Wako city, Saitama prefecture, the Kanto region. In order to reveal the particle-size-distribution of 3,6-DNBeP, the airborne particles collected in Wako city were classified by particle size. In addition, the particle-size-distribution of 3,6-DNBeP was compared to those of 1,3-, 1,6-, and 1,8-DNP isomers which are known as representative airborne contaminants.[18–20] Diesel particles and incinerator dusts, which were anticipated as sources of 3,6-DNBeP, were collected. 3,6-DNBeP and DNP isomers in diesel particles and incinerator dusts were analyzed to reveal their sources, and their amounts were compared.

MATERIALS AND METHODS

Reagents —— 3,6-DNBeP (CAS 847862-64-0) was synthesized as described previously.[14] 1,3-DNP (CAS 75321-20-9), 1,6-DNP (CAS 42397-64-8), and 1,8-DNP (CAS 42397-65-9) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Standard Reference Material (SRM) 1975 was purchased from National Institute of Standards and Technology (Gaithersburg, MD, U.S.A.). JSAC 0511 was purchased from The Japan Society for Analytical Chemistry (Tokyo, Japan). HPLC-grade acetonitrile and methanol were purchased from Nacalai Tesque (Kyoto, Japan). Silica gel (63–200 μm) was purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Sampling and Extraction of Surface Soils and Airborne Particles —— Surface soils were collected in parks located in residential areas in three metropolitan areas in the Kinki, Chukyo, and Kanto regions of Japan (Fig. 2). The soil samples were

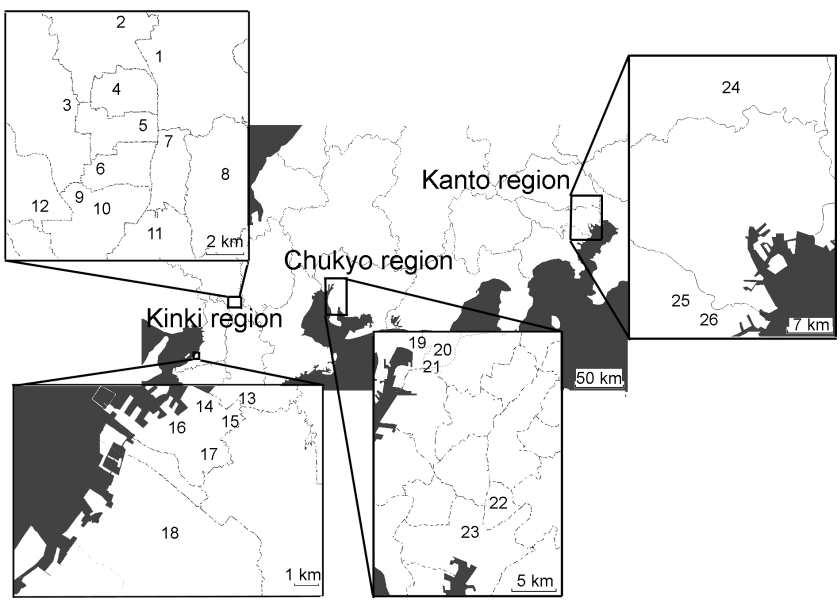


Fig. 2. Sampling Sites of Surface Soil in Three Metropolitan Areas, in the Kinki, Chukyo, and Kanto Regions of Japan
The population in Japan is concentrated in these three metropolitan areas, which have about a half of the total population of Japan.

dried at room temperature for two days and screened through a 60 mesh sieve. Fifteen grams of the sieved soil was extracted ultrasonically with 200 ml of methanol twice for 10 min each. Methanol is commonly used as extraction solvent to surface soil.[21] The extracts were filtered using Advantec Toyo (Tokyo, Japan) No. 5C filter paper, and the filtrate was evaporated to dryness.

