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Mutagenicity and Levels of 2-Phenylbenzotriazole (PBTA)-type Mutagens in Sewage Effluent, River Water, Sediment and Drinking Water Collected from the Yodo River System, Japan

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In order to assess the potential hazards to human health and aquatic ecosystem, we examined the mutagenic activity of sewage effluents, river waters, sediments and drinking water collected from the Yodo River system, Japan. We also compared the levels of mutagenic activity with the levels of 2-phenylbenzotriazole (PBTA)-type mutagens formed from corresponding dinitrophenylazo dyes *via* reduction and subsequent chlorination. We assessed mutagenicity in the *O*-acetyltransferase-overexpressing frameshift strain YG1024 of Salmonella with S9 mix. Sixty-six samples among 133 adsorbates (50%) obtained by the blue rayon hanging method collected from 1996 to 2005 were classified as extreme mutagenicity with more than 100,000 revertants per g blue rayon equivalent (BRE). The average mutagenicity of both sewage effluents and river waters at sites located below sewage plants was 382,400 revertants per g BRE ($n = 86$), which was 4.4 times as higher than the downstream river waters (87,900 revertants per g BRE, $n = 47$). PBTA-1 was detected in 33 samples among 76 (43%), and PBTA-2 was detected in 66 samples among 76 (87%), however, the concentration of these compounds fluctuated widely among the samples. Average concentrations of PBTA-1 and PBTA-2 were also much higher in sewage effluents and river waters at sites located below sewage plants ($n = 50$, PBTA-1, 24.1 ng/g BRE; PBTA-2, 88.4 ng/g BRE) than they were in downstream river water samples ($n = 26$, PBTA-1; 1.3 ng/g BRE, PBTA-2; 19.7 ng/g BRE). PBTA-1 and PBTA-2 accounted for 6% and 26% on average, respectively, of the total mutagenicity in all samples analyzed. Based on the concentrations of the PBTA-type mutagens and the effluent volume discharged from three sewage plants, we estimated that ~5 kg/year of PBTA-type mutagens including PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-6, PBTA-7 and PBTA-8, were discharged from three sewage plants into rivers. Further studies showed that these PBTA-type mutagens in river water might not easily accumulate in the river sediment and these PBTA-type mutagens were not detected in drinking water. In the final study, we monitored quantitatively the mutagenic potency of water samples collected at twelve sites, including six sites mentioned above, from the Yodo River system using Sep-Pak C18 cartridge columns and Blue-Chitin columns. The average mutagenic activities recovered by these columns were 10,000 and 5,800 revertants/L. These findings demonstrate that the Yodo River system has been continually and heavily polluted with not only polycyclic planar mutagens, but also by a wide range of chemical mutagens released from sewage plants located along the tributaries for many years.

Key words: *Salmonella typhimurium* YG1024, 2-phenylbenzotriazole (PBTA)-type mutagen, sewage effluent, river water, the Yodo River system

Introduction

A wide range of genotoxic/mutagenic compounds are released from industrial, agricultural and domestic sources into surface waters such as rivers, lakes and the sea. Mutagenicity/genotoxicity tests of complex mixtures such as surface waters using variety of bioassays demonstrate that these environmental mixtures contain many unidentified and unregulated toxicants that may

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be carcinogenic and pose a health risk of unknown magnitude (1–4). Analyses of surface waters are necessary to identify areas potentially contaminated by genotoxic compounds from the different sources (5–12). Although many attempts to identify the chemicals responsible for the mutagenicity/genotoxicity of river waters have been reported, only a limited number of new mutagens have been identified. Increased efforts are needed to identify and characterize the likely vast array of unidentified mutagens in surface waters using bioassay-directed chemical analysis. Such work would help to clarify the post-emission transport and fate of these identified toxicants for risks of adverse effects to humans and indigenous biota.

