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Occurrence and Origin of Mutagenicity in Soil and Water Environment

Tetsushi Watanabe[1*], Takeshi Ohe[2] and Teruhisa Hirayama[1]

[1]Department of Public Health, Kyoto Pharmaceutical University,
5 Nakauchicho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan
[2]Department of Food and Nutrition, Kyoto Women's University
35 Kitahiyoshicho, Imakumano, Higashiyama-ku, Kyoto 605-8501, Japan

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Surface soil and surface water are repositories of chemicals released into the environment, and contaminations of surface soil and river water with mutagens were first reported in the 1970s. However, the identity of major mutagens in surface soil and river water remained unclear for more than two decades. Recently, we have identified some nitrated polycyclic aromatic hydrocarbons (nitro-PAHs), e.g., dinitropyrene isomers and 3-nitrobenzanthrone, as major mutagens in the organic extracts of surface soil that showed strong mutagenicities toward *Salmonella typhimurium* TA98 in the absence of a mammalian metabolic system (S9 mix), using a bioassay-directed fractionation method. Moreover, we identified new potent mutagens from substances adsorbed on a blue cotton (blue rayon) from river water samples, which showed strong mutagenicity toward *S.typhimurium* YG1024 with or without the S9 mix. One group was the phenylbenzotriazole (PBTA)-type mutagens, which were detected in river water samples collected at sites below textile dyeing factories. The other group has a dichlorobiphenyl skeleton, i.e., 4-amino-3,3'-dichloro-5,4'-dinitrobiphenyl, and was isolated from a river water sample contaminated with effluent from chemical plants treating polymers and dye intermediates. Some of the nitro-PAHs detected in surface soil, such as PBTA-type mutagens, and 4-amino-3,3'-dichloro-5,4'-dinitrobiphenyl are novel compounds. Up to approximately 50% of the total mutagenicity of extracts from surface soils and river waters was accounted for by nitro-PAHs, PBTA-type mutagens, or 4-amino-3,3'-dichloro-5,4'-dinitrobiphenyl. However, major mutagens in most types of surface soil and river water with high mutagenicity remain unknown. Because environmental mutagens may play some role in the development of diseases such as cancer, their identification is an important step for understanding the risks to indigenous biota and human health. Further effort to identify these major mutagens must be made.

[*]E-mail: watanabe@mb.kyoto-phu.ac.jp

1. Introduction

An unfortunate consequence of industrialization and urbanization is the emergence of environmental pollution. Large amounts of known/unknown harmful substances are released into the atmosphere, lithosphere, and hydrosphere by a wide range of human activities, e.g., industrial, domestic and agricultural activities, and diverse mutagenic/genotoxic compounds are included among these environmental contaminants. For instance, various mutagenic/carcinogenic polycyclic aromatic hydrocarbons (PAHs) and nitrated derivatives of PAHs (nitro-PAHs) were detected in exhaust gas from motor vehicles and in airborne particles.[1,2] Most of these airborne substances eventually reach the surface of the lithosphere and hydrosphere through deposition. Large amounts of pesticides are applied to agricultural lands, and some of them are released into surface water with runoff.[3–5] In agricultural soil and surface water, the possibility that non-genotoxic pesticides are converted to genotoxic derivatives by microorganisms or photochemical reactions cannot be ruled out. Many kinds of industrial wastes and effluents were demonstrated to be mutagenic/genotoxic *in vitro* and *in vivo*,[6–9] and partially treated or untreated industrial wastes and effluents have been discharged into surface waters, e.g. rivers and lakes.[10,11] These facts indicate that the surface soil and surface water, are repositories of chemicals released into the environment, and surface soil and river water can be contaminated with mutagenic/genotoxic substances. Indeed, recent studies revealed that river water[10] and surface soil[12] in many countries are contaminated with mutagenic/genotoxic substances, and various mutagenic/genotoxic chemicals have been detected. Identifying major mutagens/genotoxins is very important to clarify the sources of these toxic chemicals and to efficiently minimize contamination levels.

In this report, we summarize the mutagens that have been recently detected and identified in surface soil and river water in Japan. The putative sources and formation mechanisms of these mutagens are also described.

2. Mutagens in Surface Soil

2.1 *Occurrence of mutagenicity in soil*

In many studies, the extracts of soil samples collected from forests,[13] roadsides,[13–17] agricultural land,[13,18,19] and residential sites[20] in urban districts[21] and industrial areas[14] were shown to exhibit mutagenicity and/or DNA damaging activity. Mutagenic PAHs were detected in soil samples from several cities in Japan,[14–16] but the contribution of the PAHs to the total mutagenicity of the soil extracts was less than a few percent.[15,16]

To clarify the mutagenic potencies of surface soil in Japan, we collected a total of 544 samples; 106 to 111 soil samples from urban/suburban sites in five geographically different regions (Hokkaido, Kanto, Chubu, Kinki and Kyushu regions) between 1996 and 2003. The organic extracts from these soil samples were examined using the *Salmonella* mutagenicity assay in the presence and absence of a mammalian system (S9 mix).[22] Tables 1 and 2 show the summary of the sampling sites and the results of mutagenicity assays of the surface soil samples, respectively. Five hundred thirty three (98%) of the 544 surface soil samples showed mutagenicity toward either *Salmonella typhimurium* TA98 or TA100 strain with or

Table 1
Sampling sites of surface soils.[22]

Region	Prefecture	Locations
Hokkaido		Abashiri, Asahikawa, Chitose, Ishikari, Kitohiroshima, Kitami, Kushiro, Muroran, Noboribetsu, Sapporo (Chuo-ku, Kita-ku, Toyohira-ku, Nishi-ku, Shiroishi-ku, Minami-ku, Teine-ku, Atsubetsu-ku)
Kanto	Tokyo	Tokyo (Minato-ku, Shibuya-ku, Bunkyo-ku, Nerima-ku, Setagaya-ku, Koto-ku), Hachioji, Higashikurume, Higashimurayama
Chubu	Aichi	Chita, Handa, Hekinan, Ichinomiya, Kariya, Nagoya (Minamiku, Moriyama-ku, Kita-ku, Mizuho-ku, Naka-ku, Minato-ku, Nishi-ku, Higashi-ku, Nakamura-ku, Nakagawa-ku, Atsuta-ku), Nishio, Okazaki, Shinshiro, Toyohashi
	Gifu	Gifu, Kaizu, Nakatsugawa, Ogaki
	Shizuoka	Inasa, Shimizu, Shizuoka
	Mie	Ichishi, Mie, Tsu, Yokkaichi
Kinki	Kyoto	Joyo, Kuze, Kyoto (Kamigyo-ku, Yamashina-ku, Fushimi-ku, Ukyo-ku, Sakyo-ku), Nagaokakyo, Sonobe, Uji
	Osaka	Ibaraki, Izumi, Katano, Minoo, Osaka (Sumiyoshi-ku, Minato-ku, Chuo-ku, Konohana-ku, Nishi-ku, Suminoe-ku, Joto-ku, Higashinari-ku), Sakai, Suita, Takaioshi, Takatsuki, Toyonaka
	Hyogo	Amagasaki, Ashiya, Itami, Kobe (Higashinari-ku, Chuo-ku, Nada-ku, Nishi-ku), Nishinomiya, Takarazuka
	Nara	Gose, Nara, Tenri, Yamatokoriyama
	Shiga	Koga, Kusatsu, Moriyama, Omihachiman, Otsu
Kyushu	Fukuoka	Dazaifu, Fukuoka (Hakata-ku, Higashi-ku), Kitakyushu, Kurume, Omuta, Yame
	Saga	Saga, Tosu
	Oita	Oita
	Kumamoto	Kumamoto
	Nagasaki	Nagasaki
	Miyazaki	Miyazaki
	Kagoshima	Kagoshima
(Other)	Hiroshima	Hiroshima
	Yamaguchi	Yamaguchi

without S9 mix. The mutagenic potencies of surface soil extracts varied widely among sampling sites. Eight soil samples induced more than 10,000 revertants per gram of soil, and 7 of them showed the highest potencies toward TA98 without S9 mix. These samples were collected from Takatsuki and two sites in Osaka city in Osaka prefecture, Uji in Kyoto prefecture, Kobe in Hyogo prefecture, and Hekinan in Aichi prefecture. The soil samples collected from Muroran in Hokkaido showed the highest mutagenicity toward TA100 with S9 mix. Moreover, major mutagens in surface soil vary with sampling sites. In a review of published data on the mutagenicity of soil, White and Claxton[12] divided the compiled data on *Salmonella* mutagenic potencies into three site categories, i.e., rural/agricultural, urban/