Airborne particles were collected from the tops of buildings in residential areas in Nagoya city, Aichi prefecture and Wako city, Saitama prefecture, Japan. The airborne particles in Nagoya city were collected on quartz filters at a flow rate of 1 m^3/min with a high volume air sampler for 24 hr and extracted with 120 ml of methanol using an ultrasonic apparatus for 20 min. Methanol is commonly used as extraction solvent to airborne particles as the most effective solvent.[22] The airborne particles in Wako city were collected on quartz filters at a flow rate of 0.556 m^3/min with an Andersen type high volume sampler for one week in order to classify the particle sizes of the airborne particles; <1.1, 1.1–2.0, 2.0–3.3, 3.3–7, and >7 μm. The filters with particle sizes more than 1.1 μm were extracted with 200 ml once and 100 ml twice of benzene/ethanol (3/1), and those with less than 1.1 μm were extracted with 400 ml once and 300 ml twice of benzene/ethanol (3/1) using an ultrasonic apparatus for 10 min each time. Benzene/ethanol is also often used for extraction solvent to airborne particles.[20, 22] All airborne particle extracts were filtered through No. 5C filter papers, and evaporated to dryness.

Sampling and Extraction of Diesel Particles and Incinerator Dusts —— SRM 1975 is a dichloromethane extract of particles from a diesel engine used for industrial forklifts.[23] Diesel particles No.1 were collected on Teflon-coated filters from an Isuzu engine A that is used for general motor vehicles. Sampling was carried out at a constant condition of 1050 rpm and 80 load, using a high-volume air sampler connected to the vent of the engine, directly. The sampling volume was 4.6 m^3. Incinerator dusts No.1–4 are bottom ash collected from the bottom in four different industrial incinerators. JSAC 0511 was fly ash collected from a wood pulp incinerator using bag filters.[24] Diesel particles and incinerator dusts were extracted ultrasonically with 200 ml of chloroform twice for 10 min each. Chloroform is apt to be used as extraction solvent to diesel particles.[25] Chloroform was also used to extract incinerator dusts as the possible sources of 3,6-DNBeP. The extracts were filtered through No. 5C filter papers and the filtrates were evaporated to dryness.

Clean-up of Extracts from Surface Soil, Airborne Particles, Diesel Particles, and Incinerator Dusts —— Organic extracts from surface soil, airborne particles, diesel particles, and incinerator dusts were dissolved in 1 ml of chloroform, and three aliquots were applied to three open columns (220 mm × 10 mm *id.*) that were filled with silica gel activated for 18 hr at 160°C and then deactivated using distilled water (7.4%, w/w). The extracts were eluted with 20 ml of *n*-hexane, 20 ml of *n*-hexane/toluene (9/1, v/v), 20 ml of *n*-hexane/toluene (2/1, v/v), 20 ml of *n*-hexane/toluene (1/1, v/v), and 30 ml of toluene. 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers were eluted in toluene fraction. Toluene fractions were evaporated to dryness, and the residues were dissolved in 0.5 ml of 70% acetonitrile. Then, 0.45 ml of each sample solution was applied to a Cosmosil 5C_{18}-MS-II column (5 μm particle size, 250 mm × 4.6 mm *id.*, Nacalai Tesque) for HPLC with 70% acetonitrile as the mobile phase at a flow rate of 0.7 ml/min. Because 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers were eluted at retention times of 32.1, 19.5, 17.5, and 16.8 min, respectively, the fractions from 14.8 to 22.5 min and from 30.1 to 35.1 min were collected as DNP and 3,6-DNBeP fractions, respectively. The 3,6-DNBeP fractions were dissolved in 0.5 ml of 90% methanol, and 0.45 ml of each sample solution was applied to a Luna 5 μ Phenyl-Hexyl column (5 μm particle size, 250 mm × 4.6 mm *id.*, Phenomenex, Torrance, CA, U.S.A.) for HPLC with 90% methanol as the mobile phase at a flow rate of 0.7 ml/min. The fractions from 23.3 to 28.3 min were collected as 3,6-DNBeP fractions, because 3,6-DNBeP was eluted at a retention time of 25.3 min. On the other hand, the DNP fractions were dissolved in 85% methanol, and 0.45 ml of the sample solution was applied to the Luna 5 μ Phenyl-Hexyl column for HPLC with 85% methanol as the mobile phase at a flow rate of 0.7 ml/min. The elutes from 19.5 to 30.3 min were collected as DNP fractions, because 1,3-, 1,6-, and 1,8-DNP isomers were eluted at retention times of 27.3, 21.5, and 22.9 min, respectively. HPLC procedures were carried out at 30°C. Elutes were monitored for UV absorption.

