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H. 知的財産権の出願・登録状況 (予定を含む。)
1. 特許取得
なし
2. 実用新案登録
なし
3.その他
なし

別紙４

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ

雑誌

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Mutagenicity of surface soil from residential areas in Kyoto city, Japan, and identification of major mutagens

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Abstract

To clarify the mutagenic potential of surface soil in residential areas in Kyoto city, surface soil samples were collected twice or three times from 12 sites, and their organic extracts were examined by the Ames/*Salmonella* assay. Almost all (>92%) samples showed mutagenicity in TA98 without and with S9 mix, and 8/25 (32%) samples showed high (1000–10,000 revertants/g of soil) or extreme (>10,000 revertants/g of soil) activity. Moreover, to identify the major mutagens in surface soil in Kyoto, a soil sample was collected at a site where soil contamination with mutagens was severe and continual. The soil extract, which showed potent mutagenicity in TA98 without S9 mix, was fractionated by diverse column chromatography methods. Five major mutagenic constituents were isolated and identified to be 1,6-dinitropyrene (DNP), 1,8-DNP, 1,3,6-trinitropyrene (TNP), 3,9-dinitrofluoranthene (DNF), and 3,6-dinitrobenzo[*e*]pyrene (DNBeP) by co-chromatography using high performance liquid chromatography and spectral analysis. Contribution ratios of 1,6-DNP, 1,8-DNP, 1,3,6-TNP, 3,9-DNF, and 3,6-DNBeP to total mutagenicity of the soil extract in TA98 without S9 mix were 3, 10, 10, 10, and 6%, respectively. These nitroarenes were detected in surface soil samples collected from four different residential sites in other prefectures, and their contribution ratios to soil mutagenicity were from 0.7 to 22%. These results suggest that surface soil in residential areas in Kyoto was widely contaminated with mutagens and there were some sites where surface soils were heavily polluted. 1,6-DNP, 1,8-DNP, 1,3,6-TNP, 3,9-DNF, and 3,6-DNBeP may be major mutagenic constituents that contaminate surface soil in Kyoto and other residential areas.

Keywords: Surface soil; Mutagenicity; 1,6-Dinitropyrene; 1,8-Dinitropyrene; 1,3,6-Trinitropyrene; 3,9-Dinitrofluoranthene; 3,6-Dinitrobenzo[*e*]pyrene

1. Introduction

Diverse mutagenic/carcinogenic compounds have been released into ambient air from anthropogenic sources such as industrial power plants [1,2], furnaces

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for metal smelting [3,4], municipal incinerators [5], and engines of motor vehicles [6–8]. Epidemiological studies have shown that air pollution tends to be associated with the incidence of lung cancer [9–15]. Compounds released into air eventually deposit on the ground and can be accumulated in surface soil; therefore, surface soil is thought to be a promising material for monitoring environmental pollution with mutagens and carcinogens. Indeed, many studies showed that organic extracts of soils from roadsides [16–19], parks [19,20], agricultural land [21–23], residential sites [24,25], and so forth exhibited mutagenicity and/or DNA damaging activity. In a review of published data on the mutagenicity of soil, White et al. [26] divided the compiled data on *Salmonella* mutagenic potencies into three site categories, i.e. rural/agricultural, urban/suburban, and industrial, and compared their geometric mean values. Analysis of variance revealed a significant relationship between site category and mutagenic potency in TA98 and TA100 with and without the mammalian metabolic system (S9 mix). The geometric mean mutagenicity values of urban/suburban and industrial sites were higher than those of rural/agricultural sites.

In previous studies [27,28], we found that surface soils in five geographically different regions of Japan, i.e. Hokkaido, Kanto, Chubu, Kinki, and Kyushu regions, were largely polluted with mutagens and that organic extracts of soil samples from some sites showed remarkably high potency in TA98 without S9 mix. Moreover, mutagenic potencies of soil samples from three metropolises of Japan, i.e. Tokyo, Nagoya, and Osaka, and their environs, in TA98 without S9 mix were significantly correlated with the amount of 1,3-, 1,6-, and 1,8-dinitropyrene (DNP) isomers. These DNP isomers are among the most potent bacterial mutagens identified so far in the literature [29] and are carcinogenic in mice [30] and rats [31]. The mean value of the total percent contributions of these DNP isomers to soil mutagenicity was about 25% [25,26]. Recently, a potent mutagen was isolated from a highly mutagenic surface soil sample, which was collected in Takatsuki in Osaka prefecture in May 2002, and was determined to be 3,6-dinitrobenzo[*e*]pyrene (DNBeP) [32]. 3,6-DNBeP is a novel chemical, and its mutagenic activity was comparable to that of DNP isomers. The contribution ratio of 3,6-DNBeP to the total mutagenicity of the soil extract was 15%.

Kyoto is Japan's seventh largest city, with a population of about 1.5 million, and one of the cities in the Hanshin Industrial Region. Electronics is a sizable heavy industry in the city. Kyoto is one of the most historic places in Japan, and tourism is a large base of Kyoto's economy. More than 40 million tourists visit Kyoto every year. Kyoto is an inland city and is surrounded on three sides, i.e. north, east, and west, by mountains. In a previous study [25], a few soil samples were randomly collected from a residential area in Kyoto city between February 1999 and November 2000, and one of the samples showed potent activity in TA98 with and without S9 mix. Although these results suggest that surface soil in Kyoto might be highly contaminated with mutagens, comprehensive assessment of the mutagenicity levels of surface soil in Kyoto has not been performed. In this study, to clarify the contamination levels of surface soil in residential areas in Kyoto city with mutagens, surface soil samples were collected twice or three times from 12 sites, and their organic extracts were examined for mutagenicity by the Ames/*Salmonella* assay. Moreover, to reveal the chemical structures of major mutagenic constituents, a large amount of a surface soil sample was collected from a site where soil contamination with mutagens was severe and persistent, and its organic extract was fractionated by diverse column chromatography methods. Five chemicals, which accounted for 39% of the mutagenicity of the soil extract in total, were identified. The distribution of these mutagens was also investigated in other highly mutagenic surface soil samples collected from four different residential sites in other prefectures.

2. Materials and methods

2.1. Materials

1,6-DNP (CAS 42397-64-8) and 1,8-DNP (CAS 42397-65-9) were purchased from Sigma Chemical Co. (St. Louis, MO). 3,9-Dinitrofluoranthen (DNF) (CAS 22506-53-2), 1,3,6-trinitropyrene (TNP) (CAS 75321-19-6), and 3,6-DNBeP (CAS 847862-64-0) were synthesized by methods described previously [32–34]. High performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of analytical grade.