Table 2
Summary of results of mutagenic activities of surface soil samples from five regions in Japan.[22)]

	Mutagenic activity (revertants/g, soil)			
	TA100		TA98	
	–S9 mix	+S9 mix	–S9 mix	+S9mix
n	544	544	544	544
positives	382	477	523	529
max	50,379	30,554	195,241	43,342
min	0	0	0	0
min(p)	5	38	16	21
median	135	357	216	376
mean	305	712	1,014	761
standard deviation	2,181	1,693	8,703	2,282
positive ratio (%)	70	88	96	97
data distribution				
$>10^5$	0	0	1	0
$10^4 – 10^5$	1	2	7	3
$10^3 – 10^4$	13	86	35	66
$10^2 – 10^3$	304	360	389	412
$<10^2$	64	29	91	48
0	162	67	21	15

The mean and standard deviation were arithmetically calculated.

suburban, and industrial, and compared their geometric mean values. The geometric mean value of the published data in TA98 without S9 mix for urban/suburban sites ($N = 219$) was 430 revertants/g of soil, and this value was much higher than that for rural/agricultural sites ($N = 125$, geometric mean value = 57 revertants/g of soil). The mutagenic potencies of the seven soil samples collected from Takatsuki, Osaka, Uji and other cities in Japan, i.e., more than 10,000 revertants/g of soil, are considerably higher than the geometric mean value of the soil samples from urban/suburban sites in many countries. These results indicate that surface soil is largely contaminated with mutagens in Japan, and that there are some sites where the contamination levels are very high.

2.2 *Identification of mutagenic chemicals in soil*

To clarify the major mutagens in the soil samples, we prepared organic extracts (3.4 g) from 4.4 kg of soil collected from Sumiyoshi-ku in Osaka and separated the compounds by bioassay-directed fractionation using column chromatography and the *Salmonella*/mutagenicity assay.[(23)] After four steps in the separation process, two major mutagens were isolated. On the basis of the structural analysis of these two mutagens, they were identified as 1,6- and 1,8-dinitropyrene (DNP) isomers. The ratios of the contributions of 1,6- and 1,8-DNP isomers to the mutagenicity of the soil extract from Sumiyoshi-ku in TA98 without S9 mix were 13 and 29%, respectively. This was the first study on the detection of DNP isomers in soil. The chemical structures and mutagenic potencies of nitro-PAHs detected in surface soil, including 1,3-, 1,6-, and 1,8-DNP isomers, are shown Fig. 1. To clarify the contamination levels of surface soil with 1,3-, 1,6-, and 1,8-DNP isomers, a highly sensitive

quantification method for the detection of DNP isomers in soil was developed,[15] and 35 soil samples collected from various districts of a megalopolis in Japan, which consists of Tokyo, Nagoya, Osaka and their adjacent cities, were analyzed.[25,26] Table 3 shows the summary of the amounts of DNP isomers and mutagenicities of surface soils in TA98 without S9 mix. DNP isomers were detected in all soil samples examined, and the following ranges of per gram of soil were detected: for 1,3-DNP, from 6 to 3,270 pg; for 1,6-DNP, from 11 to 5,587 pg; and for 1,8-DNP, from 10 to 6,809 pg. As shown in Table 3, the contribution ratios of 1,3-, 1,6-, and 1,8-DNP isomers to the total mutagenicities of the soil extracts toward TA98 without S9 mix were from 0.2 to 12%, from 0.3 to 12%, and from 0.5 to 28%, respectively.

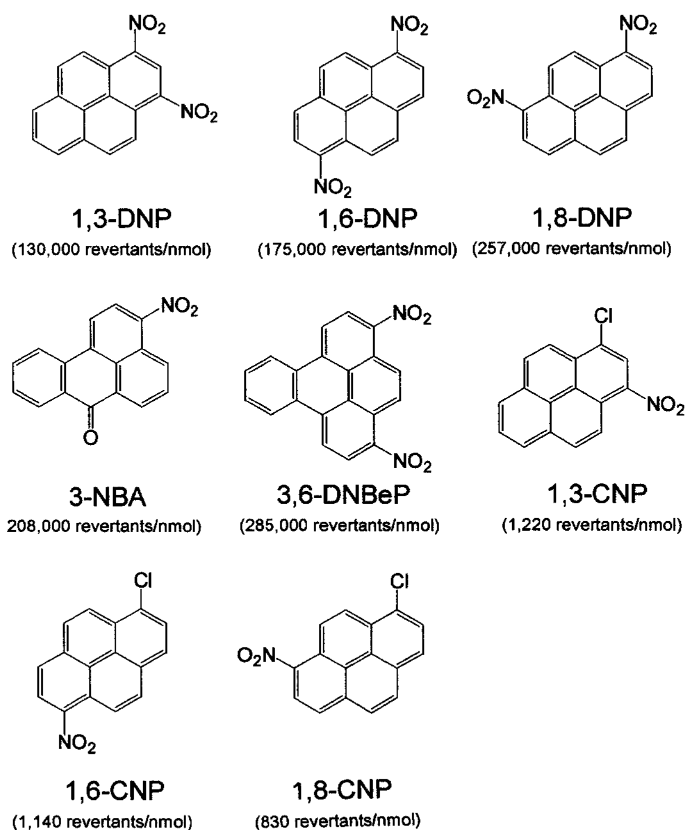

Fig. 1. Chemical structures of nitro-PAHs detected in surface soil. Values in parenthesis show the mutagenic activity in *Salmonella typhimurium* TA98 without S9 mix.
DNP: dinitropyrene, NBA: nitrobenzanthrone, DNBeP: dinitrobenzo[*e*]pyrene, CNP: chloronitropyrene

Table 3
Amounts of 1,3-, 1,6- and 1,8-dinitropyrene (DNP) isomers in soil and their contributions to mutagenicities of organic extracts from soil in *Salmonella typhimurium* TA98 without S9 mix.

Sampling site	Mutagenicity	Amount of DNP (pg/g of soil)[b]			Contribution ratio of DNP (%)[c]			Reference
	(revertants/g of soil)[a]	1,3-	1,6-	1,8-	1,3-	1,6-	1,8-	
Kanto region								
Tokyo								
Sinagawa-ku	319	25±3	34±1	125±4	3	4	30	[26]
Higashimurayama	438	17 ±2	14 ±1	17 ±2	2	1	3	[26]
Hachioji	380	21 ±2	21 ±2	30 ±1	2	2	6	[26]
Chubu region								
Aichi prefecture								
Nagoya	180	12 ±4	16±6	13 ±2	3	3	6	[26]
Hekinan-1	34,300	2,437±31	4,209 ±156	4,369 ±469	3	5	10	[26]
Hekinan-2	46,800	3,270±383	5,587 ±315	6,809 ±50	3	5	11	[26]
Gifu prefecture								
Gifu	260	51±6	82±8	77 ±5	8	12	22	[26]
Kinki region								
Kyoto prefecture								
Uji 1	3,300	318 ±51	633 ±47	863±101	4	7	20	[26]
Uji 2	4,797	624±24	1,031±106	1,181±189	5	8	19	[25]
Osaka prefecture								
Higashiosaka	248	29 ±10	25±13	61±22	4	4	19	[26]
Izumi	1,598	62±1	83±1	56±3	2	2	3	[25]
Kishiwada	1,627	7±1	11±1	10±1	0.2	0.3	0.5	[25]
Osaka Abeno-ku	3,506	818±3	1,082±74	1,174±56	9	12	26	[25]
Osaka Fukushima-ku	579	76±10	118±14	175±13	5	8	23	[25]
Osaka Higashinari-ku	1,627	22±3	32±2	53±5	0.5	0.7	2.5	[25]

(Continued)