HPLC Analysis of 3,6-DNBeP —— The analytical system consisted of a Shimadzu LC-10ADvp pump, Rheodyne 7125 sample injector (loop, 1 ml), Jasco

RO-1561 reaction oven, Jasco NP pak RL reducer column (35 mm × 4.6 mm *id.*, Jasco, Tokyo, Japan), Shimadzu CTO-10Avp column oven, and Jasco FP-1520S fluorescence detector. The NP pak RL reducer column was packed with alumina (<150 μm of particle size) coated with platinum (Pt).

Samples of 3,6-DNBeP fractions were dissolved in 85% ethanol, and 0.2 ml of each solution was injected into a Wakosil PAHs column (5 μm particle size, 250 mm × 4.6 mm *id.*, Wako Pure Chemical, Osaka, Japan) as a stationary phase, connected with the NP pak RL reducer column, continuously. After separation from interfering peaks with the Wakosil PAHs column, 3,6-DNBeP was reduced to 3,6-diaminobenzo[*e*]pyrene (DABeP) by on-line reduction using the NP pak RL column at 80°C to be detected using the fluorescence detector. Elution was carried out with 95% methanol at a flow rate of 0.7 ml/min. The detection excitation and emission wavelengths were 319 and 443 nm, respectively. Authentic 3,6-DNBeP as a standard was also dissolved in 85% ethanol injected at more than three doses into a column in order to draw calibration curves. The calibration curves were drawn with the peak heights of 3,6-DABeP on the chromatograms. HPLC procedure was carried out at 30°C.

HPLC Analysis of 1,3-, 1,6-, and 1,8-DNP Isomers —— 1,3-, 1,6-, and 1,8-DNP isomers were analyzed as described previously.[26–28] Samples of DNP fractions were dissolved in 50% ethanol, and 0.2 ml of each solution was injected into a Cosmosil $5C_{18}$-AR-II column (5 μm particle size, 250 mm × 4.6 mm *id.*, Nacalai Tesque) as the stationary phase, and connected with the NP pak RL reducer column, continuously. Three DNP isomers were separated using the Cosmosil $5C_{18}$-AR-II column and reduced to the corresponding diaminopyrene (DAP) isomers by on-line reduction using the NP pak RL column at 80°C to detect the fluorescence. Elution was carried out with 85% methanol at a flow rate of 0.7 ml/min. The detection excitation and emission wavelengths were 375 and 450 nm, respectively. Authentic DNP isomers were dissolved in 50% ethanol, and 0.2 ml of each solution was injected at more than three doses into a column in order to draw calibration curves. The calibration curves were drawn with the peak heights of 1,3-, 1,6-, and 1,8-DAP isomers on the chromatograms. HPLC procedure was carried out at 30°C.

Quality Assurance —— The relative standard division ($n = 4$) of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers was less than 2.2%. Under the analytical conditions described above, 3,6-DNBeP and DNP isomers standards were injected into the columns and the calibration graphs showed good linearity ($r^2 > 0.9999$) in the range of 0.2–200 pg.

3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers standards 500 pg were applied on silica gel, COSMOSIL $5C_{18}$-MS-II and Luna 5 μ Phenyl-Hexyl columns. After elutes were collected, amounts of 3,6-DNBeP and DNP isomers in the elutes were compared to each standard. The recoveries of 3,6-DNBeP and three DNP isomers on each column were shown in Table 1. The recoveries were enough high in more than 92%, perpetually.

When the extracts were applied on the silica gel columns, each extract was divided to three aliquot

Table 1. Recoveries and Retention Times of 3,6-DNBeP and Three DNP Isomers on Each Purification Step

Step	Column	Compound	Retention time (min)	Recovery (%)
1	Silica gel column	3,6-DNBeP	—[a]	93
		1,3-DNP	—[a]	98
		1,6-DNP	—[a]	98
		1,8-DNP	—[a]	100
2	Cosmosil $5C_{18}$-MS-II	3,6-DNBeP	32.1[b]	98
		1,3-DNP	19.5[b]	98
		1,6-DNP	17.5[b]	98
		1,8-DNP	16.8[b]	99
3	Luna 5 μ Phenyl-Hexyl	3,6-DNBeP	25.3[c]	100
		1,3-DNP	27.3[d]	92
		1,6-DNP	21.5[d]	94
		1,8-DNP	22.9[d]	93

a) 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers were eluted in toluene fraction. *b*) Elution was carried out with 70% acetonitrile at a flow rate of 0.7 ml/min. *c*) Elution was carried out with 90% methanol at a flow rate of 0.7ml/min. *d*) Elution was carried out with 85% methanol at a flow rate of 0.7 ml/min.

in order to confirm the accuracy. Quantification results were shown as "mean value ± standard division" (n = 3).