2.2. Collection of surface soil in Kyoto prefecture and preparation of organic extracts

Sample collection and preparation of organic extracts were performed by the method described previously [27]. Soil samples were collected from parks in residential areas of Kyoto and Mukomachi cities in Kyoto prefecture between December 2003 and January 2006. The locations of the sampling sites are shown in Fig. 1. At each sampling site, soil samples were collected twice or three times. Soil from the surface of the ground to about 10 cm deep was dug up, mixed thoroughly, and

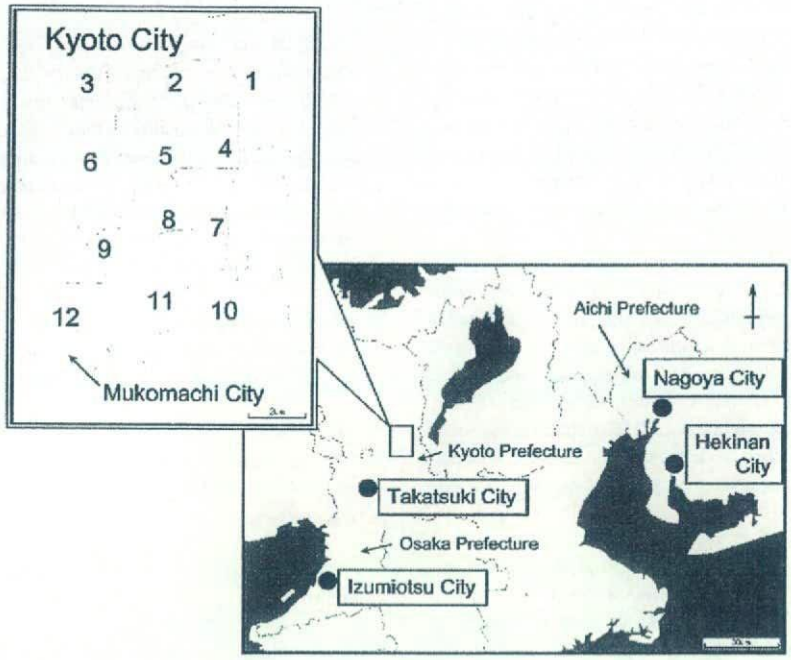

Fig. 1. Geographic locations of the sampling sites in Kyoto, Aichi, and Osaka Prefectures, Japan.

collected. After 2 days' drying at room temperature, soils were screened through a 60-mesh sieve. The sieved soil (15 g) was extracted twice with methanol using an ultrasonic apparatus (185 W) for 10 min. The extracts were combined, filtered, and evaporated to dryness for the mutagenicity test.

2.3. Isolation of mutagens in surface soil from Kyoto

For isolation of mutagens in surface soil from Kyoto, an organic extract was prepared from soil, which was collected at site No. 9 (Minami Ward 1) in April 2005, with Soxhlet apparatus and fractionated with a Sephadex LH-20 column, a silica gel column, and an Ultra pack ODS column as described previously [32]. In brief, soil extracts were applied to the Sephadex LH-20 column (27 mm × 800 mm) and eluted with chloroform/methanol (1:1). An aliquot of each 15 ml fraction was tested for mutagenicity. Mutagenic fractions, which were eluted at elution volumes of 240–330 ml, were combined and evaporated. The residue was applied to a silica gel column (11 mm × 300 mm) for low-pressure liquid chromatography (LPLC) and eluted with *n*-hexane, *n*-hexane/toluene, toluene, chloroform, and methanol. Aliquots of 15 ml were collected, and mutagenic fractions with elution volumes of 630–750 ml were combined and evaporated. The residue was applied to the Ultra pack ODS column (11 mm × 300 mm, Yamazen Corp., Osaka, Japan) and eluted with acetonitrile/water. Aliquots 6 ml were collected. Fractions at elution volumes of 18–96 ml and 156–198 ml were designated Fr. A and Fr. B, respectively, and evaporated to dryness.

Fr. A was dissolved in 50% tetrahydrofuran and applied to a COSMOSIL $5C_{18}$ AR-II column (10 mm × 250 mm, Nacalai Tesque Inc.) for HPLC. The materials were eluted with a gradient system of methanol in distilled water: 0–60 min, 75%; 60–70 min, a linear gradient of 75–100%; 70–90 min, 100%, at a flow rate of 3 ml/min. Aliquots of 3 ml were collected. Fractions with retention times of 33–37 and 44–48 min were designated Fr. A-1 and Fr. A-2, respectively, and evaporated. Fr. A-1, dissolved in 45% tetrahydrofuran, was applied to a Luna 5 μ Phenyl-Hexyl column (10 mm × 250 mm, Phenomenex, Torrance, CA) for HPLC and then eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, a linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 3 ml/min. Aliquots of 3 ml were collected, and fractions with retention times at 27–29 and 36–38 min were designated Fr. A-1-1 and Fr. A-1-2, respectively, and evaporated. Fr. A-1-1 and Fr. A-1-2 were separately dissolved in 45% tetrahydrofuran and applied to a Inertsil ODS-EP column (4.6 mm × 250 mm, GL Science Inc., Tokyo, Japan) for HPLC and then eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, a linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. Fractions of 0.7 ml were collected. Two mutagenic compounds (compounds I and II) were isolated from Fr. A-1-1 as follows: compound I at a retention time of 40 min

and compound II at a retention time of 43 min. Another mutagen (compound III) was obtained from Fr. A-1-2 at a retention time of 33 min. Fr. A-2, dissolved in 45% tetrahydrofuran, was applied to the Luna 5 μ Phenyl-Hexyl column and eluted by the method used for Fr. A-1. Fractions with retention times of 26–28 min were evaporated to dryness. The residue was dissolved in 45% tetrahydrofuran and applied to the Inertsil ODS-EP column. By eluting with the gradient system used for Fr. A-1-1, a mutagen (compound IV) was isolated at a retention time of 39 min.

Fr. B was dissolved in 75% tetrahydrofuran and applied to the COSMOSIL $5C_{18}$ AR-II column. The materials were eluted with a gradient system of methanol in distilled water: 0–60 min, 87%; 60–70 min, a linear gradient of 87–100%; 70–90 min, 100%, at a flow rate of 3 ml/min. Aliquots of 3 ml were collected. Fractions with retention times of 34–37 min were evaporated and redissolved in 45% tetrahydrofuran to apply to the Luna 5 μ Phenyl-Hexyl column. Elution was performed with a gradient system of acetonitrile in distilled water: 0–60 min, 62%; 60–70 min, a linear gradient of 62–100%; 70–90 min, 100%, at a flow rate of 3 ml/min. Fractions with retention times of 42–44 min were evaporated. The residue, dissolved in 50% tetrahydrofuran, was applied to the Inertsil ODS-EP column and eluted with a gradient system of acetonitrile in distilled water: 0–60 min, 70%; 60–70 min, a linear gradient of 70–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. A mutagen (compound V) was isolated at a retention time of 50 min.

All HPLC procedures were carried out at 30 °C, and the eluates were monitored for UV absorption at 254 nm using an SPD M10Avp diode array detector (Shimadzu Co., Kyoto, Japan). An aliquot of each fraction of the eluate was used for mutagenicity assay.

2.4. HPLC analysis of nitroarenes

Authentic 1,6-DNP, 1,8-DNP, 3,6-DNBeP, 3,9-DNF, and 1,3,6-TNP were dissolved in ethanol and analyzed by HPLC. Each sample was injected into the Inertosil ODS-EP column and eluted with the following solution: 60% acetonitrile for 1,6-DNP, 1,8-DNP, 3,9-DNF, and 1,3,6-TNP; 70% acetonitrile for 3,6-DNBeP. These five standards were also applied to the Luna 5 μ Phenyl-Hexyl column and eluted under the following conditions: 60% acetonitrile for 1,6-DNP, 1,8-DNP, 3,9-DNF, and 1,3,6-TNP; 62% acetonitrile for 3,6-DNBeP. All HPLC procedures were performed at 30 °C. Elution was conducted at a flow rate of 0.7 ml/min. Eluents were monitored with the Shimadzu SPD-M10Avp diode array detector.

2.5. Spectral measurement

UV absorption spectra were measured with the Shimadzu LC-VP HPLC system using an SPD M10Avp diode array detector. Electron impact mass spectra (EI-MS) were measured at 70 eV using a Shimadzu QP5050A mass spectrometer with a direct inlet system.

2.6. Mutagenicity test

All of the samples were dissolved in dimethyl sulfoxide and assayed for mutagenicity by the preincubation method [35] using *Salmonella typhimurium* TA98 and TA100 [36]. The S9 mix contained 0.05 ml of S9, prepared from livers of male Sprague-Dawley rats treated with phenobarbital and β-naphthoflavone, in a total volume of 0.5 ml. The mutagenic potencies of samples were calculated from linear portions of the dose–response curves, which were obtained with three or four doses and duplicate plates at each dose. The slope of the dose–response curve was adapted as the mutagenic potency. When the samples induced twofold increases over the average yield of spontaneous revertants and showed well-behaved concentration-response patterns, the samples were judged positive.