Table 3 Continued

Sampling site	Mutagenicity	Amount of DNP (pg/g of soil)[b]			Contribution ratio of DNP (%)[c]			Reference
	(revertants/g of soil)[a]	1,3-	1,6-	1,8-	1,3-	1,6-	1,8-	
Osaka Higashisumiyoshi-ku	130	16±2	18±1	20±1	5	5	12	[25]
Osaka Higashiyodogawa-ku	2,710	460±29	813±31	963±126	7	12	27	[25]
Osaka Ikuno-ku	334	102±9	87±6	87±7	12	10	20	[25]
Osaka Joto-ku	1,136	132±71	226±83	305±107	5	8	20	[25]
Osaka Kita-ku	4,232	656±33	941±16	990±61	6	9	18	[25]
Osaka Minato-ku	5,963	1,526±82	1,772±73	2,092±165	10	12	27	[25]
Osaka Naniwa-ku	224	27±4	41±1	31±2	5	7	10	[25]
Osaka Nishi-ku	1,420	88±4	166±2	66±4	2	5	4	[25]
Osaka Suminoe-ku	6,075	659±66	1,191±124	1,119±120	4	8	14	[25]
Osaka Sumiyoshi-ku 1	6,740	1,653±93	1,928±71	2,360±133	10	11	27	[26]
Osaka Sumiyoshi-ku 2	9,780	2,683±346	3,069±383	3,646±557	11	12	28	[26]
Sakai 1	3,073	526±108	804±110	1,083±102	7	10	27	[25]
Sakai 2	1,302	9±1	13±2	15±1	0.3	0.4	0.9	[25]
Sakai 3	4,092	204±26	327±30	367±41	2	3	7	[25]
Suita	103	6±1	11±2	21±2	2	4	15	[25]
Takatsuki	2,408	115±15	236±34	343±65	2	4	11	[25]
Takaishi	2,581	192±21	282±36	440±68	3	4	13	[25]
Hyogo prefecture								
Amagasaki	691	60±10	117±17	156±10	3	7	17	[25]
Kobe Sumiyoshi-ku	10,200	1,120±111	1,849±120	2,573±237	4	7	20	[26]
Kobe Nagata-ku	496	65±3	127±16	127±2	5	10	20	[25]

[a]The organic extracts were obtained from 15 g of soil (<250 mm) using an ultrasonic extractor.
[b]Values were corrected for recoveries during the purification process and are shown as mean ± SD ($n = 3$).
[c]The mutagenic potencies of DNP isomers that were used to calculate the contribution ratios were as follows: 1,3-DNP, 390 revertants/ng; 1,6-DNP, 390 revertants/ng; and 1,8-DNP 760 revertants/ng. The mutagenicities of DNP isomers were additive in the soil extracts.

The mutagenic potencies of the soil samples toward TA98 without S9 mix correlated significantly with the amounts of DNP isomers, and the correlation coefficients were as follows: $r = 0.86$ ($p<0.001$) for 1,3-DNP; $r = 0.94$ ($p<0.001$) for 1,6-DNP; $r = 0.93$ ($p<0.001$) for 1,8-DNP; and $r = 0.92$ ($p<0.001$) for the total of the DNP isomers. Nitro-PAHs, including 1,3-, 1,6-, and 1,8-DNP isomers, are produced by the incomplete combustion of organic materials such as fossil fuels[27,28] and are emitted into ambient air.[29,30] The 1,3-, 1,6-, and 1,8-DNP isomers are potent bacterial mutagens[2] and are carcinogenic to experimental animals.[31,32] The International Agency for Research on Cancer (IARC) has classified 1,6- and 1,8-DNP isomers as possible human carcinogens (group 2B).[33]

3-Nitrobenzanthrone (NBA) is also a powerful mutagen for bacteria[34] and mammalian cells.[35,36] Recently, we have detected 3-NBA, which was found in diesel exhaust and airborne particles, [34] in surface soil.[25,37] Table 4 shows the amounts of 3-NBA in surface soil and the contribution ratios of 3-NBA to the total mutagenicities of the soil extracts toward TA98 without S9 mix. The amounts of 3-NBA ranged from 144 to 1158 pg/g of soil, and the contribution ratios of 3-NBA ranged from 2 to 38%.[25] No significant correlation was found between the mutagenic potencies and the amounts of 3-NBA.

We prepared 1.8 g of organic extract from the 2.2 kg of surface soil collected from Takatsuki in Osaka prefecture to identify the major mutagens.[38] In our assessment of the mutagenicity of surface soil in Japan, a soil sample from Takatsuki showed extremely high mutagenicity toward TA98 without S9 mix. Using bioassay-directed fractionation, a new powerful mutagen in addition to the 1,6- and 1,8-DNP isomers was isolated. On the basis of the structural analysis of this mutagen and a synthetic study, this compound was identified as 3,6-dinitrobenzo[*e*]pyrene (DNBeP). The ratio of the contribution of 3,6-DNBeP to the total mutagenicity of the soil extract from Takatsuki toward TA98 with S9 mix was 15%.

Table 4
Amounts of 3-NBA in soil and its contributions to mutagenicities of organic extracts from soil toward *Salmonella typhimurium* TA98 without S9 mix.[25)]

Sampling site	Mutagenicity (revertants/g of soil)[a]	Amount of 3-NBA (pg/g of soil)[b]	Contribution ratio of 3-NBA (%)[c]
Kyoto prefecture			
Uji	4,797	144±11	2
Osaka prefecture			
Izumi	1,598	809±5	38
Kishiwada	1,627	295±64	14
Osaka Higashiyodogawa-ku	2,710	229±12	6
Osaka Nishi-ku	1,420	274±12	15
Sakai 2	1,302	158±49	9
Takatsuki	2,408	1,158±177	36
Hyogo prefecture			
Kobe Nagata-ku	496	163±33	25

[a]The organic extracts were obtained from 15 g of soil (<250 mm) using an ultrasonic extractor.
[b]Values were corrected for recoveries during the purification process and are shown as mean±SD ($n = 3$).
[c]The mutagenic potency of 3-NBA used to calculate the contribution ratios was 756 revertants/ng. The mutagenicity of 3-NBA was additive in the soil extracts.

3,6-DNBeP showed strong mutagenicity toward TA98, and its potency was comparable to those of DNP isomers and 3-NBA, which are among the strongest bacterial mutagens reported so far in the literature. In addition to the soil sample from Takatsuki, 3,6-DNBeP was detected in other soil samples (*n* = 4) examined thus far. These soil samples were collected from four different cities, namely, Izumiotsu and Takaishi in Osaka prefecture and Nagoya and Hekinan in Aichi prefecture, and were found to show strong mutagenicity toward TA98 without S9 mix during the assessment of the mutagenicity of surface soil in Japan. 3,6-DNBeP accounted for 22–29% of the total mutagenicity in these soil extracts toward TA98 without S9 mix. These results suggest that 3,6-DNBeP is a major mutagen in surface soil and may be a major contaminant of surface soil in Japan.

Recently, it has been reported that the chlorination and nitration of pyrene occurred in the presence of a metal oxide such as titanium oxide under xenon lamp irradiation.[(39,40)] Because metal oxides are contained in surface soil, the chlorination and nitration of pyrene could occur in surface soil. To clarify whether the chloronitro-derivatives of pyrene exist in surface soil, three chloronitropyrene isomers, i.e., 1-chloro-3-nitropyrene (1,3-CNP), 1,6-CNP, and 1,8-CNP, were synthesized, and surface soil samples collected from Kyoto city in Kyoto prefecture were analysed for their presence. 1,3-, 1,6-, and 1,8-CNP isomers were detected in the ranges from 9.2 to 13.8 pg, from 3.4 to 4.6 pg, and from 4.3 to 5.0 pg/g soil (*n* = 2), respectively.[(41)] These CNP isomers showed mutagenicity toward TA98 without S9 mix, and the potencies of 1,3-, 1,6-, and 1,8-CNP isomers were 1,220, 1,140, and 830 revertants/nmol, respectively.

Nitro-PAHs, including DNP isomers and 3-NBA, are produced by the incomplete combustion of organic materials, e.g., fossil fuels, and are emitted into ambient air. Some nitro-PAHs are formed by the atmospheric reactions of parent PAHs and nitrogen oxides. 3,6-DNBeP and CNP isomers may also be produced through an incomplete combustion process and atmospheric reactions, because their parent PAHs, i.e., benzo[*e*]pyrene and pyrene, are among the abundant chemicals in exhaust particles from diesel engines and ambient air. To clarify the sources of 3,6-DNBeP and CNP isomers in surface soil, the quantification of these chemicals in airborne particles over extensive areas and in exhaust particles from motor vehicles is necessary. Studies on the formations of 3,6-DNBeP and CNP isomers under environmental conditions are also required.