Mutagenicity Test of Surface Soil —— The extracts of surface soils were dissolved in dimethyl sulfoxide and assayed by the preincubation method[29] using *S.* typhimurium TA98 without S9 mix. The mutagenic potencies of samples were calculated from linear portions of the dose-response curves obtained with three or four doses and duplicated plates at each dose. The slope of the dose-response curves was adapted as the mutagenic potency. When the samples induced two-fold increases over the average of spontaneous revertants and showed well-behaved concentration-response patterns, the samples were judged positive. The mutagenic potency of 3,6-DNBeP used to calculate the contribution ratio was 1.357 revertants/pg.

RESULTS

Determination of 3,6-DNBeP in Surface Soil and Airborne Particles Using Fluorescence Detection

Surface soil was collected in Kyoto and Osaka prefectures (the Kinki region), Aichi prefecture (the Chukyo region), and Saitama and Kanagawa prefectures (the Kanto region). Since 3,6-DNBeP was detected in Kyoto, Izumiotsu, Nagoya, and Hekinan cities in the previous study,[14,17] sampling was carried out at their environs. Airborne particles were collected in Aichi prefecture (the Chukyo region) since 3,6-DNBeP was detected in the surface soil collected in Aichi prefecture.[14] Surface soil and airborne particles were extracted with methanol. After clean-up using silica gel column chromatography and HPLC, 3,6-DNBeP fractions from extracts of surface soil and airborne particles were analyzed by HPLC coupled with on-line reduction and a fluorescence detector. Typical chromatograms of authentic 3,6-DNBeP and 3,6-DNBeP in surface soil and airborne particles are shown in Fig. 3A, B, and C, respectively. 3,6-DNBeP was reduced using the NP pak RL column to be detected as 3,6-DABeP at a retention time of 11.4 min on the chromatograms. Table 2 shows the amounts of 3,6-DNBeP detected in surface soils and the contribution ratios of 3,6-DNBeP to the mutagenicity of surface soil. 3,6-DNBeP was detected in all surface soil samples in the range of 8–975 pg/g of soil, and the mutagenic contribution ratios were quite high in the range of 4.5–75.1%. Table 3 shows the amounts of 3,6-DNBeP detected in airborne particles collected in Nagoya city. 3,6-DNBeP was detected in all airborne particles collected in three sampling sites, Nagoya city in the range of 19–76 fg/m^{3}after the correction by recoveries.

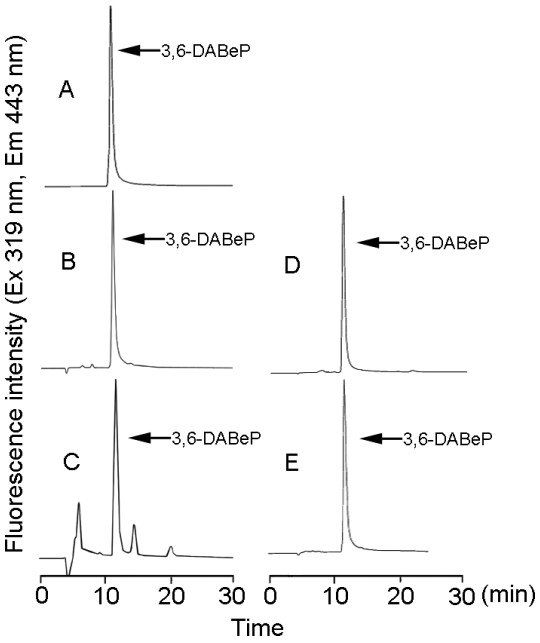


Fig. 3. Chromatograms of 3,6-bNBeP

Typical chromatograms of authentic 3,6-DNBeP (A), and 3,6-DNBeP in surface soil (B), airborne particles (C), diesel particles (D), and incinerator dusts (E). After purification with the silica gel column and two HPLC columns, 3,6-DNBeP injected into the Wakosil PAHs column. 3,6-DNBeP was reduced by on-line reduction with the NP pak RL, and detected as 3,6-DABeP by a fluorescence detector. The chromatograms were monitored with the excitation and emission wavelengths of 319 and 443 nm, respectively. Because 3,6-DNBeP in each extract was purified efficiently by the three-step purification, few interfering peaks were observed nearby the peaks of 3,6-DABeP.