2.7. Detection of mutagens in surface soils from Aichi prefecture (Hekinan and Nagoya) and Osaka prefecture (Izumiotsu and Takatsuki)

Organic extracts from surface soil samples collected in Hekinan in January 2001, Nagoya in January 2001, and Izumiotsu in February 2002 were also prepared by Soxhlet extraction [32]. In addition, soil sample was collected in Takatsuki in January 2003, and the organic extract (3.0 g) was prepared from sieved soil (3.6 kg). These four Soxhlet extracts were successively fractionated with the Sephadex LH-20, silica gel, and the Ultra pack ODS columns as described for the sample from Kyoto. An aliquot of each fraction was examined for mutagenicity. Mutagenic fractions from the four samples, which corresponded to Fr. A for the sample from Kyoto, were evaporated to dryness. For the sample from Takatsuki, a fraction corresponding to Fr. B of the sample from Kyoto was also obtained. The residues were further separated by the same fractionation methods, using the COSMOSIL $5C_{18}$ AR-II, the Luna 5 μ Phenyl-Hexyl, and Inertsil ODS-EP columns, as described for the sample from Kyoto.

3. Results

3.1. Mutagenicity of surface soil in Kyoto

Surface soil samples were collected from 11 sites in Kyoto city and one site in its neighboring city, Mukomachi. Fig. 1 shows the location of sampling sites. At each site, soil samples were collected twice or three times between December 2003 and January 2006. Table 1 summarizes the mutagenicity of the soil samples toward *S. typhimurium* TA98 and TA100. Almost all, i.e. 23/25 (92%) and 24/25 (96%), samples showed mutagenicity in TA98 without and with S9 mix, respectively. On the other hand, 14/25 (56%) and 18/25 (72%) of the samples were positive in TA100 without and with S9 mix, respectively. Six (24%) and two (8%) sam-

Table 1
Mutagenicities of organic extracts from surface soil collected in Kyoto and Mukomachi cities

Site No.	Sampling site	Sampling date	Mutagenicity (revertants/g of soil)[a]			
			TA98		TA100	
			−S9 mix	+S9 mix	−S9 mix	+S9 mix
Kyoto city						
1	Sakyo Ward	December 2004	3780	4930	395	801
		January 2006	ND[b]	53	ND	ND
2	Kamigyo Ward	December 2003	995	675	142	276
		December 2004	ND	ND	ND	ND
3	Ukyo Ward 1	December 2003	833	407	88	250
		December 2004	796	791	78	432
4	Nakagyo Ward 1	December 2003	10,060	857	700	843
		December 2004	157	47	ND	156
5	Nakagyo Ward 2	December 2003	3087	2237	299	616
		December 2004	102	48	ND	ND
6	Ukyo Ward 2	December 2003	833	407	88	250
		December 2004	796	791	78	432
7	Shimogyo Ward 1	December 2003	448	636	122	695
		December 2004	96	39	ND	ND
8	Shimogyo Ward 2	December 2003	245	155	ND	199
		December 2004	288	83	ND	ND
9	Minami Ward 1	December 2004	2904	3546	360	1082
		April 2005	5329	5363	1013	1999
		January 2006	12,870	4264	2316	2,620
10	Fushimi Ward	December 2003	4893	1612	601	1759
		December 2004	143	67	ND	ND
11	Minami Ward 2	December 2003	3184	1707	441	893
		December 2004	191	51	ND	ND
12	Mukomachi city	December 2003	204	141	ND	345
		December 2004	83	93	ND	82

[a] Organic extracts were obtained from 15 g of soil (<250 mm) with an ultrasonic extractor. The slope (revertants/g of soil) of the dose–response curve was calculated by least-squares linear regression from the first linear portion of the dose–response curve.
[b] ND = not detected.

ples showed high (1000–10,000 revertants/g of soil) and extreme (more than 10,000 revertants/g of soil) mutagenicity, respectively, in TA98 without and/or with S9 mix. For TA100, four samples (16%) were highly mutagenic without and/or with S9 mix, but no sample was extreme. Soil samples were collected three times at site No. 9 (Minami Ward 1) and all samples showed high or extreme mutagenicity in TA98 and TA100. Other five samples, which showed high or extreme mutagenicity in TA98 and/or TA100, were collected from five sites. The highest mutagenic potency was detected for the sample collected at site No. 9 in January 2006, and the extracts induced 12,870 revertants in TA98 per gram of soil equivalent without S9 mix.

3.2. *Isolation and identification of mutagens in surface soil in Kyoto*

To identify the major mutagens in surface soil, organic extract was prepared from the soil samples collected at site No. 9. This soil extract, which showed potent mutagenicity in TA98 without S9 mix (13,640 revertants/mg of extract), was fractionated by diverse column chromatography methods by monitoring the mutagenicity of the fractions in TA98 without S9 mix. First, the soil extract was applied to a Sephadex LH-20 column. Mutagenicity was detected in several fractions, and the most potent mutagenicity was observed in the fraction with elution volumes of 210–330 ml. This mutagenic frac-

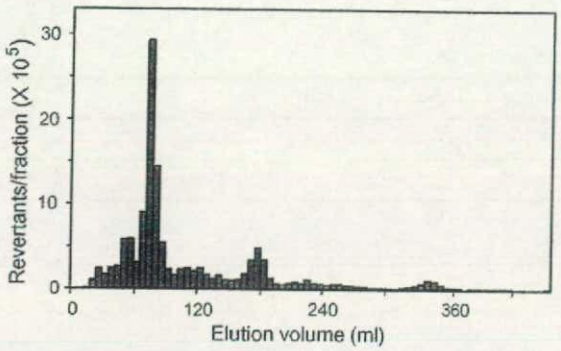

Fig. 2. LPLC profile of mutagens in the sample collected at site No. 9, Minami Ward 1, in Kyoto. LPLC was performed on an Ultra pack ODS column. The mutagenicity of each 6 ml fraction was assayed in *S. typhimurium* TA98 without S9 mix.

tion was separated by LPLC using a silica gel column, and fractions with potent mutagenicity were obtained at elution volumes of 540–795 ml. Mutagenicity of these highly active fractions eluted from the silica gel column corresponded to 71% of the total mutagenicity of the soil extract. This fraction was applied to an Ultra pack ODS column for LPLC. Fig. 2 shows the mutagenicity profile of the eluate. Many fractions showed mutagenicity, and high potencies were detected in fractions with elution volumes of 18–96 ml and 156–198 ml. These two fractions were designated as Fr. A and Fr. B, respectively. The contribution ratios of Fr. A and Fr. B to the total mutagenicity of the soil extract were 48 and 9%, respectively.