3. Mutagens in River Water

3.1 *Concentration method of mutagens in water*

Pelon *et al*.[(42)] reported on the mutagenicity of Mississipi River water samples using *Salmonella typhimurium* strains TA98 and TA100 using the *Salmonella*/mutagenicity assay developed by Ames *et al*. in 1975.[(43)] Since then many researchers[(44–52)] have reported the mutagenicites of river water in the world, and many varieties of concentration methods, including liquid-liquid extraction, solid phase extraction and other types of column chromatography, have been used. Among those reports, the Yodo River system belonged to the group with potent mutagenicity.[(51,52)] However, the sources of mutagenic contamination and the identification of mutagens in the Yodo River system have not yet been clarified. One major reason involved difficulty in collecting sufficient amounts of chemicals necessary for

the identification of the structures of mutagens in river water. In 1983, Hayatsu *et al*.[53] developed the blue cotton, a solid matrix with covalently linked copper phthalocyanine trisulfonate, that can selectively adsorb polycyclic planar molecules with three or more fused rings. In 1990, Sakamoto and Hayatsu[54] reported that they hung a blue rayon, which was employed as the support for the ligand and contains 2–3 times more ligand than the blue cotton, in a plastic net in the Yodo River system for one day and demonstrated that this method was easy to perform and enabled the mutagenicity monitoring of river water. This result showed that the blue rayon hanging method was suitable for collecting large amounts of target chemicals with polycyclic planar structures, dissolved at ppt levels flowing in the river. They also demonstrated that at least four mutagenic compounds other than polycyclic aromatic hydrocarbons were included in the adsorbates on the blue rayon. On the other hand, Sayato *et al*. [55,56] reported that the highly sensitive detection of mutagenicity in river water could be effectively achieved by combining the blue cotton as an adsorbent and the new *Salmonella* tester strains as a sensitive bioassay. They found that the activity of the subfractions, followed by Sephadex G-25 gel chromatography, was greatly increased by the addition of metabolic activation, particularly in the *O*-acetyltransferase-overproducing strain YG1024 and suggested that S9-activated mutagenic aromatic amines were present in the Katsura River, one tributary of the Yodo River.

3.2 *Identification of mutagenic chemicals in river water*

3.2.1 2-*Phenylbenzotriazole(PBTA)-type mutagens*

For the elucidation of the chemical structures of mutagens in the Yodo River system that show high mutagenicity toward the *O*-acetyltransferase-overproducing strain, our group hung 3 kg of blue cotton nine times between January and June, 1996, at a site below two sewage treatment plants in the Nishitakase River, a tributary of the Yodo River.[57] We isolated 1.1 mg of mutagenic compound I and 1.2 mg of mutagenic compound II by various column chromatography methods, which accounted for 21% and 17% of the total mutagenicities, respectively. The structure of compound I was finally determined to be 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), and that of compound II was 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), as determined from the X-ray analysis, UV spectra, mass spectra, and ^{1}H-NMR spectra of both compounds and/or the debrominated derivative of both compounds. PBTA-1 and PBTA-2 are newly identified potent mutagens, inducing 3,000,000 and 3,200,000 revertants of YG1024 per microgram, respectively, in the presence of metabolic activation.[57,58]

Using the same method, our group identified 2-[2-(acetylamino)-4-[(2-hydroxyethyl)-amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3) at a site below the sewage treatment plant in the Nikko River in Aichi and 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4) at the same site in the Nishitakase River in Kyoto.[59,60]

2-[(2-Bromo-4,6-dinitrophenyl)azo]-5-[bis(2-methoxyethyl)amino]-4-methoxyacetoanilide, 2-[(2-bromo-4,6-dinitrophenyl)azo]-5-[*N*-(2-cyanoethyl)ethylamino-4-methoxyacetanilide, and 5-amino-2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-

methoxyacetanilide, the precursors of PBTA-1, PBTA-2 and PBTA-4, respectively, are listed as chemicals used in the textile industry as disperse dyes, although they are not widely used. 2-[(2-Bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[(2-hydroxyethyl)amino]acetanilide, a precursor of PBTA-3 was not listed as an industrial material. The Color Index name Disperse Blue 79:1 (2-[(2-bromo-4,6-dinitrophenyl)azo-5-[bis(2-acetoxyethyl)amino]-4-methoxyacetanilide) is widely used in dye works throughout the world and has the 2-[2-bromo-4,6-dinitrophenylazo]-4-methoxyacetanilide moiety in its structure, similar to the precursors of PBTA-1, PBTA-2 and PBTA-4. We synthesized 2-[4-[bis(2-acetoxyethyl)amino]-2-(acetylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-5) from Disperse Blue 79:1 by reduction with sodium hydrosulfite and subsequent chlorination with sodium hypochlorite. We found that the bis(2-acetoxyethyl)amino group of PBTA-5 was easily hydrolyzed in alkaline conditions, and 2-[2-(acetylamino)-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6) was formed in high yield; PBTA-6 was found at high levels in five rivers, whereas the level of PBTA-5 in the samples was less than the detection limit.[61]

We made a comprehensive survey of the levels and behavior of PBTA-type mutagens in effluents discharged from a sewage treatment plant located along the bank of the Uji River, a tributary of the Yodo River, using both the blue rayon column method and the blue rayon hanging method. We showed that PBTA-type mutagens were discharged from the sewage treatment plant into the tributary of the Yodo River on a day-to-day basis as shown in Fig. 2.[62] We observed a trend of lower levels of PBTA-type mutagens, and correspondingly lower effluent mutagenicity, on Mondays in comparison with those observed on Tuesday through Sunday. In conclusion, we suggest that PBTA-type mutagens originate from industrial sources on weekdays, considering the time delay for the treatment of dye factory discharges in the sewage treatment plant. Moreover, we detected high levels of PBTA-6 but we could not detect PBTA-5.[62] These findings strongly suggest that the bis(2-acetoxyethyl)amino group of PBTA-5 was hydrolyzed to bis(2-hydroxyethyl)amino group in the textile dyeing and/or wastewater treatment process in the factories or sewage treatment plants, and the levels of PBTA-5 were consequently not detected in river water.

The precursors of PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5 and PBTA-6 have the 2-(2-bromo-4,6-dinitrophenyl)azo-4-methoxyacetanilide moiety in their structures, and this moiety is thought to be required for the formation of PBTA–type mutagens (Table 5). We further synthesized PBTA-7 and PBTA-8 from disperse Blue 291 and Disperse Blue 373, respectively, and detected both PBTA-7 and PBTA-8 in river water samples collected from two geographically different areas of Japan.[63] The chemical structures of PBTA-type mutagens identified so far are shown in Fig. 3. For detecting the presence of PBTA-type mutagens for wide-scale screening, we used the blue rayon hanging method because of its simplicity and convenience. The levels of PBTA-type mutagens determined by this method can be usually expressed as the amounts adsorbed per one gram of blue rayon. Therefore, the blue rayon hanging method is a semiquantitative determination method. For quantitative analysis, we performed the blue rayon column method by packing the blue rayon in the glass column. Furthermore, Moriwaki *et al.* developed a rapid analysis method for the simultaneous determination of eight kinds of PBTA-type mutagen in river water based on LC/MS/MS.

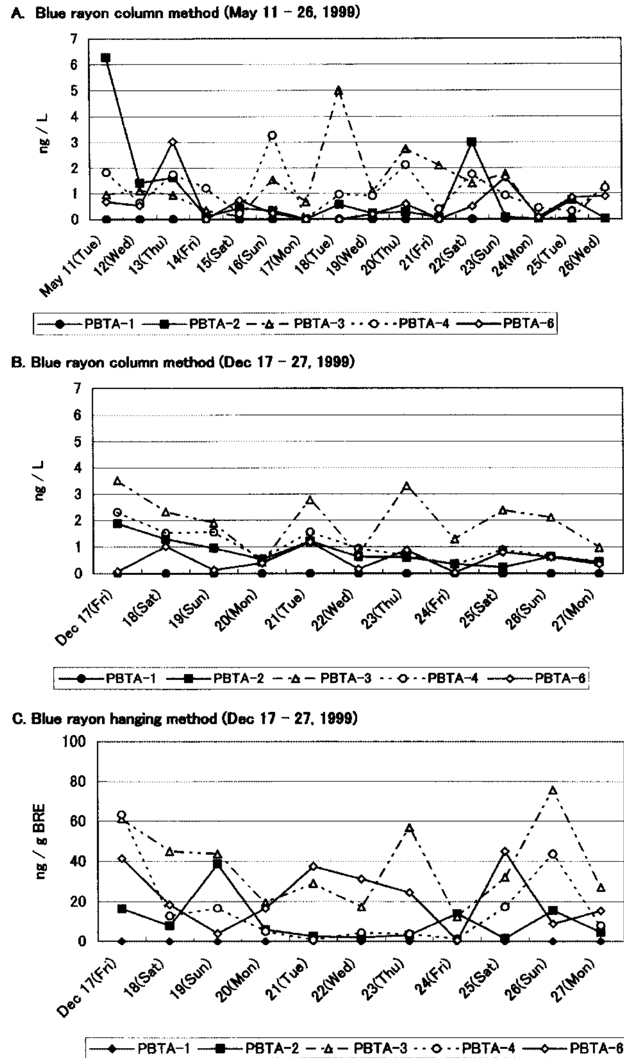

Fig. 2. Daily changes in levels of PBTA-type mutagens discharged from a sewage treatment plant analyzed using blue rayon column method and blue rayon hanging method.