In 1997 and 1998, we identified the chemical structures of two novel potent mutagens with a 2-phenylbenzotriazole structure that accounted for 21% and 17%, respectively, of the total mutagenicity of the adsorbates collected at sites below a sewage plant in the Yodo River system, Japan, by blue cotton hanging method developed by Sakamoto and Hayatsu (12). These two mutagens were confirmed to be 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)-amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), and 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and they were further synthesized from the corresponding dinitrophenylazo-type dyes, which were used for industrial purposes, by reduction with sodium hydrosulfite followed by chlorination with sodium hypochlorite mainly used in sewage plants for disinfection purposes (13–15). We also demonstrated that a major source of PBTA-1 and PBTA-2 in the Yodo River system was effluents from sewage plants and that discharged mutagens were diluted and/or decomposed while moving down the river (16). It was further suggested that other PBTA-type compounds besides PBTA-1 and PBTA-2 might also contribute to the mutagenicity of the river water samples, because various kinds of dinitrophenylazo-type dyes were used as industrial materials. Between 2000 and 2002, six PBTA-type compounds, i.e. 2-[2-(acetylamino)-4-[(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-3), 2-[2-(acetylamino)-4-amino-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-4), 2-[4-[bis(2-acetoxyethyl)-amino]-2-(acetylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-5), 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6), 2-[2-(acetylamino)-4-(diethylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-7) and 2-[2-(acetylamino)-4-(diallylamino)-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-8) were confirmed and were suggested to be formed from the corresponding dinitrophenylazo dyes *via* reduction with sodium hydrosulfite and subsequent chlorination with sodium hypochlorite (17–20). In fact, these PBTA-type compounds with the exclusion PBTA-5 were detected in concentrates collected at sites downstream from municipal sewage plants or textile-dying factories along several rivers flowing in geographically different areas in Japan (16–23). The Yodo River system is an especially important one in Japan, serving as the main source of drinking water for more than 17 million people living in the Osaka area (24).

In the first part of this paper we demonstrate the mutagenicity and levels of PBTA-1 and PBTA-2 in sewage effluents and river waters collected from the Yodo River system between 1996 and 2005. Subsequently, we estimated the concentrations of eight kinds of PBTA-type mutagens discharged from sewage plants into the Yodo River system. To make a comprehensive survey of the occurrence of PBTA-type mutagens in surface waters, we also surveyed the level of mutagenicity and PBTA-type mutagens in river sediments and drinking water.

In this report, we used blue rayon to collect organic pollutants in water samples such as sewage effluents, river water and drinking water. Blue rayon is a solid matrix with covalently linked copper phthalocyanine trisulfonate that can selectively adsorb polycyclic planar molecules with three or more fused rings and enables easy monitoring of water pollution with these chemicals by hanging the rayon in a plastic net in the flowing river for one day or packing blue rayon into glass column. Furthermore, Blue-Chitin columns allow highly efficient quantitative concentration of polycyclics in river water (25–28). On the other hand, adsorption on Amberlite XAD resins and Silica C18 is the most commonly applied method for concentrating organic substances from different kinds of water samples in aquatic environment. It is known that they can generally adsorb a broad class of organic substances, including polycyclic aromatic hydrocarbons, arylamines, nitro-compounds, quinolines, anthraquinones and so on (29). In the latter part of this paper we report here the comparative analysis of mutagenicity monitoring of the Yodo River system, using two different solid matrixes, one with Silica C18 (Sep-Pak C18 cartridges) and the other with covalently linked phthalocyanine trisulfonate (Blue-Chitin columns).

Materials and Methods

Materials: Eight PBTA-type mutagens (PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7 and PBTA-8) shown in Fig. 1 were synthesized according to methods reported previously (13–15, 17–20). Blue rayon and Blue-Chitin column were obtained from