Particle-size-determination of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Airborne Particles Collected in Wako City

3,6-DNBeP was detected in every particle size, *i.e.* <1.1, 1.1–2.0, 2.0–3.3, 3.3–7, and >7 μm, collected in Wako city in the range of 188–530 fg/m^3, and the total amount was 1433 fg/m^3 (Table 4). The highest particle-size-distribution in airborne particles of 3,6-DNBeP was observed in 2.0–3.3 μm (530 fg/m^3, 37.0%). The second and third highest distributions were observed in 3.3–7.0 μm (273 fg/m^3, 19.1%) and >7 μm (244 fg/m^3, 17.0%) sized particles, respectively.

Typical chromatograms of authentic three DNP isomers and those in airborne particles are shown in Fig. 4A and B, respectively. Peaks of 1,3-, 1,6-, and 1,8-DNP isomers, detected as corresponding DAP isomers, were observed at retention times of 18.5,

Table 2. Amount of 3,6-DNBeP and Mutagenicity in Surface Soil

	Sampling site	Sampling date	Amount[a] MV ± S.D. (pg/g of soil)	Mutagenicity[b] (revertants/g of soil)	Contribution[c] (%)
Kinki region					
	Kyoto prefecture				
	Kyoto city				
1	Sakyo ward	18 Nov. 2006	29 ± 1	124	31.7
2	Kita ward	18 Nov. 2006	97 ± 29	479	25.7
3	Ukyo ward	18 Nov. 2006	270 ± 40	1840	19.9
4	Kamigyo ward	21 Dec. 2007	20 ± 1	98	27.7
5	Nakagyo ward	18 Nov. 2006	46 ± 14	401	15.6
6	Shimogyo ward	18 Nov. 2006	8 ± 5	203	5.3
7	Higashiyama ward	5 Apr. 2008	25 ± 3	460	7.4
8	Yamashina ward	5 Apr. 2008	671 ± 115	1210	75.3
9	Minami ward-1[d]	25 Dec. 2004	355 ± 17	2900	16.6
10	Minami ward-2[d]	25 Dec. 2004	347 ± 81	2860	16.5
11	Fushimi ward	18 Nov. 2006	44 ± 7	194	30.8
12	Nishikyo ward	18 Nov. 2006	60 ± 9	591	13.8
	Osaka prefecture				
13	Takaishi city	19 Nov. 2005	149 ± 21	743	27.2
14	Izumiotsu city-1	19 Nov. 2005	975 ± 266	6370	20.8
15	Izumiotsu city-2	19 Nov. 2005	72 ± 23	625	15.6
16	Izumiotsu city-3	19 Nov. 2005	30 ± 12	913	4.5
17	Izumiotsu city-4	19 Nov. 2005	54 ± 1	501	14.6
18	Kishiwada city	19 Nov. 2005	181 ± 14	627	39.2
Chukyo region					
	Aichi prefecture				
	Nagoya city				
19	Minato ward	8 Dec. 2005	93 ± 7	447	28.2
20	Minami ward-1	8 Dec. 2005	187 ± 11	669	37.9
21	Minami ward-2	8 Dec. 2005	313 ± 108	913	46.5
22	Takahama city	25 Nov. 2005	25 ± 1	155	21.9
23	Handa city	25 Nov. 2005	43 ± 9	354	16.5
Kanto region					
	Saitama prefecture				
24	Wako city	30 Nov. 2005	34 ± 5	211	21.9
	Kanagawa prefecture				
	Kawasaki city				
25	Nakahara ward	8 Dec. 2005	51 ± 7	401	17.3
26	Kawasaki ward	2 Apr. 2005	82 ± 28	1100	10.1

a) Amounts were corrected by the recovery of each purification step. *b*) Mutagenicity of 3,6-DNBeP was tested in *S.* typhimurium TA98 without mammalian metabolic system (S9 mix). *c*) The mutagenic contribution ratios of 3,6-DNBeP to surface soils were calculated as 1.357 revertants/pg of 3,6-DNBeP. It is supposed that mutagens in each sample don't interact. *d*) The data were reported in the previous study.[17]