Table 5
Chemical names of precursors of emerging mutagens detected in river water.

Chemical name of precursor	Mutagen (Identification year)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-[bis(2-methoxyethyl)amino]-4-methoxyacetoanilide (C.I. Name : Disperse Blue 301, CAS No. : 26377-33-3)	PBTA-1(1997)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-[*N*-(2-cyanoethyl)ethylamino]-4-methoxyacetoanilide (C.I. Name : -, CAS No. : 22578-86-5)	PBTA-2 (1998)
2-[(2-bromo-4,6-dinitrophenyl)azo]-4-methoxy-5-[(2-hydroxyethyl)-amino]acetanilide (C.I. Name : -, CAS No. : 26021-21-6)	PBTA-3 (2000)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-amino-4-methoxyacetanilide (C.I. Name : -, CAS No. : 53950-22-4)	PBTA-4 (2001)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-[bis(2-acetoxyethyl)amino]-4-methoxyactanilide (C.I. Name : Disperse Blue 79:1, CAS No. : 3618-72-2)	PBTA-5[a] (2001)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-[bis(2-acetoxyethyl)amino]-4-methoxyacetanilide (C.I. Name : Disperse Blue 79:1, CAS No. : 3618-72-2)	PBTA-6 (2001)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-(diethylamino)-4-methoxyacetanilide (C.I. Name : Disperse Blue 291, CAS No. : 56548-64-2)	PBTA-7(2002)
2-[(2-bromo-4,6-dinitrophenyl)azo]-5-(diallylamino)-4-methoxyacetanilide (C.I. Name : Disperse Blue 373, CAS No. : 51868-46-3)	PBTA-8 (2002)
3,3'-dichlorobenzidine(CAS No. : 91-94-1) or 3,3'-dichloro-4,4'-dinitrobiphenyl	ADDB(2002)

C.I. Name : Color Index Name
CAS No. : CAS RegistryNumber
[a] PBTA-5 has not been detected in river water up to the time of publication.

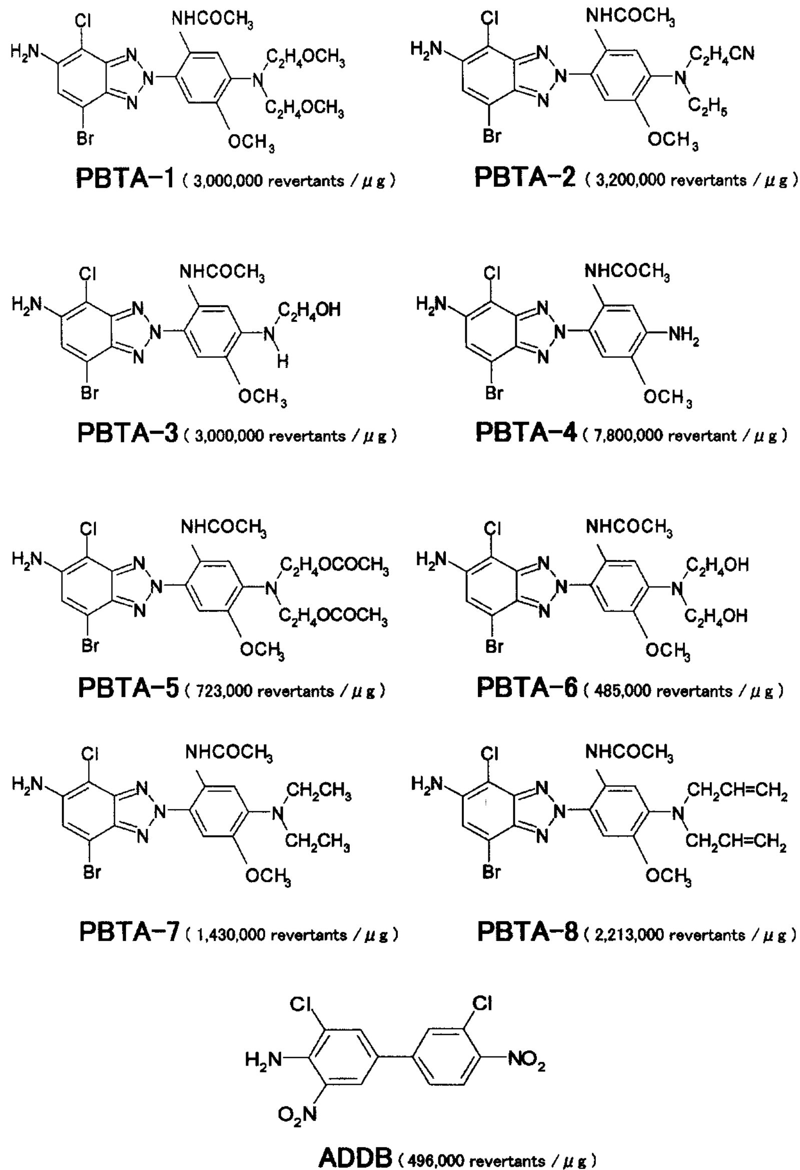

Fig. 3. Chemical structures of emerging mutagens detected in river water. Values in parenthesis show the mutagenic activity toward *Salmonella typhimurium* YG1024 with or without S9 mix. The value for ADDB shows the mutagenic activity without S9 mix. PBTA-5 has not been detected in river water up to the time of publication.

Table 6 shows the summary of levels of PBTA-type mutagens detected in river water in Japan using these three different analysis methods and the contribution ratio (%) of each PBTA-type mutagen to the total mutagenicity. Many water samples were taken at sites downstream from sewage treatment plants, the abundant effluents of which from textile dyeing factories are treated, as well as downstream from domestic wastewater or at the outlets of sewage treatment plants. Because dinitrophenylazo dyes such as Disperse Blue 79:1 are very popular in dye works worldwide, it is plausible that the formation of PBTA-type mutagens from azo-dyes results in pollution not only in river water in Japan but also in rivers in other countries, wherever there are textile-related industries.

3.2.2 *4-Amino-3,3'-dichloro-5,4'-dinitrobiphenyl (ADDB)*

We also found that extracts from blue rayon adsorbates obtained at sites along the Waka River in Wakayama showed potent mutagenic activity toward YG1024 without S9 mix, and in the process we monitored many river water samples throughout Japan.[22] As in the case of the identification of PBTA-type mutagens, we obtained 1.1 g of concentrates using 3 kg of blue cotton at the site, which showed the strongest direct-acting mutagenicity in the monitoring process described.[67] We further collected 0.6 mg of the mutagen from the mutagenic fraction accounting for approximately 50% of the total mutagenicity associated with the blue cotton adsorbate, separating the fraction by low-pressure liquid chromatography using a column packed with COSMOSIL $40C_{18}$-PREP followed by HPLC on a semipreparative TSK-GEL-120A column, a LUNA 5μ phenyl-hexyl column or an STR ODS-II column, monitoring the mutagenicity toward *S. typhimurium* YG1024 without S9 mix. This compound, 4-amino-3,3'-dichloro-5,4'-dinitrobiphenyl (ADDB), was identified as a major mutagen in the Waka River. It contains a dichlorobiphenyl moiety with nitro and amino substitution groups and could have a coplanar conformation because it has no substituents in the 2- or 2'-positions as shown in Fig. 3; it is a novel compound exerting strong mutagenicity in the absence of metabolic activation. We also demonstrated that this newly identified compound activated the human aryl hydrocarbon receptor-mediated transport in the lac Z reporter gene assay with an efficiency almost equal to that of β-naphtoflavone, well-known to be a synthetic aryl hydrocarbon receptor agonist. Consequently, these observations suggest that this compound may disrupt the endocrine system *in vivo*. We estimated that this compound was formed unintentionally via the postemission modification of drainage water containing 3,3'-dichlorobenzidine or 3,3'-dichloro-4,4'-dinitrobiphenyl, both of which are known to be raw materials for the manufacture of polymers and dye intermediates in chemical plants. We also suggested that this compound is discharged from chemical plants close to the sampling site in the Waka River. We are now analyzing this mutagen, as well as its suggested precursors, namely, 3,3'-dichlorobenzidine and 3,3'-dichloro-4,4'-dinitrobiphenyl, and we are also investigating its probable formation pathway.