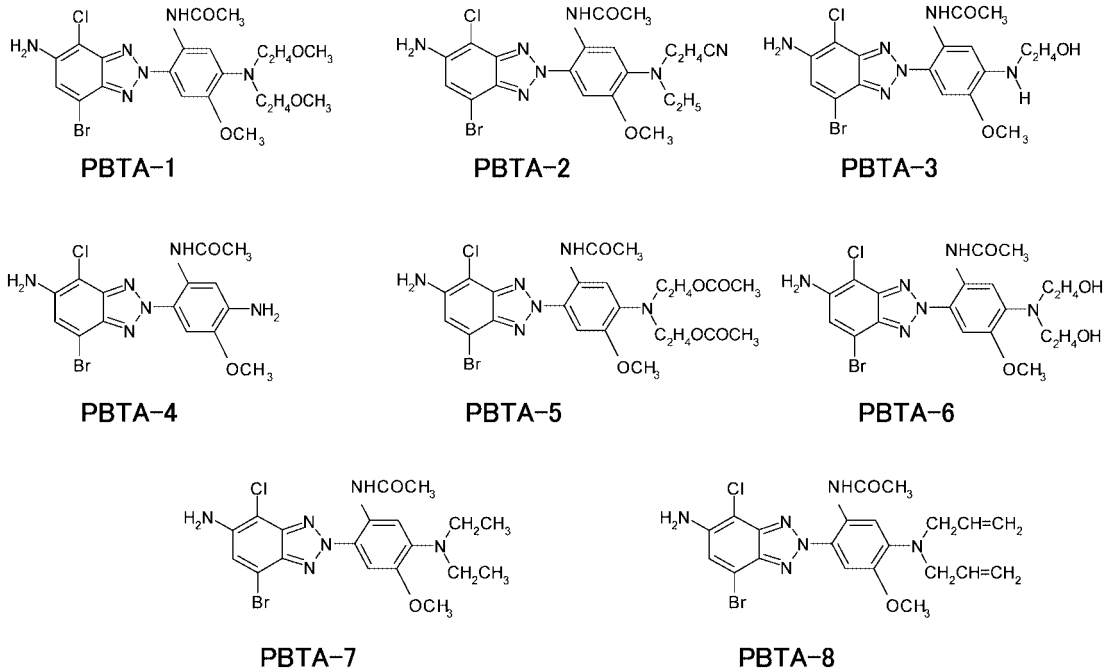

Fig. 1. Structure of 2-phenylbenzotriazole (PBTA)-type mutagens.

Funakoshi Pharmaceutical Co. Ltd. (Tokyo, Japan), and Sep-Pak C18 cartridges were obtained from Waters Co. Ltd. (Milford, Ireland). High-performance liquid chromatography (HPLC) grade methanol and acetonitrile, 2-aminoanthracene and 3-amino-1-methyl-5*H*-pyrido[4, 3-*b*]indole (Trp-P-2) were purchased from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals and reagents were of analytical-reagent grade.

Sample collection and extraction procedures: Sample collection and extraction procedure for sewage effluents, river waters, sediments and drinking waters are shown in Table 1. As shown in Fig. 2, the Yodo River system is mainly composed of the Yodo River (Y) and tributaries of the Uji River (U), Katsura River (KA), Nishitakase River (N), and the Kizu River (KI). The annual mean flow rate of the Yodo River system was ca. 240 m^3/s in 1995 (30). Sampling collection sites are also shown in Fig. 2. All extracts of samples prepared by the following method were stored at −20°C until assayed for mutagenicity and characterized for the levels of PBTA-type mutagens.

Sewage effluents and river waters—Blue rayon hanging method (Exp. 1): Water samples were collected at six sites: two outlets of the sewage plants (Site U-1 and KA-1), two river waters at sites located below sewage plants (Site N-1 and Site N-2), and their two downstream river waters (Site KA-2; 5 km downstream from SP 2 and Y-2; 10 km downstream from Site KA-2). At each point, 5 g of blue rayon in a meshed plastic bag was hung for 24 h between 1996 and 2005 according to the method by Sakamoto and Hayatsu (12). After hanging, blue rayon was washed with distilled water several times. The materials adsorbed to blue rayon were extracted by shaking in 200 mL of methanol/ammonia water (50:1, v/v) or methanol for 20 min three times. The eluates were combined and were evaporated to dryness under reduced pressure, and the residues were dissolved in 2 mL of 75% methanol to prepare a sample to be subjected to the mutagenicity assay and for quantification of PBTA-1 and PBTA-2.