13.3, and 16.0 min, respectively. Table 4 shows the particle-size-distribution of three DNP isomers in airborne particles collected in Wako City. 1,3-, 1,6-, and 1,8-DNP isomers were detected in the range of 5–60, 8–34, and 1–20 fg/m^3, respectively, and the total amounts of the three DNP isomers were 93, 76, and 58 fg/m^3, respectively. The highest particle-size-distribution of 1,3-, 1,6-, and 1,8-DNP isomers was observed in <1.1 μm (60, 34, and 20 fg/m^3, and 64.5%, 44.7%, and 34.5%, respectively).

Determination of 3,6-DNBeP in Diesel Particles and Incinerator Dusts Using Fluorescence Detection

Diesel particles extracts SRM 1975, purchased from National Institute of Standards and Technology, is the extract of particle collected from a diesel engine used for industrial forklift.[23] Diesel parti-

cles No.1 was collected from a diesel engine used for general automobiles. Incinerator dusts No.1–4 were bottom ash, and JSAC0511 was fly ash.[24] The chloroform extracts of diesel particles and incinerator dusts were analyzed by HPLC coupled with on-line reduction and a fluorescence detector after purification. Typical chromatograms of 3,6-DNBeP in diesel particles and incinerator dusts are shown in Fig. 3D and F, respectively. Peaks of 3,6-DNBeP, detected as 3,6-DABeP, were observed at a retention time of 11.4 min without any interfering peaks nearby.

3,6-DNBeP was detected in all samples analyzed in the ranges of 29–90 ng/g of diesel particle and 3–5900 pg/g of incinerator dust. Tables 5 and 6 show the amounts of 3,6-DNBeP detected in diesel particles and incinerator dusts, respectively.

Determination of 1,3-, 1,6-, and 1,8-DNP Isomers in Diesel Particles and Incinerator Dusts with Fluorescence Detection

Typical chromatograms of three DNP isomers in diesel particles and incinerators dusts are shown in Fig. 4 C and D, respectively. Peaks of 1,3-, 1,6-, and 1,8-DNP isomers, detected as corresponding DAP, were observed at retention times of 18.5, 13.3, and 16.0 min, respectively. Three DNP isomers were purified using the silica gel column and two HPLC columns. Tables 5 and 6 show the amounts of three DNP isomers detected in diesel particles and incinerator dusts. 1,3-, 1,6-, and 1,8-DNP isomers were detected at the amounts of 4, 5, and 5 ng/g of diesel particle, respectively, and detected in the range of ND–5, ND–5, and ND–5 pg/g of incinerator dust, respectively.

Table 3. Amount of 3,6-DNBeP in Airborne Particles Collected in Nagoya City

Sampling site	Sampling date	Amount[a] (fg/m^3 of airborne) MV ± S.D.
Chukyo region		
Aichi prefecture		
Nagoya city-1	27 Jan. 2005	76 ± 5
Nagoya city-2	31 Jan. 2005	48 ± 13
Nagoya city-3	1 Feb. 2005	19 ± 6

a) Amounts were corrected by the recovery of each purification step.

