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Mutagenicity and PAH Contents of Soil in Forests or Planted Areas in Japan

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Abstract We investigated the behavior of mutagenic substances in the soil of forests or planted areas. Mutagenicity and concentration was examined for 16 types of PAHs in soil samples collected at a depth of 1 m in 10 forests in Iwate, Ibaraki, Tokyo, Kanagawa, Yamanashi and Shizuoka prefectures in Japan. Mutagenicity and PAHs were detected mostly in soil from the surface to a depth of 30 cm when strains TA100, TA98 and YG1024 were used. In addition, a significant correlation was not found between the concentration of BaP, and specific mutagenic activity (TA98 without S9mix, $r = 0.285$).

Keywords Mutagenicity · PAH · Soil · Depth

We have been investigating the state of contamination of environmental media by carcinogenic substances through mutagenicity tests, a type of biological assay. Mutagenicity of airborne particle, river water, and surface soil near roads in urban areas in Japan was measured, and a number of samples were found to have mutagenicity (Goto et al. 2000; Endo et al. 2004). Various soil samples were found to have mutagenicity in various degrees. For example, in central city areas, soil collected from underground as deep as 1 m was mutagenic (Takagi et al. 2006), and sand in the sandboxes of general residential areas also exhibited mutagenicity (Takagi et al. 2008).

This study focused on measuring the mutagenicity of soil in places considered to be less mutagenic than those studied previously, namely, in forests covered with leaf mold and in planted areas, where contamination sources were barely found other than air-polluting falling matter. Taking into consideration the possibility that mutagenic substances seep underground, soil samples were collected not only from the surface layer but also from underground at several depth levels (down to 1 m), and mutagenicity at each depth was examined. Furthermore, the concentration of PAHs, which are generally contained in airborne particles, in the relevant soil was also measured to examine the relationship between mutagenicity and PAHs.

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Materials and Methods

During the period from March 2006 to August 2007, we collected soil samples at six depth levels in steps of 20 cm (Surface: 0 to ~10 cm deep, 20 cm: 10–30 cm, 40 cm: 30–50 cm, 60 cm: 50–70 cm, 80 cm: 70–90 cm, 100 cm: 90–100 cm) in 10 wooded or planted sites (Table 1), where the soil can be dug by hand, in Ibaraki (Wild Bird Forest on the river bank of the Tone River, site A; Ryugasaki Forest Park, B; Akebono Grove in Toride City, C; backyard of a farm in Inashiki City, D; forest within Yukari-no-mori in Tsukuba City, H), Tokyo (grove in Nozuta Park (E) and grove in Yakushiike Park (F), in Machida City),

Table 1 Outline of each sampling site in Japan

Sampling site	Prefecture	City	Location
A	Ibaraki	Toride	Forest
B	Ibaraki	Ryugasaki	Forest park
C	Ibaraki	Toride	Grove
D	Ibaraki	Inashiki	Backyard of farm
E	Tokyo	Machida	Grove in garden park
F	Tokyo	Machida	Grove in park
G	Yamanashi	Minami-tsuru	Grove around country villa
H	Ibaraki	Tsukuba	Forest
I	Shizuoka	Kamogun	Forest
J	Iwate	Iwate	Forest in university

Yamanashi (grove around country villas in the Yamanakako area, G), Shizuoka (forest in Kamogun, I), and Iwate (forest next to the seminar house of Morioka College,) prefectures.

Each collected soil sample was dried in a desiccator at room temperature for approximately 1 week. A total of 60 dried samples were sieved with a 60-mesh sieve, and extracted with methanol (for testing residual agricultural chemicals) by ultrasonication (Goto et al. 2000; Endo et al. 2004). The extract was filtered (with no. 5C, ADVANTEC, Tokyo) to recover precisely 90% of the methanol used for extraction, which was then concentrated in a rotary evaporator. The condensate was poured into a sample vial (8 mL), and methanol was distilled off under a nitrogen gas stream to obtain a tarry matter. The tarry substance was stored at −80°C until the day of the mutagenicity test.

The Ames (preincubation) method (Maron and Ames 1983; Yahagi et al. 1977) was used for the mutagenicity tests. Tester strains were the TA100 and TA98 *Salmonella typhimurium* strains and the YG1024 strain (Watanabe et al. 1990), which had been prepared by introducing the acetyltransferase high-productivity gene to the parent TA98 *S. typhimurium* strain, and tests were conducted with and without the addition of a metabolic activation system (S9mix). Mutagenic activity was calculated by the dose-response relationship of the obtained test results.