Sewage effluents and river waters—Blue rayon column method (Exp. 2): The blue rayon column method was used for the estimation of PBTA-type mutagen levels discharged from sewage plants into river water, according to the method described previously. Ten liter of water samples was collected at two outlets (Sites U-1 and KA-1) of two sewage plants SP 2 and SP 3, treating effluents from the textile dyeing plants, and at Site N-2 receiving effluents from two sewage plants, SP 1 and SP 2. Each water sample was passed through a glass column (25 mm × 300 mm) packed with 2 g of blue rayon at the flow rate of 30 mL/min at room temperature. After passing the water sample through the column, the blue rayon was washed with distilled water several times and excess water was absorbed onto paper towels. Adsorbed materials were then extracted by shaking the blue rayon in 100, 100, and 50 mL respectively, of methanol three times for 20 min each, because it was suggested that bis(2-acetoxyethyl)amino group of PBTA-5 was hydrolyzed to the bis(2-hydroxyethyl)amino group in alkaline solution (19). The extracts were

Table 1. Sample collection and extraction method for water environmental samples in the Yodo River system

Experiment No.	Sample	Sampling period	Sampling site*	Collection and extraction method
Exp. 1	Sewage effluent and river water	March 1996–October 2005	U-1, KA-1, KA-2, N-1, N-2 and Y-2	Blue rayon hanging method
Exp. 2	Sewage effluent and river water	November and December 1999, and December 2001	U-1, KA-1 and N-2	Blue rayon column method
Exp. 3	Sediment	March and September 2002	U-1 and Y-2	Ultrasonication
Exp. 4	Drinking water	April and July 2002	Y-6	Blue rayon column method
Exp. 5	Sewage effluent and river water	June, July and October 2004, and May, July, October and December 2005	U-1, U-2, KA-1, KA-2, N-1, N-2, KI-1, Y-1, Y-2, Y-3, Y-4 and Y-5	Sep-Pak C18 cartridge column and Blue-Chitin column method

*Sampling sites are shown in Fig. 2.