Fr. A was further separated by HPLC using a COSMOSIL $5C_{18}$ AR-II column. Potent mutagenicity was observed in fractions with retention times of 33–37 and 44–48 min, which were designated as Fr. A-1 and Fr. A-2, respectively. Fr. A-1 was further separated on a Luna 5 μ Phenyl-Hexyl column, and two highly mutagenic fractions were obtained at retention times of 27–29 and 36–38 min. These two mutagenic fractions, designated as Fr. A-1-1 and Fr. A-1-2, respectively, were separated on an Inertsil ODS-EP column. For Fr. A-1-1, UV absorption peaks, which showed potent mutagenicity, were observed at retention times of 40 and 43 min, and these peak fractions were designated as compound I and compound II, respectively (Fig. 3(a)). In a similar way, another UV absorption peak fraction (compound III), which showed strong mutagenicity, was isolated from Fr. A-1-2 at a retention time of 33 min, as shown in Fig. 4(a). Fr. A-2 was also separated by HPLC on the Luna 5 μ Phenyl-Hexyl column. A highly mutagenic fraction was obtained at retention times of 26–28 min, and this mutagenic fraction was further separated on the

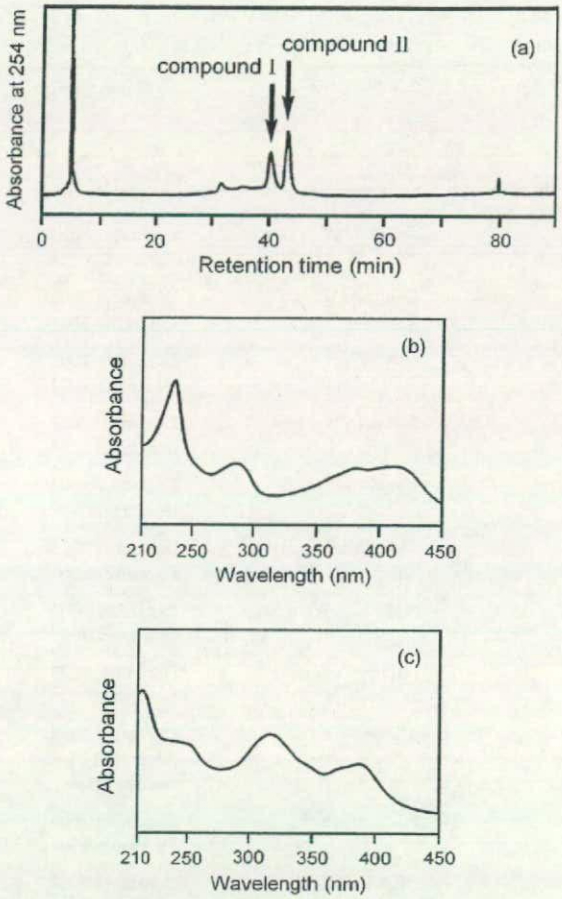

Fig. 3. HPLC profile (a) and UV absorption spectra (b and c) of compounds I and II isolated from surface soil from site No. 9, Minami Ward 1, in Kyoto. HPLC was performed on an Inertsil ODS-EP column, and eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. UV absorption peaks corresponding to compounds I and II are indicated by arrows in the HPLC profile (a). UV absorption spectra of compounds I and II are shown in (b) and (c), respectively.

Inertsil ODS-EP column. As shown in Fig. 5(a), a UV absorption peak, which showed strong mutagenicity, was observed at retention times of 39 min, and this mutagen was designated as compound IV.

Fr. B was applied to the COSMOSIL $5C_{18}$ AR-II column, and potent mutagenicity was observed in the fraction with retention times of 34–37 min. This mutagenic fraction was further separated on the Luna 5 μ Phenyl-Hexyl column. Resulting fractions with retention times of 42–44 min, which showed potent mutagenicity, was further applied to the Inertsil ODS-EP column, and a mutagen (compound V) was isolated as a UV absorption peak at a retention time of 50 min (Fig. 6(a)). Contribu-

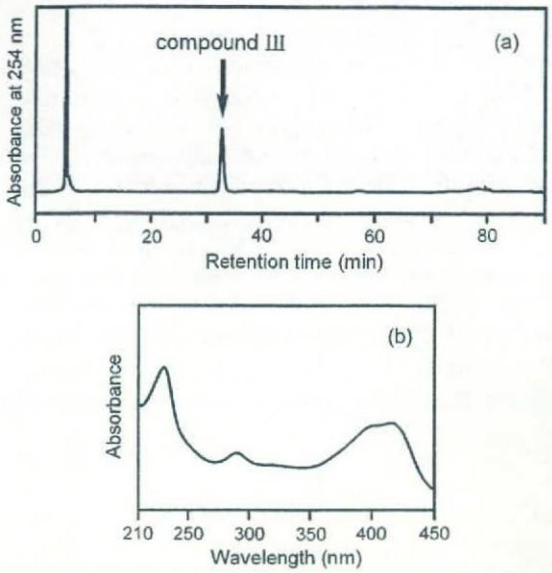

Fig. 4. HPLC profile (a) and UV absorption spectrum (b) of compound III isolated from surface soil from site No. 9, Minami Ward 1, in Kyoto. HPLC was performed on an Inertsil ODS-EP column, and eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. UV absorption peak corresponding to compound III is indicated by an arrow in the HPLC profile (a).

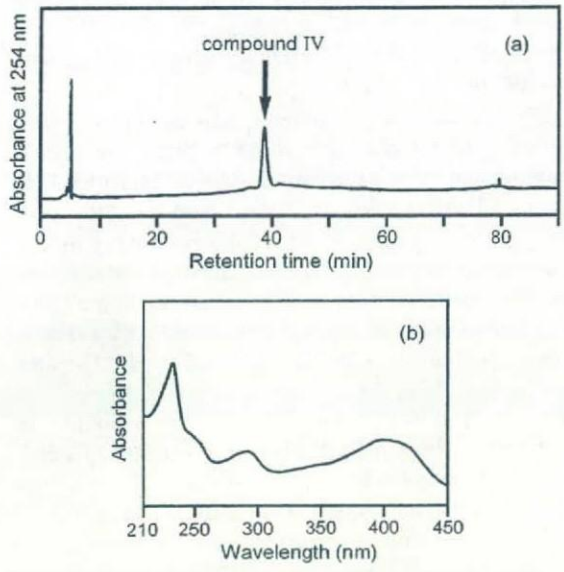

Fig. 5. HPLC profile (a) and UV absorption spectrum (b) of compound IV isolated from surface soil from site No. 9, Minami Ward 1, in Kyoto. HPLC was performed on an Inertsil ODS-EP column, and eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. UV absorption peak corresponding to compound IV is indicated by an arrow in the HPLC profile (a).

tion ratios of compounds I–V to the total mutagenicity of soil extracts in TA98 without S9 mix were 3, 10, 10, 10, and 6%, respectively.

3.3. Identification of compounds I–V

UV absorption spectra of compounds I, IV, and V, which are shown in Figs. 3(b), 5(b), and 6(b), were consistent with those of 1,6-DNP, 1,8-DNP, and 3,6-DNBeP, respectively. Retention times of compounds I, IV, and V on the Inertsil ODS-EP column were found to be the same as those of 1,6-DNP, 1,8-DNP, and 3,6-DNBeP, respectively. Moreover, retention times of 1,6-DNP, 1,8-DNP, and 3,6-DNBeP on the Luna 5 μ Phenyl-Hexyl column were 28, 27, and 43 min, respectively. These retention times corresponded to those of mutagenic fractions from with compounds I, IV, and V were isolated. As shown in Fig. 7, molecular ion peaks of compounds II and III were observed at *m/z* 292 and 337, respectively. Mass spectra of compounds II and III were consistent with those of authentic 3,9-DNF and 1,3,6-TNP. UV absorption spectra of compounds II and III, which are shown in Figs. 3(c) and 4(b), were consistent with those of 3,9-DNF and 1,3,6-TNP, respectively. Retention times of compounds II and III on the Inertsil ODS-EP column and the Luna 5 μ Phenyl-Hexyl column were found to coincid with those of 3,9-DNF and 1,3,6-TNP, respectively. On the basis of these results, compounds I–V were concluded to be 1,6-DNP, 3,9-DNF, 1,3,6-TNP, 1,8-DNP, and 3,6-DNBeP, respectively. The chemical structures of these nitroarenes are shown in Fig. 8.