The extracted solution (equivalent to 3.2 g of soil) was dispensed in a vial, and the methanol was distilled off under a nitrogen gas stream to obtain a tarry substance, to which 250 μL dichloromethane was added and ultrasonic waves irradiated for dissolution. The obtained solution was supplemented with 10 μL deuterated PAH 16 component solution as internal standard (IS, 16 types of PAHs subjected to analysis by US EPA Method 8310, ES-2528, CIL), and measurement was carried out using a gas chromatograph mass spectrometer (GC/MS) (5973 N/6890, Agilent) (Nakajima et al. 2007) by internal standard method. BPX-5 (30 m, 0.25 mm I.D., 0.30 um F.T.) was used as the column, helium was used as the carrier gas, and 1 μL of sample was injected by the split-less method, and quantification was performed in SIM mode. Sixteen kinds of PAH cocktail (48905-U, Supelco) was diluted appropriately and it was used as a standard. The peak shapes of the internal standards of each sample were good. The deviation of retention time of IS was within 0.1 min.

Results and Discussion

A total of 60 samples were collected from 6 depth levels at 10 sites covered with leaf mold, where human-induced contamination by factory emissions and waste was inconceivable. As shown in Fig. 1, in many cases, samples collected from surface soil and those collected from a depth of 20 cm exhibited mutagenicity, that samples collected from deeper soil barely had mutagenicity, and that the YG1024 strain had relatively high mutagenicity.

Figure 1a shows the mutagenic activities (revertants/g soil) of the TA100 strain. As shown by the figure, although some surface samples did not exhibit mutagenicity when S9mix was not added, every surface sample exhibited mutagenicity when S9mix was added (mean ± SD: TA100 with S9mix, 18 ± 8; TA100 without S9mix, 8 ± 7). Samples collected from deeper soil exhibited no mutagenicity in many cases (mutagenicity detection ratio of samples collected from 40 cm or deeper soil: 20% or lower). The mutagenic activity values obtained this time were much lower than those obtained by previous investigations conducted using samples collected from soil down to 1 m deep in places near the city center (Takagi et al. 2006) (TA100 with S9mix: 76 ± 23; TA100 without S9mix: 107 ± 40), and the detection ratio was also found to be lower (20% or lower from 40 cm or deeper soil). In the previous tests, when S9mix was added, the results varied depending on the sampling point, with lower or higher mutagenic properties detected, unlike the case where S9mix was not added. Meanwhile, the surface samples collected this time exhibited higher mutagenicity in most cases when S9mix was added, which indicates that there was not much difference between samples collected at 10 different sampling sites.

Figure 1b, c summarize the results of mutagenicity tests conducted using the TA98 and YG1024 strains, respectively. The mutagenic activities of the surface soil collected this time were as follows: mean ± SD: TA98 with S9mix, 13 ± 10; TA98 without S9mix, 8 ± 9; YG1024 with S9mix, 95 ± 62; YG1024 without S9mix, 54 ± 50. Comparison between the results obtained this time and those of the previous tests (Takagi et al. 2006) (TA98 with

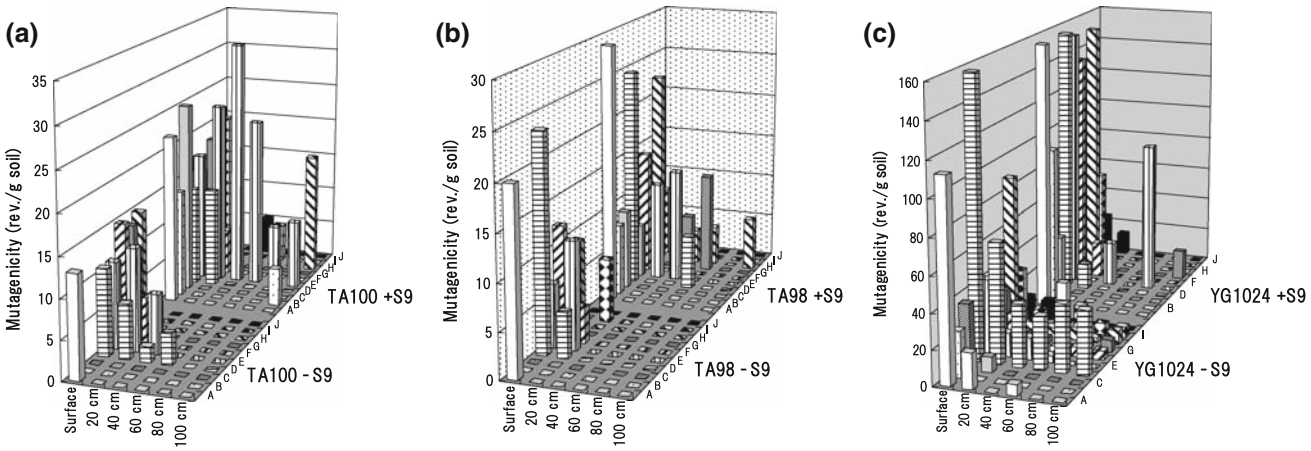

Fig. 1 Mutagenicity of soil samples by depth at each sampling point. **a** TA100, **b** TA98, **c** YG1024