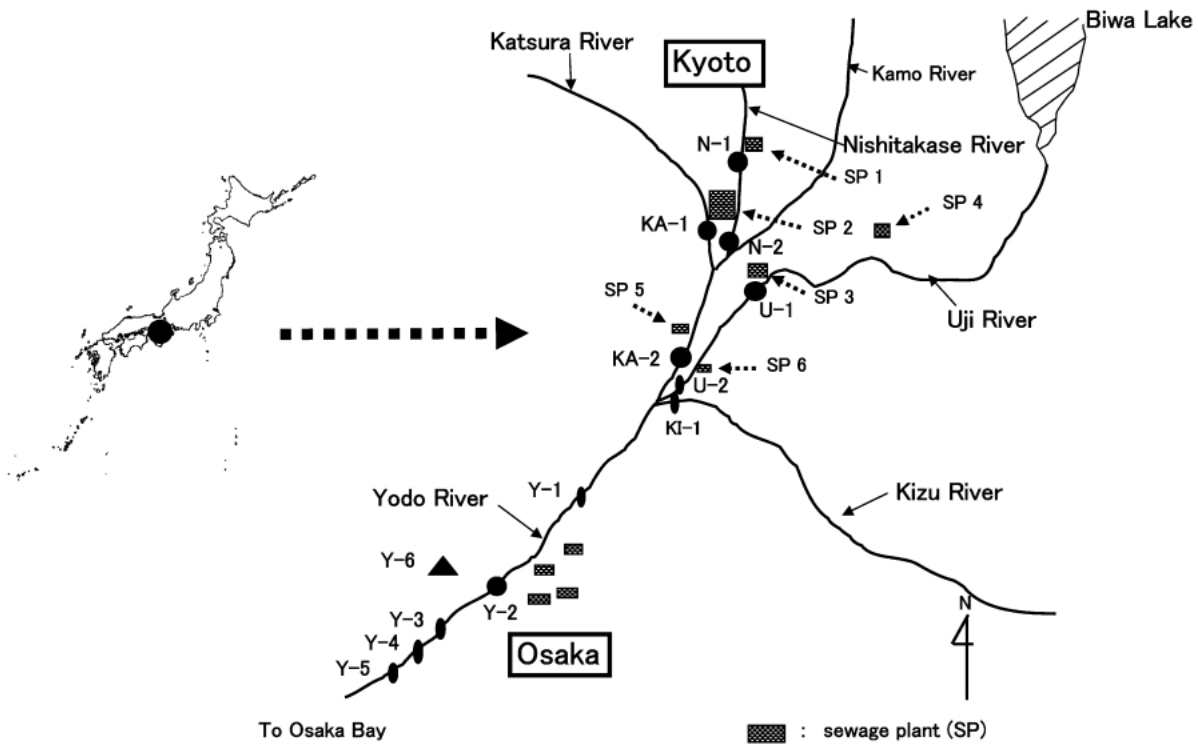

Fig. 2. Sampling locations in the Yodo River system. SP 1–6 shows the place of sewage plants, which their discharges flow into the Nishitakase, Katsura and Uji Rivers. Treatment capacities are: SP 1, 114; SP 2, 1,047; SP 3, 155; SP 4, 140; SP 5, 199 and SP 6,132 × 10^3 m^3 per day.

combined and evaporated to dryness, and the residue was dissolved in 2 mL of 75% methanol. A portion of the extract equivalent to 7 L was used for the purification and determination of eight kinds of PBTA-type mutagens and another portion equivalent to 3 L was evaporated to dryness and the residue was dissolved in dimethylsulfoxide for Salmonella mutagenicity assay.

River sediments (Exp. 3): River sediments were collected at Sites U-1 and Y-2 in the Yodo River system on May 31 and September 6, 2002. The sediments were spread on paper towel and allowed to stand to dryness for 3 days at room temperature in the dark. The dried sediments were screened through a 60-mesh sieve (250 μm) to remove large gravel and trash. The sieved sediment (30 g, dry basis) was extracted with each 300 ml of methanol or a mixture of methanol/benzene (1:1) by triplicate ultrasonication. The combined extracts were reduced to dryness under reduced pressure. Condensed sample solutions were used for the determination of PBTA-type mutagens and the Salmonella mutagenicity assay.

Drinking water (Exp. 4): Each 30 L of drinking water was collected in three pre-cleaned polyethylene bottles at Ibaraki City (Site Y-6 in Fig. 2), whose source for drinking water is located close to Site Y-2 of the Yodo River system. Sampling was performed on April

and July 2002, respectively, and each drinking water sample was passed through a glass column (25 mm × 300 mm) packed with 2 g of blue rayon at the flow rate of 30 mL/min at room temperature after de-chlorination. Each blue rayon adsorbate was treated as mentioned above for the Salmonella mutagenicity assay and the determination of PBTA-type mutagens.

Mutagenicity monitoring using Sep-Pak C18 cartridge column and Blue-Chitin column (Exp. 5): Water samples were collected at twelve sites shown in Fig. 2 on 3 June, 8 July and 16 October 2004, and 26 May, 21 July, 14 October and 9 December 2005. Each water sample (3 L) was passed through Sep-Pak C18 cartridge column and Blue-Chitin column at the flow rate of 10 mL/min at room temperature. Both columns were then washed with 20 mL distilled water and eluted with 2 mL of dimethyl sulfoxide. Both dimethyl sulfoxide eluates obtained here were used for mutagenicity assays with *S. typhimurium* YG1024 in the presence of S9 mix.