3.4. Detection of nitroarenes in surface soil collected in other prefectures

To investigate the distribution of 1,6-DNP, 1,8-DNP, 3,9-DNF, 1,3,6-TNP, and 3,6-DNBeP in surface soil in other residential areas, organic extracts of soil samples collected in different four cities were examined. In a previous study [32], we analyzed soil samples collected in Izumiotsu, Hekinan, and Nagoya between January 2001 and February 2002, which showed potent mutagenicity in TA98 without S9 mix (1240–14,460 revertants/mg of extract), and 3,6-DNBeP was detected from all soil samples. These three soil samples were examined for 1,6-DNP, 1,8-DNP, 3,9-DNF, and 1,3,6-TNP in this study. The organic extract of a surface soil sample collected in Takatsuki in January 2003 showed strong mutagenicity toward TA98 without S9 mix (337,800 revertants/mg of extract) and was also examined for all

five nitroarenes in this study. These four soil extracts were fractionated by the method used for the soil sample from Kyoto, monitoring the mutagenicity of the fractions in TA98 without S9 mix.

Potent mutagenicity was detected in the fractions with elution volumes similar to those of highly mutagenic fractions for the sample from Kyoto on Sephadex LH-20 and silica gel columns. These active fractions were subsequently separated with the Ultra pack ODS column. Fractions corresponding to Fr. A from all soil samples and Fr. B from the sample from Takatsuki on the Ultra pack ODS column showed potent mutagenicity. These active fractions were further separated by HPLC using COSMOSIL $5C_{18}$ AR-II, Luna 5 μ Phenyl-Hexyl, and then Inertsil ODS-EP columns under the same conditions used for the sample from Kyoto. Potent mutagenicity was found in the fractions corresponding to compounds I–IV in each fractionation step for all samples. Mutagens corresponding to compounds I–IV were eluted at retention times of 40, 43, 33, and 39 min, respectively, as single UV absorption peaks on the Inertsil ODS-EP column. The retention times and UV absorption spectra of these peak fractions coincided with those of 1,6-DNP, 3,9-DNF, 1,3,6-TNP, and 1,8-DNP, respectively. On the basis of these results, the mutagens corresponding to compounds I–IV isolated from soil samples from four cities other than Kyoto were concluded to be 1,6-DNP, 3,9-DNF, 1,3,6-TNP, and 1,8-DNP, respectively. A mutagen corresponding to compound V was isolated from Fr. B prepared from the sample from Takatsuki and was deduced to be 3,6-DNBeP by consistency of the retention time on HPLC using the Luna 5 μ Phenyl-Hexyl and the Inertsil ODS-EP columns and UV absorption spectrum of the mutagen and those of the authentic compound.

The amounts of 1,6-DNP, 1,8-DNP, 3,9-DNF, 1,3,6-TNP, and 3,6-DNBeP in soil extracts from soil samples and their percent contributions to the total mutagenicity of the extracts are shown in Table 2. The amounts of nitroarenes were from 0.14 to 66.26 ng/mg of extract. The highest levels of nitroarenes were detected in the soil sample collected in Takatsuki. Percent contributions of each nitroarene ranged from 0.7 to 22%. For the soil sample from site No. 9 in Kyoto, percent contributions of 1,3,6-TNP, and 3,9-DNF were relatively high, and the values were the same as that of 1,8-DNP, i.e. 10%. In contrast, for the soil sample from Takatsuki, the percent contribution of 1,8-DNP was particularly high, i.e.

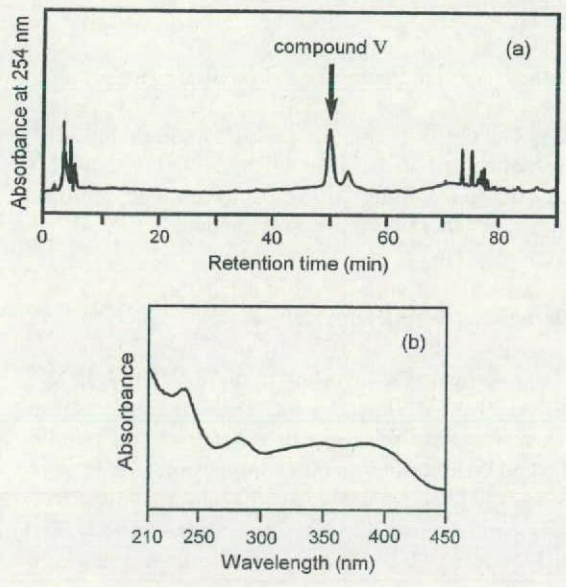

Fig. 6. HPLC profile (a) and UV absorption spectrum (b) of compound V isolated from surface soil from site No. 9, Minami Ward 1, in Kyoto. HPLC was performed on an Inertsil ODS-EP column, and eluted with the following gradient system of acetonitrile in distilled water: 0–60 min, 60%; 60–70 min, linear gradient of 60–100%; 70–90 min, 100%, at a flow rate of 0.7 ml/min. UV absorption peak corresponding to compound V is indicated by an arrow in the HPLC profile (a).

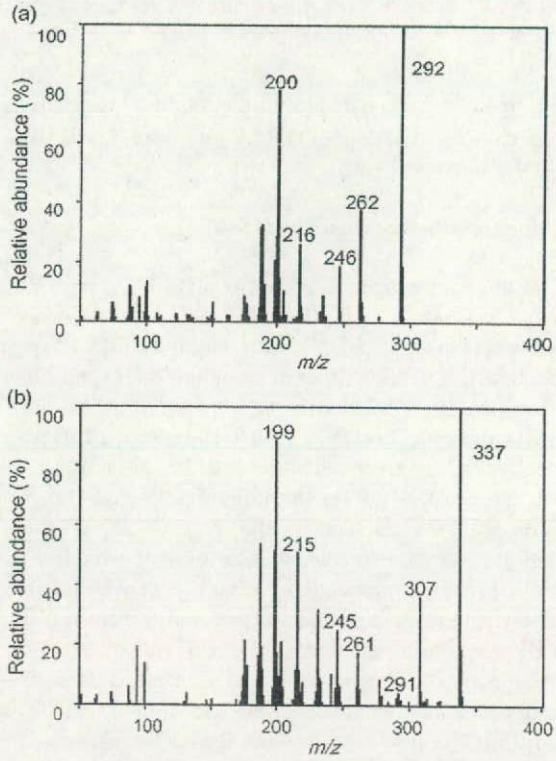

Fig. 7. Electron impact mass spectra of compounds II and III isolated from surface soil from site No. 9, Minami Ward 1, in Kyoto.

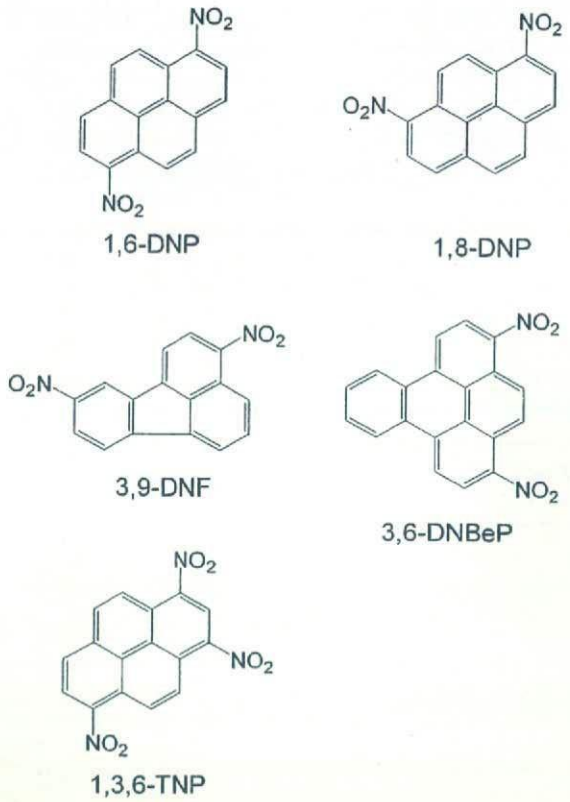

Fig. 8. Chemical structures of 1,6- and 1,8-dinitropyrene (DNP) isomers, 3,9-dinitrofluoranthene (DNF), 1,3,6-trinitropyrene (TNP), and 3,6-dinitrobenzenzo[*e*]pyrene (DNBeP).