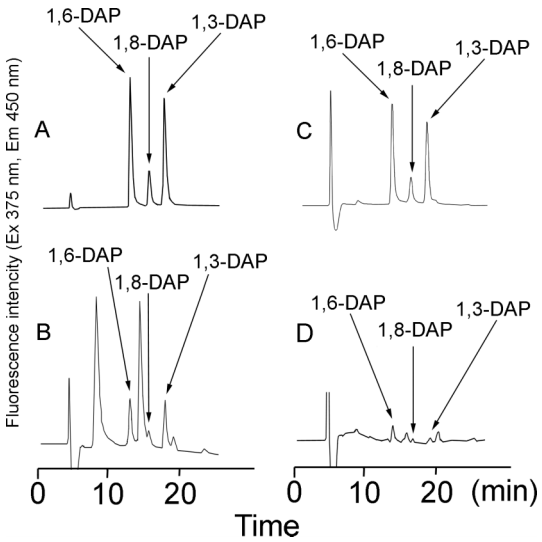


Fig. 4. Chromatograms of 1,3-, 1,6-, and 1,8-DNP isomers

Typical chromatograms of authentic 1,3-, 1,6-, and 1,8-DNP isomers (A), and 1,3-, 1,6-, and 1,8-DNP isomers in airborne particles (B), diesel particles (C), and incinerator dusts (D). After purification with the silica gel column and two HPLC columns, 1,3-, 1,6-, and 1,8-DNP isomers were injected into the Cosmosil 5C_{18}-AR-II column. Three DNP isomers were reduced by on-line reduction with the NP pak RL, and detected as 1,3-, 1,6-, and 1,8-DAP isomers by a fluorescence detector. The chromatograms were monitored with excitation and emission wavelengths of 375 and 450 nm, respectively.

Table 4. Particle-size-distribution of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Airborne Particles Collected in Wako City

Particle size (μm)	Amounts[a] (fg/m^3 of airborne) 3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
> 7	244 ± 7	6 ± 3	12 ± 5	14 ± 7
3.3–7	273 ± 134	10 ± 2	9 ± 1	8 ± 2
2.0–3.3	530 ± 55	5 ± 1	13 ± 1	15 ± 0
1.1–2.0	198 ± 100	12 ± 6	8 ± 10	1 ± 0
< 1.1	188 ± 40	60 ± 7	34 ± 10	20 ± 1
total	1433	93	76	58

a) Amounts were corrected by the recovery of each purification step.

Table 5. Amounts of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP isomers in diesel particle

	Amounts[a] (ng/g of diesel particle)			
	3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
No. 1	29 ± 7	4 ± 3	5 ± 4	5 ± 4
SRM1975	90 ± 7	—[b]	—[b]	—[b]

a) Amounts were corrected by the recovery of each purification step. *b*) — : Not analyzed.

Table 6. Amounts of 3,6-DNBeP and 1,3-, 1,6-, and 1,8-DNP Isomers in Incinerator Dust

	Amounts[a] (pg/g of incinerator dust)			
	3,6-DNBeP MV ± S.D.	1,3-DNP MV ± S.D.	1,6-DNP MV ± S.D.	1,8-DNP MV ± S.D.
Bottom Ash				
No. 1	5900 ± 1200	5 ± 6	5 ± 5	2 ± 0
No. 2	164 ± 3	2 ± 1	3 ± 1	5 ± 2
No. 3	15 ± 3	ND[b]	ND[b]	ND[b]
No. 4	3 ± 0	ND[b]	ND[b]	ND[b]
Fly Ash				
JSAC 0511	51 ± 10	3 ± 0	4 ± 1	ND

a) Amounts were corrected by the recovery of each purification step. *b*) ND : Not detected.

DISCUSSIONS

3,6-DNBeP was detected in all surface soil samples collected in three metropolitan areas in Japan. Moreover, 3,6-DNBeP was detected in all surface soil samples collected in 11 wards of Kyoto city. These results indicated that the three metropolitan areas in Japan were contaminated with 3,6-DNBeP, and that the contamination was not restricted in particular sites. The amounts of 3,6-DNBeP in surface soil were 8–975 pg/g of soil, and the contribution ratio of 3,6-DNBeP to the mutagenicity of surface soil toward *S.* typhimurium TA98 without S9 mix was in the range of 4.5–75.1%. The mean value of the mutagenic contribution ratio of 3,6-DNBeP was quite high at 17.3%. Significant differences were not observed in the mutagenic contribution ratios between the three metropolitan areas. The correlation between the mutagenicity of surface soil (X pivot) and amounts of 3,6-DNBeP in surface soil (Y pivot) is shown in Fig. 5. The slope was 0.1468 and the coefficient of correlation was quite high at 0.8653. These results suggested that there was a positive correlation between the mutagenicity of surface soil and the amounts of 3,6-DNBeP in surface soil, and that 3,6-DNBeP was a major mutagen in surface soil.