S9mix: 96.3 ± 36.4; TA98 without S9mix: 160.3 ± 101.1) (YG1024 with S9mix: 948.1 ± 759.2; YG1024 without S9mix: 833.2 ± 467.6) indicates that mutagenicity of samples collected this time was much lower and that the detection ratio was also lower, as demonstrated by the results of the tests on the TA100 strain. The results of tests using the TA98 and YG1024 strains (Fig. 1b, c) revealed that mutagenicity of surface soil was high, that the deeper the soil, the lower the mutagenicity, and that about 70%–80% of all revertants found in samples was collected from the soil from the surface down to a depth of 30 cm. With regard to samples E and G tested using the TA100 strain, those collected from deeper soil exhibited slightly higher mutagenicity compared to the surface samples, but the difference was negligible.

As in the case of the previous tests, higher mutagenic activity was obtained with the YG1024 strain compared to the TA100 or TA98 strain, which suggests that nitro- or amino-group mutagenic substances were contained at high rates. Furthermore, surface samples exhibited higher mutagenicity when S9mix was added, whereas the samples collected from soil 10 cm or deeper exhibited lower mutagenicity if S9mix was added, which suggests the existence of mutagenic substances such as nitroarenes. Sample D did not exhibit particularly high mutagenicity compared with other samples when the TA98 or TA100 strain was used. However, relatively high mutagenicity was detected even deep under the ground when the YG1024 strain was used with S9mix, which suggests the existence of nitroarenes. Since sampling point D is relatively close to a field, the effect of agricultural chemicals and fertilizers should be examined.

We measured the concentration of 16 PAHs, major air contaminants that can be analyzed relatively easily, in the 60 samples collected this time. Table 2 lists the analysis results for the samples collected at sampling site A.

Among the 16 PAHs, the following five were contained in relatively high concentration (10 ng/g soil or higher in surface soil): fluoranthene (Fluo), pyrene, chrysene, phenanthrene, and benzo(b)fluoranthene. The measurement results for samples collected at 10 sampling sites are summarized as follows: Four-cyclic PAHs, such as Fluo and pyrene, were contained in high concentrations (Fluo exhibited the highest concentration at 9 points out of 10 and the second highest concentration at the remaining point, and pyrene exhibited the second highest concentration at 8 points out of 10 and the third highest concentration at the remaining 2 points). The ratio of existence of 16 PAHs exhibited approximately the same tendencies (patterns), which suggests that their existence was affected by similar contamination sources. Among the 16 PAHs, the content of benzo(a)pyrene (BaP), which has the highest carcinogenic and mutagenic properties, was 4.97 ng/g soil (surface soil in Table 2), which was approximately 15% of that of Fluo (mean of surface soil at 10 points: 16.0% ± 5.0%).

On the other hand, the samples collected from deeper soil exhibited extremely low PAH content compared with the surface soil samples, many of them falling below the minimum limit of detection. As in the case of mutagenicity test results (Fig. 1a, c), if surface samples exhibited high PAH content, deeper soil also exhibited a certain level of content, but PAHs were detected mostly (98%) within the range from the surface down to a depth of 30 cm. Table 3 lists the concentration of BaP, from among the PAH data obtained this time. The table indicates that the BaP concentration in the surface soil at 10 points fell within the range from 0.23 to 8.28 ng/g soil (average: 2.99 ± 2.34 ng/g soil), and that the concentration decreased significantly below the surface, with approximately 90% of the contents detected in the soil from the surface down to a depth of 30 cm. Since PAHs examined