4. Conclusions

Recently, several nitro-PAHs and other chemicals, i.e., PBTA-type and dichlorodinitrobiphenyl-type compounds, have been identified as major mutagens in

Table 6
Levels of PBTA-type mutagens detected in river water and contribution ratios to mutagenicity of river water samples.
1. Blue rayon/cotton hanging method followed by HPLC

Mutagen	Sampling site	Level[a] (ng/g BC or BR)	Contribution ratio (%)	Reference
PBTA-1	Nishitakase River	47	21	[57]
	Uji River	ND	0	[62][b]
PBTA-2	Nishitakase River	44	17	[58]
	Uji River	2–39	0.3–28	[62][b]
PBTA-3	Nishitakase River	22	NA	[59]
	Katsura River	35	NA	[59]
	Uji River	12–76	6–51	[62][b]
	Nikko River	140	NA	[59]
	Mawatari River	ND–33	0–17	[59]
	Asuwa River	ND–59	0–21	[59],[64]
	Kitsune River	ND–27	0–9	[64]
PBTA-4	Nishitakase River	32	NA	[60]
	Uji River	0.7–63	1–43	[62][b]
	Uji River	33	NA	[60]
	Nikko River	21	NA	[60]
	Mawatari River	0.6–3	0.5–2	[64]
	Asuwa River	ND–6	0–9	[64]
	Kitsune River	ND–15	0–7	[64]
PBTA-6	Nishitakase River	21	3	[61]
	Katsura River	3	0.6	[61]
	Uji River	0.5–45	0.2–14	[62][b]
	Uji River	0.5–134	13	[63]
	Mawatari River	1–122	0.3–17	[64]
	Asuwa River	ND–468	0–39	[63],[64]
	Kitsune River	ND–32	0–3	[64]
	Tobei River	80	2	[61]
PBTA-7	Katsura River	4–51	6–7	[63]
	Uji River	8–55	6–16	[63]
	Mawatari River	3	0.5	[63]
	Asuwa River	4	1	[63]
	Kitsune River	55–101	9–16	[63]
PBTA-8	Katsura River	0.2–15	0.6–4	[63]
	Uji River	2–31	7	[63]
	Asuwa River	1	0.6	[63]
	Kitsune River	20–49	7–15	[63]

[a]Recoveries are not considered.
[b]Data on effluents from sewage treatment plant
ND: not detectable
NA: not available

2. Blue rayon column method followed by HPLC

Mutagen	Sampling site	Level[a] (ng/L)	Contribution ratio (%)	Reference
PBTA-1	Katsura River	ND– 0.5	0–10	[65]
	Nishitakase River	0.03–1.9	0.2–21	[65]
	Kamo River	ND	0	[65]
	Yodo River	ND	0	[65]
	Uji River	ND	0	[62][b]
PBTA-2	Katsura River	ND– 0.9	0–16	[65]
	Nishitakase River	ND–2.3	0–6	[65]
	Kamo River	ND	0	[65]
	Yodo River	ND–0.1	0– 2	[65]
	Uji River	ND– 6.3	0–17	[62][b]
PBTA-3	Uji River	0.02– 5.0	0.7–58	[62][b]
PBTA-4	Uji River	ND–3.3	0–54	[62][b]
PBTA-6	Uji River	ND–3.0	0–11	[62][b]

[a]The amounts of mutagens were corrected for the recoveries of the compounds during the purification process in the river water samples; PBTA-1, 56%; PBTA-2, 56%; PBTA-3, 50%; PBTA-4, 42%; PBTA-6, 50%

[b]Data on effluents from sewage treatment plant

ND: not detectable

NA: not available

3. Solid-phase extraction followed by LC/MS/MS

Mutagen	Sampling site	Level[a] (ng/L)	Contribution ratio (%)	Reference
PBTA-1	Katsura River	ND	NA	[66]
	Nishitakase River	ND	NA	[66]
PBTA-2	Katsura River	16	NA	[66]
	Nishitakase River	17	NA	[66]
PBTA-3	Katsura River	1.6	NA	[66]
	Nishitakase River	4.1	NA	[66]
PBTA-4	Katsura River	3.5	NA	[66]
	Nishitakase River	7.2	NA	[66]
PBTA-6	Katsura River	0.82	NA	[66]
	Nishitakase River	ND	NA	[66]
PBTA-7	Katsura River	ND	NA	[66]
	Nishitakase River	ND	NA	[66]
PBTA-8	Katsura River	0.28	NA	[66]
	Nishitakase River	0.10	NA	[66]

[a]The amounts of mutagens were corrected for the recoveries of the compounds during the purification process in the river water samples; PBTA-2, 74%; PBTA-3, 72%; PBTA-4, 94%; PBTA-6, 72%; PBTA-8, 62%

ND: not detectable

NA: not available

surface soil and river water in several sites in Japan. Most of these mutagens are novel chemicals and are thought to be formed unintentionally through industrial process and/or by the reaction of parental chemicals with environmental factors after their emissions into the environment. The quantification of the levels of these mutagens in the environment, e.g., surface soil, ambient air and surface water, and the determination of their other biological activities including mutation mechanisms and carcinogenicity, are necessary to estimate the impact of these mutagens on indigenous biota and human health.

Although many chemicals were detected in the environment, major mutagens in most types of soil and surface water with potent mutagenicity remain unknown worldwide. Further efforts on the identification of major mutagens must be performed for a better comprehension of risk and for the development of effective methods for preventing environmental pollution by these mutagens.

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References

1 Grimmer, G. (1983): *Environmental carcinogens: Polycyclic aromatic hydrocarbons*. CRC Press, Boca Raton, FL.
2 Tokiwa, H. and Onishi, Y. (1986): Mutagenicity and carcinogenicity of nitroarenes and their sources in the environment. *CRC Crit. Rev. Toxicol.* **17**: 23–60.
3 Aguilar, C., Ferrer, I., Borrull, F., Marce, R.M. and Barcelo, D. (1999): Monitoring of pesticides in river water based on samples previously stored in polymeric cartridges followed by online soil-phase extraction-liquid chromatography-diode array detection and confirmation by atmospheric pressure chemical ionization mass spectrometry. *Anal. Chim. Acta* **386**: 237–248.
4 de Almedia Azevedo, D., Lacorte, S., Vinhas, T., Viana, P. and Barcelo, D. (2000): Monitoring of priority pesticides and other organic pollutants in river water from Portugal by gas chromatography-mass spectrometry and liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr., A* **879**: 13–26.
5 Mitobe, H., Ibaraki, T., Tanabe, A., Kawata, K., Sakai, M. and Kifune, I. (1999): Variation of pesticides in a river flowing through paddy fields area. *Kankyo Kagaku* **9**: 311–320.
6 McGeorge, L.J., Louis, J.B., Atherholt, T.B. and McGarrity, G.J. (1985): Mutagenicity analyses of industrial effluents: Results and considerations for integration into water pollution control programs. In: M.D. Waters, S.S. Sandhu, J. Lewtas, L. Claxton, G. Strauss, S. Nesnow Eds.: *Short-Term Bioassays in the Analysis of Complex Environmental Mixtures, IV*, Plenum Press, New York, N.Y., pp. 247–257.
7 Houk, V.S. (1992): The genotoxicity of industrial wastes and effluents. *Mutat. Res.* **277**: 91–138.
8 White, P.A., Rasmussen, J.B. and Blaise, C. (1996): Comparing the presence, potency and potential hazard of genotoxins extracted from a broad range of industrial effluents. *Environ. Mol. Mutagen.* **27**: 116–139.
9 Claxton, L.D., Houk, V.S. and Hughes, T.J. (1998): Genotoxicity of industorial waste and effluents. *Mutat. Res.* **410**: 237–243.