3,6-DNBeP was detected in airborne particles collected at three sampling sites in Nagoya city, Aichi prefecture (the Chukyo region). The detection of 3,6-DNBeP in airborne particles collected in Kyoto and Osaka prefectures (the Kinki region), and Tokyo prefecture (the Kanto region) was reported previously.[17] These results suggested that 3,6-DNBeP was widely distributed in the air in these three metropolitan areas in Japan.

In the airborne particles collected in Wako

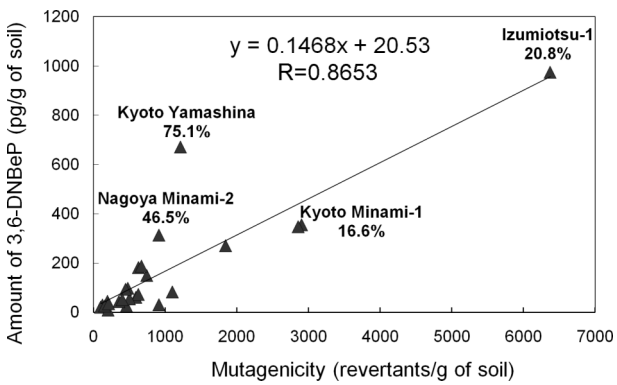


Fig. 5. Correlation between the Amount of 3,6-DNBeP in Surface Soil and Mutagenicity of Surface Soil Collected in Three Metropolitan Areas Toward *S.* typhimurium TA98 without S9 Mix

The slope was 0.1468, and the coefficient of correlation was high in 0.8653. The positive correlation between the amount of 3,6-DNBeP in surface soil and mutagenicity of surface soil was determined from this correlation graph. Representative sampling sites and their mutagenic contribution ratios (%) are noted in the graph.

city, the highest level of 3,6-DNBeP was observed in 2.0–3.3 μm sized particles, while those of the three DNP isomers were observed in <1.1 μm sized particles. However, the amount of 3,6-DNBeP (188 fg/m^3) in particles sized <1.1 μm was 3–9 times greater than those of 1,3-, 1,6-, and 1,8-DNP isomers (60, 34, and 20 fg/m^3, respectively) in this study. It is known that particles with a diameter >5 μm tend to be filtered out in the nose for the most part, and that those <1–2 μm in diameter are deposited predominantly in the alveolar regions of lung.[30] The amount of 3,6-DNBeP that shall be deposited in alveolar tissue was thought to be much greater than those of the three DNP isomers.

3,6-DNBeP was detected in both diesel particles in the range of 29–90 ng/g of diesel particle. SRM 1975 and diesel particles sample No.1 were collected from diesel engines used for industrial forklifts and general motor vehicles, respectively. These results suggested that diesel engines were one of the sources of 3,6-DNBeP, and that 3,6-DNBeP was produced by the combustion of various diesel engines used for industry and general transportation to be emitted into air and surface soil.

3,6-DNBeP was detected in all analyzed incinerator dusts in the range of 3–5900 pg/g of incinerator dust. 3,6-DNBeP was detected not only in the bottom ash of incinerator dusts but also in fly ash, which was emitted into the air directly, like diesel particles. These results suggested that 3,6-DNBeP was produced by combustion in incinerators, and emitted into air and surface soil. The five incinerator dusts analyzed in this study were collected from incinerators that had different combustion scales and temperatures. The differences between incinerators may affect the production of 3,6-DNBeP in the incinerator dusts.

NPAHs are known to be produced by the combustion of diesel engines and incinerators. The amounts of 3,6-DNBeP in diesel engine particles and incinerator dusts were higher than those of 1,3-, 1,6-, and 1,8-DNP isomers. These results suggested that 3,6-DNBeP was more easily produced by combustion in diesel engines and incinerators rather than DNP isomers. Some NPAHs are reported to be produced from PAHs and NO_X by photochemical reactions with ultraviolet rays[31] or PAHs and N_2O_5 without photoradiation[32] in the atmosphere. Because benzo[*e*]pyrene (BeP) is detected in ambient air,[33] the formation of 3,6-DNBeP from BeP and NO_X with or without photoradiation might be another source of 3,6-DNBeP in the environment.

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