Salmonella mutagenicity assay—Ames test: The mutagenic activity of the extracts was measured in using *Salmonella typhimurium* YG1024, an *O*-acetyltransferase-overexpressing derivative of the frameshift strain TA98 (31), with metabolic activation according to the method previously reported (32,33). *Salmonella typhimurium* YG1024 was kindly provided by Dr. Takehiko Nohmi from the National Institute of Health Sciences, Tokyo, Japan. The S9 mix contained 25 μL of S9 (25 mg of protein/mL), prepared from livers of male Sprague-Dawley rats intraperitoneally administered phenobarbital and 5,6-benzoflavone, at a total volume of 500 μL. Dose-response curves of samples were obtained between 10^{-4}–0.2 g blue rayon equivalent (BRE) in the blue rayon hanging method, 2–20 mL water in blue rayon column method, 0.0625–1 g sediment, 0.0625–2 L drinking water, and 20–150 mL water in the Sep-Pak C18 cartridge and Blue-Chitin column method, respectively, per plate. Mutagenic activities of test samples were calculated from the linear portions of the dose-response curves obtained with three or four nontoxic doses, and duplicate plates in two independent experiments, and results are the mean of two independent experiments. The positive controls were 2-aminoanthracene (0.1 μg/plate) and Trp-P-2 (0.01 μg/plate) in YG1024 with S9 mix. The mutagenic potencies were expressed as revertants per one g of BRE or one L of water. A positive result was refined as a reproducible and dose-related response that at least induced a two-fold increase in revertants over the control. Mutagenic activities of authentic PBTA-type mutagens, calculated from linear portions of the dose-response curves in this study were as follows: PBTA-1, 883; PBTA-2, 786; PBTA-3, 1,279; PBTA-4, 2,396; PBTA-5, 723; PBTA-6, 487; PBTA-7, 1,430 and PBTA-8, 2,213 revertants per ng.

Purification and quantification of PBTA-type mutagens by HPLC: PBTA-type mutagens were purified by HPLC on reverse-phase columns, then quantified by HPLC with UV and an electrochemical detector according to the method described previously (16, 20–22). The residue dissolved in 75% methanol was fractionated by HPLC using a Nanospace SI-1 chromatograph (Shiseido, Tokyo, Japan) on a semi-preparative TSK-GEL ODS-10A column (10 μm particle size, 7.8 × 300 mm; Tosoh Corp., Tokyo, Japan) in purification step 1. The mobile phase of 80% methanol was pumped in isocratically at a flow rate of 1.6 mL/min at ambient temperature, and eluates containing corresponding authentic PBTA-type mutagens were collected. The eluates were then reduced to 400 μL. The condensed eluate was further purified with a reverse-phase CAPCELL PAK C18 (UG120, 5 μm, 4.6 × 150 mm, Shiseido) in purification step 2. A mobile phase of 25–42% acetonitrile in 25-mM H_3PO_4/Na_2HPO_4 (pH 6.5) was pumped in at a flow rate of 0.8 mL/min at 35°C. The absorption of the eluate at 260 nm was monitored and the fractions corresponding to eight PBTA-type mutagens were separately collected and reduced to 400 μL. The condensed fraction was finally analyzed on a reverse-phase YMC-Pack ODS A column (5 μm, 4.6 × 250 mm, YMC Co. Ltd. Kyoto, Japan) with an UV detector (260 nm, Shimadzu Co. Ltd. Kyoto, Japan) and an electrochemical detector (900 mV, Irica 985, Kyoto, Japan) in a determination step. The mobile phase of 20–45% acetonitrile in 25-mM H_3PO_4/Na_2HPO_4 (pH 2.0) was pumped in at a flow rate of 0.8 mL/min at 35°C. The eluates were also monitored for the structural confirmation with the Shiseido nanospace SI-1 photodiode array detector. In cases where it was not possible to perform definitive structural confirmation, eluates were analyzed further on a YMC-Pak ODS A column with a mobile phase of 25–50% acetonitrile in 20-mM acetic acid/sodium acetate (pH 4.7) at a flow rate of 0.8 mL/min at 35°C.