10 Ohe, T., Watanabe, T. and Wakabayashi, K. (2004): Mutagens in surface waters: a review. *Mutat. Res.* **567**: 109–149.
11 Stahl, R.G.. Jr. (1991): The genetic toxicology of organic compounds in natural waters and wastewaters. *Ecotoxcol. Environ. Saf.* **22**: 94–125.
12 White, P.A. and Claxton, L.D. (2004): Mutagens in contaminated soil: a review. *Mutat. Res.* **567**: 227–345.
13 Edenharder, R., Ortseifen, M., Koch, M. and Wesp, H.F. (2000): Soil mutagens are airborne mutagens: variation of mutagenic activities induced in *Salmonella typhimurium* TA98 and TA100 by organic extracts of agricultural and forest soils in dependence on location and season. *Mutat. Res.* **472**: 23–36.
14 Nishimura, T., Goto, S., Kata, Y., Okunuki, M. and Matsushita, H. (1984): Mutagenicity and benzo[*a*]pyrene contents in soils in Tokyo. *J. Jpn. Soc. Air Pollut.* **19**: 228–238.
15 Arashidani, K., Someya, T., Yoshikawa, M. and Kodama, Y. (1992): Polynuclear aromatic hydrocarbon concentration and mutagenic activity in soils sampled at the roadsides. *J. Jpn. Soc. Air Pollut.* **27**: 190–197.
16 Tamakawa, K., Takahashi, Y., Mishima, Y., Seki, T. and Tsunoda, A. (1985): Mutagenicity and benzo[*a*]pyrene contents in soils in Sendai City; Influences of particulate substances produced by studded tires of automobiles. *Eisei Kagaku* **31**: 329–333.
17 Wesp, H.F., Tang, X. and Edenharder, R. (2000): The influence of automobile exhausts on mutagenicity of soils: contamination with, fractionation, separation, and preliminary identification of mutagens in the *Salmonella*/reversion assay and effects of solvent fractions on the sister-chromatid exchanges in human lymphocyte cultures and in the *in vivo* mouse bone marrow micronucleus. *Mutat. Res.* **472**: 23–36.
18 Goggleman, W. and Spitzauer, P. (1982): Mutagenic activity in agricultural soils. In: Carcinogens and Mutagens in the Environment (H. Stich, Ed.), Vol. 3, CRC Press, Orlando, FL, pp.178–183.
19 Brown, K.W., Donnelly, K.C., Thomas, J.C., Davol, P. and Scott, B.R. (1985): Mutagenicity of three agricultural soils. *Sci. Total Environ.* **41**: 173–186.
20 Knize, M.G., Takemoto, B.T., Lewis, P.R. and Felton, J.S. (1984): The characterization of the mutagenic activity of soil. *Mutat. Res.* **192**: 23–30.
21 Misik, M. and Micieta, K. (2002): *Tradescantia* micronucleus and Vicia chromosome anatelophase assay in monitoring of genotoxicity of urban soil. *Water, Air, Soil Pollut.* **141**: 181–187.
22 Endo, O., Goto, S., Matsumoto, Y., Sakai, S., Akutagawa, T., Asanoma, M., Hirayama, T., Watanabe, T., Tsukatani, H., Sera, N., Tada, A. and Wakabayashi, K. (2004): Mutagenicity of airborne particles, river waters and soils in Japan from 1996 to 2003. *Environ. Mutagen Res.*, **26**: 9–22.
23 Watanabe, T., Ishida, S., Minami, H., Kasai, T., Ogawa, S., Wakabayashi, K. and Hirayama, T. (1998): Identification of 1,6- and 1,8- dinitropyrene isomers as major mutagens in organic extracts of soil from Osaka. Japan. *Chem. Res. Toxicol.* **11**: 1501–1507.
24 Watanabe, T., Ishida, S., Kishiji, M., Takahashi, Y., Furuta, A., Kasai, T., Wakabayashi, K. and Hirayama, T. (1999): High-performance liquid chromatography-fluorescence determination of dinitropyrenes in soil after column chromatographic clean-up and on-line reduction. *J. Chromatogr., A* **839**: 41–48.
25 Watanabe, T., Hasei, T., Takahashi, Y., Otake, S., Murahashi, T., Takamura, T., Hirayama, T. and Wakabayashi, K. (2003): Mutagenic activity and quantification of nitroarenes in surface soil in the Kinki region of Japan. *Mutat. Res.* **538**: 121–131.
26 Watanabe, T., Goto, S., Matsumoto, Y., Asanoma, M., Hirayama, T., Sera, N., Takahashi, Y., Endo, O., Sakai, S. and Wakabayashi, K. (2000): Mutagenic activity of surface soil and quantification of 1,3-, 1,6-, and 1,8-dinitropyrene isomers in soil in Japan. *Chem. Res. Toxicol.* **13**: 281–286.

27 Nakagawa, R., Kitamori, S., Horikawa, K., Nakashima, K. and Tokiwa, H. (1983): Identification of dinitropyrenes in diesel-exhaust particles, their probable presence as the major mutagens. *Mutat. Res.* **124**: 201–211.

28 Gibson, T.L. (1983) Sources of direct-acting nitroarene mutagens in airborne particulate matter. *Mutat. Res.* **122**: 115–121.

29 Tokiwa, H., Kitamori, S., Nakagawa, R., Horikawa, K. and Matamala, L. (1983): Determination of a powerful mutagenic dinitropyrene in airborne particulate matter. *Mutat. Res.* **121**: 107–116.

30 Hayakawa, K., Murahashi, T., Butoh, M. and Miyazaki, M. (1995): Determination of 1,3-, 1,6- and 1,8-dinitropyrenes and 1-nitropyrene in urban air by high-performance liquid chromatography using chemiluminescence detection. *Environ. Sci. Technol.* **29**: 25–29.

31 Takayama, S., Ishikawa, T., Nakajima, H. and Sato, S. (1985): Lung carcinoma induction in Syrian golden hamsters by intratracheal instillation of 1,6-dinitropyrene. *Jpn. J. Cancer Res.* **76**: 457–461.

32 Imaida, K., Lee, M.S. Wang, C.Y. and King, C.M. (1991): Carcinogenicities of dinitropyrenes in the female weanling CD rat. *Carcinogenesis* **12**: 1187–1191.

33 IARC(1989): I*nternational Agency for Research on Cancer Monograph 46*, International Agency for Research on Cancer, Lyons.

34 Enya, T., Suzuki, H., Watanabe, T., Hirayama, T. and Hisamatsu, Y. (1997): 3-Nitrobenzanthrone, a powerful bacterial mutagen and suspected human carcinogen found in diesel exhaust and airborne particulates. *Environ. Sci. Technol.* **31**: 2772–2776.

35 Phousongphouang, P.T., Grosovsky, A.J., Eastmond, D.A., Covarrubias, M. and Arey, J. (2000): The genotoxicity of 3-nitrobenzanthrone and the nitropyrene lactones in human lymphoblasts. *Mutat. Res.* **472**: 93–1003.

36 Borlak, J., Hansen, T., Yuan, Z., Sikka, H.C., Kumar, S., Schmidbauer, S., Frank, H., Jacob, J. and Seidel, A. (2000): Metabolism and DNA-binding of 3-nitrobenzanthrone in primary rat alveolar type II cells, in human fetal bronchial, rat epithelial and mesenchymal cell lines. *Polycyclic. Aromat. Compd.* **21**: 73–86.

37 Murahashi, T., Watanabe, T., Otake, S., Hattori, Y., Takamura, T., Wakabayashi, K. and Hirayama, T. (2003): Determination of 3-nitrobenzanthrone in surface soil by normal-phase high-performance liquid chromatography with fluorescence detection. *J. Chromatogr., A* **992**: 101–107.

38 Watanabe, T., Hasei, T., Takahashi, T., Asanoma, M., Murahashi, T., Hirayama, T. and Wakabayashi, K. (2005): Detection of a novel mutagen, 3,6-dinitrobenzo[*e*]pyrene, as a major contaminant in surface soil in Osaka and Aichi prefectures. Japan. *Chem. Res. Toxicol.* **18**: 283–289.

39 Sugiyama, H., Watanabe, T. and Hirayama, T. (2000): The mutagenic activity of the crude extract obtained from silicon dioxide and titanium dioxide with pyrene and sodium chloride under xenon lamp irradiation. *J. Health Sci.* **46**: 214–218.

40 Sugiyama, H., Watanabe, T. and Hirayama, T. (2000): Nitration of pyrene in metallic oxides as soil components in the presence of indoor air, nitrogen dioxide gas, nitrite, or nitrate ion under xenon irradiation. *J. Health Sci.* **47**: 28–35.