22%, and that of 1,3,6-TNP was the lowest, i.e. 0.7%, among the soil samples examined. For the sample from Izumiotsu, the contribution of 1,3,6-TNP was relatively low. The total percent contributions of five nitroarenes to these five soil extracts were from 39 to 45%.

4. Discussion

In a previous study [28], we examined the mutagenicity of 544 surface soil samples collected in five geographically different regions of Japan, i.e. Hokkaido, Kanto, Chubu, Kinki, and Kyushu regions, by the Ames assay using *S. typhimurium* TA98 and TA100 with and without S9 mix. On the basis of mutagenic potency, these soil samples were classified into four levels, i.e. low (up to 100 revertants/g of soil), moderate (100–1000 revertants/g of soil), high (1000–10,000 revertants/g of soil), and extreme (more than 10,000 revertants/g of soil). The percentage of soil samples classified as high ($n = 86$) and extreme ($n = 8$) were 16 and 1.5%, respectively. In this study, sample

Table 2
Amounts of nitroarenes and their contribution ratios of nitroarenes to the mutagenicities of organic extracts from surface soil in *S. typhimurium* TA98 without S9 mix

Sampling site	Mutagenicity (revertants/mg of extract)	Amount (ng/mg of extract)					Contribution ratio (%)					Total
		1,6-DNP	1,8-DNP	1,3,6-TNP	3,9-DNF	3,6-DNBeP	1,6-DNP	1,8-DNP	1,3,6-TNP	3,9-DNF	3,6-DNBeP	
Kyoto prefecture												
Kyoto (site No. 9)	13,640	0.89	1.75	12.29	3.81	0.93	3	10	10	10	6	39
Aichi prefecture												
Nagoya	14,460[a]	1.19	1.33	10.29	3.48	2.72	4	7	8	9	16[a]	44
Hekinan	1,240[a]	0.14	0.14	0.96	0.22	0.31	5	9	4	6	21[a]	45
Osaka prefecture												
Izumiotsu	5,280[a]	0.35	0.73	1.02	1.48	1.08	3	11	2	10	17[a]	43
Takatsuki	337,800	34.46	66.26	15.04	23.30	29.79	6	22	0.7	4	11	43.7

DNP, dinitropyrene; TNP, trinitropyrene; DNF, dinitrofluoranthene; NBeP, dinitrobenzo[*e*]pyrene.
[a] From Ref. [32].

collection was performed twice or three times at 12 sites, and 25 surface soil samples were collected. Almost all samples showed mutagenicity in TA98 with and without S9 mix, and eight (32%) samples showed high or extreme mutagenicity. Three soil samples collected at site No. 9 (Minami Ward 1) showed high or extreme mutagenicity in TA98, and the other highly or extremely mutagenic soil samples were collected at five sites. These results suggest that surface soil was extensively contaminated with mutagens in the residential area in Kyoto, and there were some sites where contamination levels were high. The pollution of surface soil at site No. 9 was thought to be severe and persistent.

As major mutagenic constituents, five compounds were isolated from surface soil samples from site No. 9, and were identified to be 1,6-DNP, 1,8-DNP, 1,3,6-TNP, 3,9-DNF, and 3,6-DNBeP. Many researchers have reported the biological activity, including carcinogenicity, of these nitroarenes, which are highly mutagenic in TA98 without S9 mix; inducing 175,000 revertants/nmol of 1,6-DNP [29], 257,000 revertants/nmol of 1,8-DNP [29], 65,500 revertants/nmol of 1,3,6-TNP [34], 104,000 revertants/nmol of 3,9-DNF [33], and 285,000 revertants/nmol of 3,6-DNBeP [32]. 1,6-DNP, 1,8-DNP and 1,3,6-TNP showed mutagenicity at the hypoxanthine–guanine phosphoribosyl transferase gene locus in cultured Chinese hamster ovary cells [37] and were mutagenic in Chinese hamster lung (CHL) cells for the induction of diphtheria toxin resistance [38]. 3,9-DNF induced chromosomal aberration in CHL cells [39]. 1,6-DNP, 1,8-DNP and 3,9-DNF showed carcinogenicity in experimental animals [30,31,40] and are classified as possible human carcinogens (group 2B) by the International Agency for Research on Cancer (IARC) [9,41]. 3,6-DNBeP is a novel chemical and was newly detected as a major mutagenic constituent in surface soil [32]. Except for mutagenicity in *S. typhimurium* strains, no other biological activity of 3,6-DNBeP has been reported. As 3,6-DNBeP is an extremely potent bacterial mutagen, other biological activities of 3,6-DNBeP, including carcinogenicity, should be elucidated.

All five nitroarenes identified as major mutagenic constituents in surface soil from Kyoto were found in other highly mutagenic soil samples collected in Aichi and Osaka prefectures. The amounts of the five nitroarenes ranged from 0.14 to 66.26 ng/mg of extract. The percent contributions of these nitroarenes to the total mutagenicity of soil extracts were from 0.7 to 22%. The amounts and percent contributions of these nitroarenes were comparable to those of 1,6- and 1,8-DNP isomers detected in soil samples from the Kinki Region [25]. The total percent contributions of the five nitroarenes to these five soil extracts were from 39 to 45% in this study. The results suggest that these five nitroarenes might be major mutagenic constituents of the surface soil in the five residential areas investigated in this study. Relative contribution ratios of the five nitroarenes to the mutagenicity of the five soil samples were different among soil samples. Nitroarenes are formed by incomplete combustion of organic matter, such as fossil fuels. The differences of the relative contributions of nitroarenes among soil samples might be attributed to the combustion conditions, which affect the formation of incomplete combustion products, such as different fuels and combustion temperatures at the sources of the nitroarenes. To clarify the sources, the quantification of these nitroarenes in airborne particles over extensive areas and in exhaust particles from potential origins such as the engines of motor vehicles, incinerators, and furnaces is required. The exposure levels of inhabitants to theses nitroarenes should be assessed to estimate their impact on human health and biota.

Conflict of interest

This study has been performed for academic purposes neither for companies producing chemicals that were used and/or mentioned in this study nor for the competing companies. I do not receive direct or indirect rewards from any source for writing this manuscript.

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ABSTRACT

Heavy metal pollution of soil causes biological problems, such as mutagenicity to living organisms, including human beings. However, few methods have been developed to assess metal mutagenicity in soil. To avoid metal mutagenicity, an adequate bio-monitoring method is required. In the present study, to determine if the analysis of oxidative DNA damage generated in the earthworm is a useful bio-monitoring method for soil mutagenicity, the accumulation of 8-hydroxydeoxyguanosine (8-OH-dG), a major form of oxidative DNA damage, in *Eisenia fetida* (Savigny, 1826) treated with cadmium chloride ($CdCl_2$) or nickel chloride ($NiCl_2$) was analyzed. *E. fetida* was treated with Cd (10 or 200 μg/g soil) or Ni (10 or 200 μg/g soil) for 1, 2, and 3 weeks or 3 months. After metal exposure, the metal concentration in *E. fetida* was analyzed by atomic absorption spectrometry and the 8-OH-dG accumulated in *E. fetida* was analyzed by HPLC analyses and immunohistochemistry. Atomic absorption spectrometry revealed that Cd, but not Ni, accumulated within *E. fetida*. The 8-OH-dG levels in the DNA of *E. fetida* treated with Cd for 3 months were significantly higher than those in control *E. fetida*. Moreover, immunohistochemical analyses revealed that positive signals for 8-OH-dG accumulation in seminal vesicles were detected only in *E. fetida* treated with 10 μg of Cd for 3 months. Although some points remain unresolved, a bio-monitoring system analyzing the DNA damage generated in the earthworm might be useful for the assessment of the mutagenicity of soil contaminated with various heavy metals, such as Cd.