Table 2 PAH concentration of soil samples by depth at sampling site A

Compounds (ring numbers)	PAH concentration (ng/g soil)						QL (ng/g soil)
	Surface	20 cm	40 cm	60 cm	80 cm	100 cm	
Naphthalene (2)	0.59	0.02	0.07	0.03	0.01	ND	0.01
Acenaphthylene (3)	0.78	ND[a]	ND	ND	ND	ND	0.02
Acenaphthene (3)	0.57	ND	ND	0.01	0.01	ND	0.01
Fluorene (3)	0.87	ND	0.05	0.03	0.04	0.03	0.02
Phenanthrene (3)	12.4	0.22	0.18	0.09	0.18	0.22	0.03
Anthracene (3)	0.98	ND	0.03	ND	0.04	0.10	0.03
Fluoranthene (4)	32.3	0.87	0.10	0.05	0.13	0.07	0.03
Pyrene (4)	23.7	0.74	0.05	0.04	0.04	0.05	0.03
Benz[a]anthracene (4)	9.81	0.25	ND	0.02	0.02	ND	0.02
Chrysene (4)	19.5	0.22	ND	0.12	0.01	ND	0.01
Benzo[b]fluoranthene (5)	11.7	0.42	ND	0.02	ND	ND	0.02
Benzo[k]fluoranthene (5)	4.21	0.16	ND	0.04	ND	ND	0.03
Benzo[a]pyrene (5)	4.97	0.15	ND	0.02	0.10	0.13	0.02
Indeno[1,2,3-cd]pyrene (6)	3.60	0.27	0.11	ND	ND	ND	0.05
Benzo[ghi]perylene (6)	2.42	0.11	0.02	ND	ND	ND	0.02
Dibenz[ah]anthracene (5)	0.93	0.24	0.11	ND	ND	ND	0.04
Total	129	3.65	0.72	0.48	0.59	0.60	

QL quantification limit (S/N = 10)

[a] Less than the limit of determination

Table 3 Benzo[a]pyrene concentration (ng/g soil) of soil samples by depth at each sampling site (ng/g soil)

Sampling point	Surface	20 cm	40 cm	60 cm	80 cm	100 cm
A	4.97	0.15	ND	0.02	0.10	0.13
B	2.55	ND	ND	ND	0.07	0.03
C	0.58	0.02	ND	ND	ND	0.03
D	2.75	0.05	0.04	ND	0.08	0.04
E	8.28	0.07	0.03	ND	0.03	0.07
F	3.14	1.93	0.04	0.02	0.02	0.08
G	2.78	0.12	0.06	ND	ND	0.23
H	3.46	0.05	0.03	0.03	0.02	0.03
I	1.11	0.10	0.02	0.03	0.03	0.07
J	0.23	0.04	1.27	ND	ND	ND
Mean ± SD	2.99 ± 2.34	0.28 ± 0.62	0.19 ± 0.44	0.02 ± 0.00	0.04 ± 0.04	0.08 ± 0.07

ND less than the limit of determination

this time exhibited approximately the same behavior and a considerable amount was contained in the surface soil, it is likely that the samples tested this time had been affected mainly by air pollutants.

The correlation between the 4- or more cyclic PAH concentration in soil and their mutagenicity was examined. Since mutagenicity was not detected in many cases other than in surface soil, correlation was examined for the surface soil. The coefficient of correlation of benzo(k)fluoranthene (BkF) and benzo[ghi]perylene (BghiP) exhibited relatively low values (BkF/Fluo = 0.666, BkF/benz[a]anthracene (BaA) = 0.691, BghiP/Fluo = 0.713, Bghi/BaA = 0.728), but the coefficient of correlation for the other PAHs was higher, which suggests that the contamination source and behavior of these PAHs were very similar. Under the 3-strain and 2-condition measurement conducted this time (TA100 ± S9mix, TA98 ± S9mix, and YG1024 ± S9-mix), the coefficient of correlation in mutagenicity was high between TA98 with S9mix and TA98 without S9mix ($r = 0.846, p < 0.01$), as well as in TA98 without S9mix and

YG1024 without S9mix ($r = 0.803$, $p < 0.01$). However, significant correlation was not found in all of the combinations, which suggests that multiple types of mutagenic substances existed in a mixed state.

The coefficient of correlation between 10 PAHs and mutagenicity was high between BghiP and TA100 without S9mix ($r = 0.797$, $p < 0.01$), or YG1024 with S9mix ($r = 0.638$, $p < 0.05$). The YG1024 with S9mix also exhibited significant correlation with BkF ($r = 0.679$, $p < 0.05$) and BaP ($r = 0.674$, $p < 0.05$). PAHs are promutagens that exhibit mutagenicity when S9mix is added to them. However, since mutagenicity was also found in cases where S9mix was not added, PAHs do not always account for the generation of mutagenicity. Nnitroarenes are thought about as one of the candidates of direct mutagen in these samples. As shown in Fig. 1, More numbers of revertant colonies were observed by YG1024 strain than TA98 and TA100 strains. It is strongly suggested that nitroarenes were included in this samples. Most of nitroarenes such as 1-nitropyrene and dinitropyren are direct mutagens. And, these compounds induce many revertant colonies with very small amount. These compounds have the possibility of more contributing to the mutagenicity than PAHs.

Since the contamination level of the soil samples collected this time was as low as approximately 1/100 of those collected near public roads in suburban areas (Endo et al. 2004), identifying and quantifying individual causative agents might be difficult, but we intend to take on that challenge, including the development of an applicable analysis method.

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