Results and Discussion

Salmonella mutagenicity of blue rayon extracts from sewage effluents and river waters collected between 1996 and 2005 (Exp. 1): Thirty-nine effluent samples were collected at outlets of two sewage plants (Site U-1 and KA-1 in Fig. 2); 47 river water samples were collected at sites located below sewage plants (Site N-1 and N-2 in Fig. 2); and 47 river water samples were collected at their downstream sites (Site KA-2 and Y-2 in Fig. 2) in the Yodo River system, Japan, between 1996 and 2005, using the blue rayon hanging method, which gives a one-day exposure. Table 2 summarize the mutagenic potency values analyzed by the *Salmonella* mutagenicity assay in strain YG1024 with S9 mix

Table 2. Mutagenicity of sewage effluents and river waters collected from the Yodo River system

Sampling date	Salmonella YG1024 with S9 mix (revertants/g BRE)					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
10 March 1996	—	9,100	16,400	76,300	27,400	17,700
15 June 1996	—	108,800	303,200	165,600	340,100	—
21 July 1996	—	366,600	53,500	455,700	339,500	10,900
18 September 1996	—	185,200	47,600	859,400	606,000	83,600
13 May 1997	—	—	184,000	501,500	670,200	—
13 June 1997	—	—	12,900	565,500	1,095,000	66,000
25 July 1997	—	—	24,800	16,200	303,800	5,500
7 October 1997	2,898,400	—	8,700	3,500	93,200	4,900
20 October 1999	418,100	280,400	203,600	—	450,600	23,300
9 November 1999	129,800	943,500	—	116,400	171,800	18,200
19 July 2000	702,400	549,400	8,900	23,300	170,500	2,600
8 September 2000	49,600	212,300	6,800	1,400	44,000	2,500
28 March 2001	546,500	885,900	75,100	810,400	413,000	556,200
29 March 2001	552,800	2,073,700	92,800	528,300	929,700	1,075,200
30 May 2001	593,300	618,900	135,700	720,100	2,194,500	86,400
29 May 2002	586,800	30,700	95,000	3,600	369,200	12,800
30 May 2002	698,800	33,400	98,100	3,600	760,000	21,400
31 May 2002	817,100	24,900	45,700	3,000	877,600	11,000
6 September 2002	460,100	143,900	30,300	9,100	253,100	7,600
12 September 2002	614,400	132,500	61,100	3,200	258,400	13,200
15 January 2004	63,500	103,800	37,700	—	65,800	42,000
16 July 2004	186,000	29,600	36,200	—	174,800	6,300
21 December 2004	176,700	205,700	236,200	4,900	73,000	57,000
21 July 2005	266,500	178,600	84,100	nd*	128,800	13,600
13 October 2005	136,200	77,000	42,500	28,000	87,400	50,400
Total number	18	21	24	22	25	23
Average	726,700	394,300	82,500	233,300	435,900	95,100
Median	586,800	185,200	53,500	28,000	303,800	17,700
SD	757,400	522,800	79,900	306,400	474,000	241,800
Maximum	2,898,400	2,073,700	303,200	859,400	2,194,500	1,075,200
Percentage with ''extreme mutagenicity''†	89%	71%	21%	41%	76%	9%

Samples were collected by blue rayon hanging method between 1996 and 2005.
—: Data are not available.
*not detected (< 400 revertants/g BRE).
†Percentage of samples with ''extreme mutagenicity'' among total numbers.
''extreme mutagenicity'': >100,000 revertants/g BRE according to mutagenic potency classification by Ohe *et al.* (4).

calculated from the linear regression equation in dose-response effect of blue rayon extracts. All but one sample was mutagenic, and the mutagenic activities fluctuated widely among samples. An average mutagenicity of the total 133 samples was 278,300 rev/g BRE with a maximum of 2,898,400 rev/g BRE and a median value of 98,100 rev/g BRE. Average mutagenicities of sewage effluent samples at Site U-1 and KA-1 were 726,700 and 394,300 rev/g BRE; those of river water samples at Site N-1 and N-2 located below sewage plants were 233,300 and 435,900 rev/g BRE; and those of downstream river waters at Site KA-2 and Y-2 were 82,500 and 95,100 rev/g BRE, respectively. The results showed that levels of mutagenic potency of sewage effluents (Site U-1 and KA-1) and river water samples at sites located below sewage plants (N-1 and N-2) were remarkably higher than those for river water samples collected from their downstream sites (Site KA-2 and Y-2). We (4) have classified the mutagenic potencies of samples obtained from the combination of the blue rayon hanging method as a collecting method and YG1024 strain as a bioassay system as low, moderate, high and extreme mutagenic activity. Based on this scheme, 50% of all samples had extreme mutagenicity, with more than 100,000 rev/g BRE. Samples from sewage plants and river waters at sites located below sewage plants were classified as extreme mutagenicity in high frequency as shown in Table 2. Exceedingly high mutagenicities, at 1,000,000 or more revertants per gram BRE, were found in samples collected at Site U-1 (Uji River) in October 1997, Site KA-1 (Katsura River) in March 2001, Site N-2 (Nishitakase River) in June