1. Introduction

Heavy metal pollution of soil is widespread across the globe and has caused biological problems, leading to potential toxicity to living organisms. Recent research found that the atmospheric input of heavy metals to agricultural systems also significantly contributed to metal loading in soil [1]. These complicated pathways of contamination make it difficult to avoid exposure to metals existing in our surroundings.

To avoid metal toxicity, we should first understand the features of metals and assess metal toxicity. However, the methods used for the assessment are often not sensitive enough, because metals can be toxic below the technical detection limits. To overcome this limitation, many research efforts have been made to develop detection techniques or assessment methods for metal contamination of soil. Furthermore, the toxic action of metals sometimes depends on their metabolites generated in living organisms. Thus, adequate methods to assess metal toxicity are difficult to develop.

A bio-monitoring method would be appropriate to evaluate metal toxicity, because of its sensitivity and availability for unknown metabolites [2–4]. This approach is also somewhat limited, because it could be available only for a specific combination of a living organism with certain substances. Hence, it is important to find adequate living organisms as bio-monitors for each assessment.

Recent research has indicated that the earthworm is a candidate organism as a bio-monitor for soil contaminants, because it plays an important role in the soil macrofauna biomass. The species *Eisenia*

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fetida (Savigny, 1826) is most commonly used in ecotoxicology as a useful bio-monitor for soil [5]. In particular, this species' proximity to the soil contaminants is a merit for the analysis [6,7].

Metal-induced mutagenicity is a serious problem for the diversity of living organisms and human health. To prevent mutagenicity-associated diseases, we must understand the substances around us and avoid the environmental mutagens. Therefore, the evaluation of soil mutagenicity is very important, and direct analyses of DNA damage generated in living organisms may provide precise and useful information.

It is believed that 8-hydroxy-2′-deoxyguanosine (8-OH-dG), a major form of oxidative DNA damage, may have an important role in carcinogenesis, because it causes the GC to TA transversion type point mutation [8–10]. 8-OH-dG is constantly generated in DNA and the nucleotide by reactive oxygen species (ROS). We have studied the relationship between 8-OH-dG accumulation in DNA and health-related factors, such as smoking [11], chemical agents [12–18], X-rays [19], aging [20–22], and physical exercise [23]. Therefore, it is predicted that the accumulation level of 8-OH-dG in the DNA of *E. fetida* would directly reflect the soil mutagenicity.

Of note, some heavy metals are known to affect 8-OH-dG repair systems, leading to 8-OH-dG accumulation. Heavy metals such as arsenic (As) [18,24], cadmium (Cd) [14,18,25,26], chromate (Cr) [27,28], manganese (Mn) [29], and lead (Pb) [30] inhibit 8-OH-dG excision repair activity or down-regulate the expression of 8-oxoguanine DNA glycosylase 1 (OGG1), a major repair enzyme for 8-OH-dG. Among these metals, the effects of Cd on 8-OH-dG repair systems were well documented. In particular, it was found that Cd inhibited 8-OH-dG excision repair activity or 8-OH-dGTPase activity, leading to 8-OH-dG accumulation in DNA [14,18,26,31]. This inhibitory action of Cd on the DNA repair system might be involved in Cd carcinogenesis.

In the present study, we analyzed 8-OH-dG accumulated in the DNA of *E. fetida* exposed to heavy metals, to determine if a method using earthworms as a bio-monitor is useful for the assessment of soil mutagenicity. We employed Cd and Ni as test metals, because the carcinogenic potentials of Cd and Ni have been established for humans and animals [32,33], and these metals are known to generate 8-OH-dG in DNA [14,34–36].

2. Materials and methods

2.1. Earthworms and heavy metal exposure

The *E. fetida* used in this study, which we had employed in the previous work [37], was kindly provided by Dr. C.A. Edwards (Ohio State University). They were kept in a 20-L stainless steel tank at an ambient temperature of 24 °C on mold with skim milk as a food source until heavy metal exposure. We used scrapped mold as soil, which consisted of the core of corn, rice bran, and other components and had been used for mushroom cultivation (gift from Ikesue Toyotada Shoten, Mizuma-gun, Japan). Adult organisms with a body wet weight of around 100 mg were used. Three (for a short-term experiment) or six (for a long-term experiment) individuals were kept in a 600 mL glass container containing 50 g of soil with/without heavy metal. They were exposed to 10 or 200 μg heavy metal/g soil for 1, 2, and 3 weeks or 10 μg heavy metal/g soil for 3 months (Fig. 1).

2.2. Atomic absorption spectrometry

The earthworm's body was cut into four rough segments: head region (S1), anterior body region (S2), posterior body region (S3), and tail region (S4). They were weighed under wet conditions and quickly frozen at −80 °C. The concentrations of heavy metals in each segment were determined using standard atomic absorption spectrometry. Each segment of *E. fetida* was transferred into a 100 mL kjeldahl flask and digested with a mixture of 5 mL nitric acid and 0.5 mL sulfuric acid. The flask was gently heated on a hot plate in a fume cupboard until only a few drops of clear liquid remained. After filtration, the samples were brought up to 10 mL with 0.1N nitric acid. This sample was analyzed by atomic absorption spectroscopy, with a Hitachi Model 180-80 spectrometer equipped with a graphite atomizer (Hitachi, Tokyo, Japan). The metal concentration was calculated from absorbance *versus* concentration curves prepared for each run of specimens. The metal concentration was expressed as micrograms per gram of body wet weight.

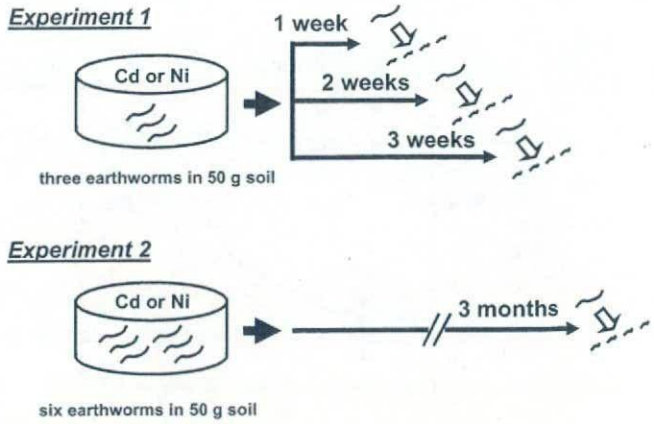

Fig. 1. Experimental design. Experiment 1 was a short-term exposure of *E. fetida* to $CdCl_2$ or $NiCl_2$. Metals (0, 0.5, or 10 mg as Cd or Ni) were added into 50 g soil per 600 mL glass container. After 0-, 1-, 2- and 3-week exposures, *E. fetida* were cut into four rough segments. We kept a total of 45 *E. fetida* (15 glass containers; 3 *E. fetida* per container). Experiment 2 was a long-term exposure of *E. fetida* to $CdCl_2$ or $NiCl_2$. Metals (0 or 0.5 mg as Cd or Ni) were added into 50 g soil per 600 mL glass container. After a 3-month exposure, *E. fetida* were cut into four rough segments. We kept a total of 54 *E. fetida* (9 glass containers; 6 *E. fetida* per container).