41 Murahashi, T., Kawabata, K., Sugiyama, H., Hasei, T., Watanabe, T. and Hirayama, T. (2004): Determination of mutagenic 1-chloro-3-, -6-, and -8-nitropyrenes in surface soil collected in Kyoto, Japan. *J. Health Sci.* **50**: 625–633.

42 Pelon, W., Whitman, B.F. and Beasley, T.W. (1977): Reversion of histidine-dependent mutant strains of *Salmonella typhimurium* by Mississippi River water samples. *Environ. Sci. Technol.* **11**: 619–623.

43 Ames, B.N., McCann, J. and Yamasaki, E. (1975): Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. *Mutat. Res.* **31**: 347–364.

44 Klekowski, Ed. and Levin, D.E. (1979): Mutagens in a river heavily polluted with paper recycling wastes : results of field and laboratory mutagen assays*Environ. Mutagenes.* **1**: 209–219.

45 Van Kreijl, C.F., Kool, H.J., De Vries, M., Van Kranen, H.J. and De Greef, E. (1980): Mutagenic activity in the rivers Rhine and Meuse in the Netherlands. *Sci. Total Environ.* **15**: 137–147.

46 Moore, R.L., Osborne, L.L. and Davies, R.E. (1980): The mutagenic activity in a section of the Sheep River, Alberta receiving a chlorinated sewage effluent. *Water Res.* **14**: 917–920.

47 Van Hoof, F. and Verheyden, J. (1981): Mutagenic activity in the River Meuse in Belgium. *Sci. Total Environ.* **20**: 15–22.

48 Kool, H.J., Van Kreijl, C. F., Van Kranen, H.J. and De Greef, J.E. (1981): The use of XAD-resins for the detection of mutagenic activity in water. I. Studies with surface water. *Chemosphere* **10**: 85–98.

49 DeMarini, D.M., Plewa, M.J. and Brockman, H.E. (1982): Use of four short-term tests to evaluate the use mutagenicity of municipal water. *J. Toxicol. Environ. Health.* **9**: 127–140.

50 Slooff, W. and Van Kreijl, C.F. (1982): Monitoring the rivers Rhine and Meuse in the Netherlands for mutagenic activity using the Ames test in combination with rat or fish liver homogenates. *Aquat. Toxicol.* **2**: 89–98.

51 Maruoka, S., Yamanaka, S. and Yamamoto, Y. (1985): Mutagenic activity in organic concentrate from Nishitakase River water in Kyoto City, and its fractions separated by using liquid-liquid fractionation and thin layer chromatography. *Mutat Res.* **19**: 249–256.

52 Maruoka, S., Yamanaka, S. and Yamamoto, Y. (1986): Isolation of mutagenic components by high-performance liquid chromatography from XAD extract of water from the Nishitakase River water, Kyoto City, Japan. *Sci. Total Environ.* **57**: 29–38.

53 Hayatsu, H., Oka, T., Wakata, A., Ohara, Y., Hayatsu, T., Kobayashi, H. and Arimoto, S. (1983): Adsorption of mutagens to cotton bearing covalently bound trisulfo-copper- phthalocyanine. *Mutat. Res.* **119**: 233–238.

54 Sakamoto, H. and Hayatsu, H. (1990): A simple method for monitoring mutagenicity of river water mutagens in Yodo River system Kyoto–Osaka, Japan. *Bull. Environ. Contam. Toxicol.* **44**: 521–528.

55 Sayato, Y., Nakamuro, K., Ueno, H. and Goto, R. (1990): Mutagenicity of adsorbates to a copper-phthalocyanine derivative recovered from municipal river water. *Mutat. Res*. **242**: 313–317.

56 Sayato, Y., Nakamuro, K., Ueno, H. and Goto, R. (1993): Identification of polycyclic aromatic hydrocarbons in mutagenic adsorbates to a copper-phthalocyanine derivative recovered from municipal river water. *Mutat. Res.* **300**: 207–213.

57 Nukaya, H., Yamashita, J., Tsuji, K., Terao, Y., Ohe, T., Sawanishi, H., Katsuhara, T., Kiyokawa, K., Tezuka, M., Oguri, A., Sugimura, T. and Wakabayashi, K. (1997): Isolation and chemical-structural determination of a novel aromatic amine mutagen in water from the Nishitakase River in Kyoto. *Chem. Res. Toxicol.* **10**: 1061–1066.

58 Oguri, A., Shiozawa, T., Terao, Y., Nukaya, H., Yamashita, J., Ohe, T., Sawanishi, H., Katsuhara, T., Sugimura, T. and Wakabayashi, K. (1998): Identification of a 2-phenylbenzotriazole (PBTA)-type mutagen, PBTA-2, in water from the Nishitakase River in Kyoto. *Chem. Res. Toxicol.* **11**: 1195–1200.

59 Shiozawa, T., Tada, A., Nukaya, H., Watanabe, T., Takahashi, Y., Asanoma, M., Ohe, T., Sawanishi, H., Katsuhara, T., Sugimura, T., Wakabayashi, K. and Terao, Y. (2000): Isolation and identification of a new 2-phenylbenzotriazole-type mutagen (PBTA-3) in the Nikko River in Aichi, Japan. *Chem. Res. Toxicol.* **13**: 535–540.

60 Nukaya, H., Shiozawa, T., Tada, A., Terao, Y., Ohe, T., Watanabe, T., Asanoma, M., Sawanishi, H., Katsuhara, T., Sugimura, T. and Wakabayashi, K. (2001): Identification of 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole (PBTA-4) as a potent mutagen in river water in Kyoto and Aichi prefectures. *Mutat. Res.* **492**: 73–80.

61 Watanabe, T., Nukaya, H., Terao, Y., Takahashi, Y., Tada, A., Takamura, T., Sawanishi, H., Ohe, T., Hirayama, T., Sugimura, T. and Wakabayashi, K. (2001): Synthesis of 2-phenylbenzotriazole-type mutagens, PBTA-5 and PBTA-6, and their detection in river water from Japan. *Mutat Res*. **498**: 107–115.

62 Morisawa, T., Mizuno, T., Ohe, T., Watanabe, T., Hirayama, T., Nukaya, H., Shiozawa, T., Terao, Y., Sawanishi, H. and Wakabayashi, K. (2003): Levels and behavior of 2-phenylbenzotriazole-type mutagens in the effluent of a sewage treatment plant. *Mutat. Res.* **534**: 123–132.

63 Watanabe, T., Shiozawa, T., Takahashi, Y., Takahashi, T., Terao, Y., Nukaya, H., Takamura, T., Sawanishi, H., Ohe, T. Hirayama, T. and Wakabayashi, K. (2002): Mutagenicity of two 2-phenylbenzotriazole derivatives, 2-[2-(acetylamino)-4-(diethyl-amino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole and 2-[2-(acetylamino)-4-(diallylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2H-benzotriazole and their detection in river water in Japan. *Mutagenesis* **17**: 293–299.

64 Watanabe, T., Takahashi, Y., Takahashi, T., Nukaya, H., Terao, Y., Hirayama, T. and Wakabayashi, K. (2002): Seasonal fluctuation of the mutagenicity of river water in Fukui, Japan, and the contribution of 2-phenylbenzotriazole-type mutagens. *Mutat. Res.* **519**: 187–197.

65 Ohe, T., Takeuchi, N., Watanabe, T., Tada, A., Nukaya, H., Terao, Y., Sawanish, H., Hirayama, T., Sugimura, T. and Wakabayashi, K. (1999): Quantification of two aromatic amine mutagens, PBTA-1 and PBTA-2, in the Yodo River system. *Environ. Health Perspect.* **107**: 701–704.

66 Moriwaki, H., Harino, H., Yoshikura, T., Ohe, T., Nukaya, H., Terao, Y., Sawanishi, H., Wakabayashi, K., Miyakoda, H. and Alary, J-F. (2004): Simultaneous determination of 2-phenylbenzotriazole-type mutagens, PBTA-1 through -8, in river water by liquid chromatography–tandem mass spectrometry. *J. Environ. Monit.* **6**: 897–902.

67 Takamura-Enya, T., Watanabe, T., Tada, A., Hirayama, T., Nukaya, H., Sugimura, T. and Wakabayashi, K. (2002): Identification of a new mutagenic polychlorinated biphenyl derivative in the Waka River, Wakayama, Japan, showing activation of an aryl hydrocarbon receptor-dependent transcription. *Chem. Res. Toxicol.* **15**: 419–425.