2.3. Quantitative analyses of 8-OH-dG levels

The 8-OH-dG levels in the S1 region of each earthworm (3-month experiment) were measured. The assay was described previously [40]. Briefly, DNA from the earthworms was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako Pure Chemical Industries, Ltd., Japan). For homogenization, a lysis solution containing 1 mM desferal (deferoxamine mesylate, Sigma Chemical Co. MO, USA) was used. The isolated DNA was digested with nuclease P1 (Yamasa Corp., Choshi, Japan) and alkaline phosphatase (Roche Diagnosis GmbH, Mannheim, Germany) to obtain a deoxynucleoside mixture. The solution was filtered with an Ultrafree-Probind filter (Millipore, Bedford, MA) and was injected into a high-performance liquid chromatography (HPLC) column (Shiseido Capcell Pak C18 MG) equipped with an electrochemical detector (ECD) (ECD-300, Eicom Co., Kyoto, Japan). The 8-OH-dG value in the DNA was expressed as the percentage of the control value.

2.4. Immunohistochemistry

Samples were fixed in Bouin's solution for 3 h in ice. After fixation, they were cut into the four rough segments as described above, immersed in 90% ethanol for 24 h to remove the picric acid, dehydrated, and embedded in paraffin. These embedded samples were sagittally sectioned at 5 μm, mounted on glass slides coated with poly-L-lysine, and subjected to either hematoxylin/eosin (HE) or immunohistochemical (IHC) staining. The avidin–biotin complex method [38] was used to detect 8-OH-dG. We used the monoclonal antibody N45.1 (Nikken SEIL, Shizuoka, Japan), which was raised against 8-OH-dG, as the first antibody and biotin-labeled goat anti-mouse IgG serum (Vector Laboratories, Inc., Burlingame, California), and streptavidin–alkaline phosphatase complex (Vector Laboratories, Inc., Burlingame, California) diluted 1:100 [39]. The substrate for alkaline phosphatase (red) was obtained from the DAKO New Fuchsin Substrate System (DAKO, Copenhagen). For negative staining, a 2% bovine serum albumin solution was used instead of the first antibody. HE or IHC staining specimens were observed by Olympus BX50 System microscopy (Olympus, Tokyo, Japan).

2.5. Statistical analysis

Values obtained in the present study were calculated to the means ± S.D. The statistical significance was calculated using the Student *t*-test. Probability values less than 0.05 were considered to indicate significant differences.

3. Results

3.1. Body wet weights of E. fetida

All *E. fetida* used in the present study were weighed under wet conditions (Fig. 2). The body wet weights of *E. fetida* treated with 200 μg Cd/g soil for 2 weeks were significantly lower (49.5% of

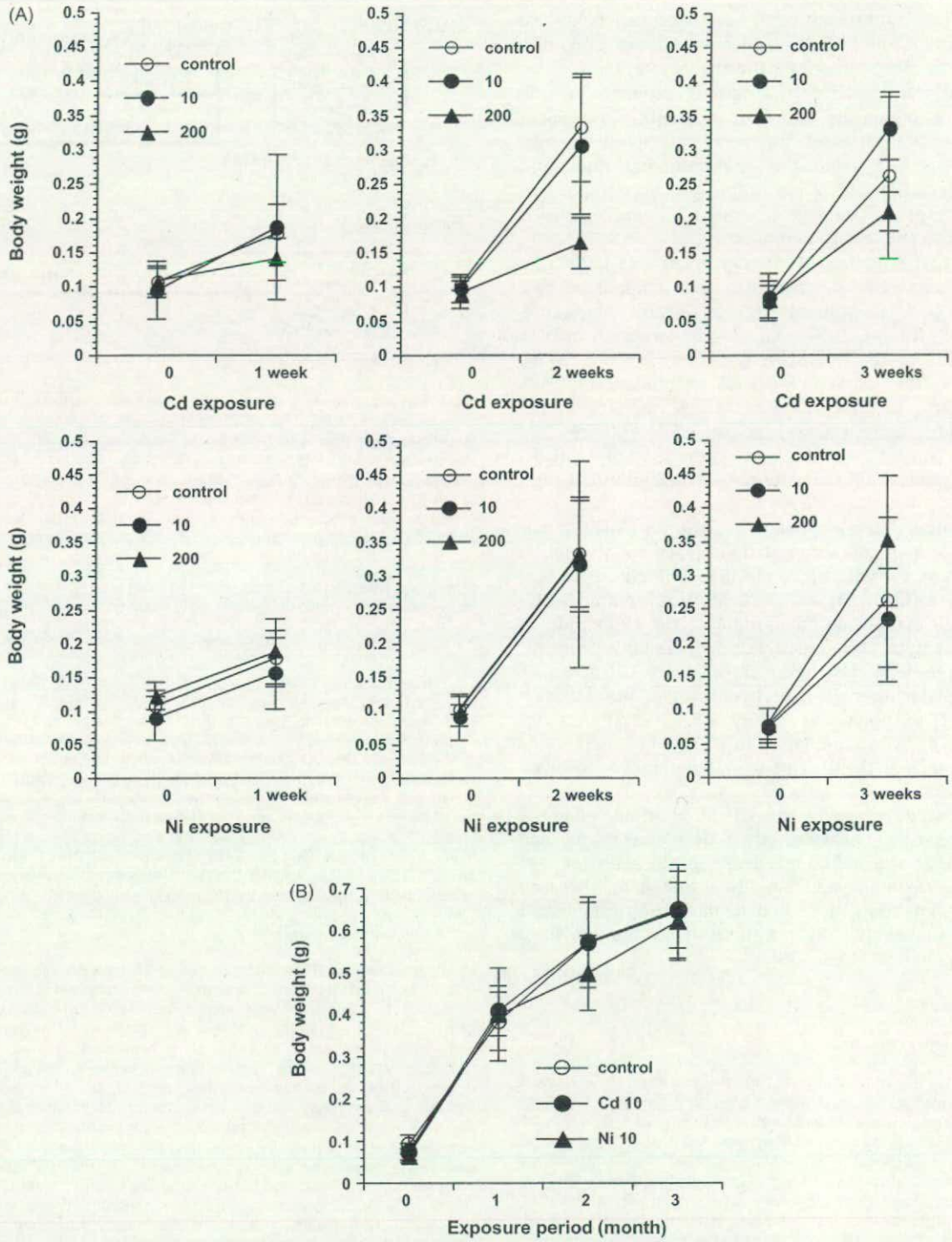

Fig. 2. All *E. fetida* were weighed under wet conditions in the short-term (A) and the long-term (B) experiments. Each data point represents the mean of six *E. fetida*. The treatment of *E. fetida* with 200 μg Cd/g soil resulted in body weight loss, suggesting Cd-induced growth inhibition. In the long-term experiment, each data point represents the mean of 16 *E. fetida*, and no significant differences between any groups were observed.

control) than those of all other 2-week exposure individuals (*versus* control, $p < 0.001$; *versus* 10 μg Cd/g soil treatment group, $p < 0.01$; *versus* 10 μg Ni/g soil treatment group, $p < 0.05$; *versus* 200 μg Ni/g soil treatment group, $p < 0.005$). The body wet weights of *E. fetida* treated with 200 μg Cd/g soil for 3 weeks were significantly lower than those of some other 3-week exposure individuals (*versus* 10 μg Cd/g soil treatment group, $p < 0.01$; *versus* 200 μg Ni/g soil treatment group, $p < 0.01$). However, no growth inhibition was observed in *E. fetida* treated with 10 μg Cd/g soil, even in a 3-month exposure. No inhibitory effects were also observed with the Ni exposure. All earthworms were alive during the experiment.

3.2. Cd and Ni concentrations in *E. fetida*

The Cd and Ni concentrations in *E. fetida* were analyzed using atomic absorption spectrometry. In a short-term experiment, Cd accumulated in the head (S1) and anterior body (S2) regions at an early stage (1 week) and in all segments at a later stage (2 and 3