Table 3. Levels of PBTA-1 and PBTA-2 and their contribution ratio to the mutagenicity of water samples in *S. typhimurium* YG1024 with S9 mix

Sampling date	Levels of PBTA-1/ng per g blue rayon equivalent					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
15 June 1996	—	7.9 (6.4)	1.0 (0.3)	104.5 (55.7)	24.4 (6.0)	nd (0)
18 September 1996	—	3.2 (1.5)	2.0 (3.7)	78.5 (8.1)	29.4 (4.3)	3.0 (3.2)
25 July 1997	—	—	2.4 (8.5)	0.5 (2.7)	1.0 (0.3)	0.2 (3.2)
7 October 1997	4.3 (0.1)	—	0.7 (7.1)	0.3 (7.6)	1.0 (0.9)	0.4 (7.2)
20 October 1999	nd (0)	nd (0)	nd (0)	—	nd (0)	—
19 July 2000	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
28 March 2001	7.6 (1.2)	53.7 (5.4)	7.4 (8.7)	76 (8.3)	211 (42.6)	3.5 (0.6)
29 March 2001	7.5 (1.2)	63.0 (2.7)	11.3 (10.8)	268 (44.8)	235 (22.3)	2.8 (0.2)
30 May 2002	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
31 May 2002	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
16 July 2004	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)	nd (0)
21 December 2004	nd (0)	9.5 (4.1)	nd (0)	nd (0)	nd (0)	nd (0)
21 July 2005	nd (0)	10.5 (5.2)	nd (0)	nd (0)	nd (0)	nd (0)
13 October 2005	nd (0)	8.2 (9.6)	nd (0)	nd (0)	nd (0)	nd (0)
Number	11	12	14	13	14	12
Average	1.76 (0.2)	13.8 (2.9)	1.77 (2.8)	40.6 (9.8)	35.8 (5.5)	0.83 (1.1)
Median	0	8.1	0	0	0	0
SD	3.14	21.3	3.39	78.1	80	1.39
Range	nd–7.6 (0–1.2)	nd–63.0 (0–9.6)	nd–11.3 (0–10.8)	nd–268 (0–44.8)	nd–235 (0–42.6)	nd–4.8 (0–7.2)
	Levels of PBTA-2/ng per g blue rayon equivalent					
	Uji River	Katsura River		Nishitakase River		Yodo River
	U-1	KA-1	KA-2	N-1	N-2	Y-2
15 June 1996	—	25.2 (18.2)	18.6 (4.8)	101.8 (48.3)	114.4 (26.4)	nd (0)
18 September 1996	—	15.1 (6.4)	4.0 (6.6)	84.0 (7.7)	50.1 (6.5)	10.1 (9.5)
25 July 1997	—	—	15.7 (49.8)	2.1 (10.2)	20.1 (5.2)	nd (0)
7 October 1997	138.8 (3.8)	—	2.2 (19.9)	1.1 (24.7)	4.0 (3.4)	nd (0)
20 October 1999	154.0 (29.0)	109.0 (30.6)	79.0 (30.5)	—	200.0 (34.9)	—
19 July 2000	54.1 (6.1)	54.8 (7.8)	5.5 (48.6)	2.3 (7.8)	6.6 (3.0)	nd (0)
28 March 2001	31.9 (4.6)	279.2 (24.8)	33.8 (35.4)	83.9 (8.1)	109.6 (20.9)	19.6 (2.8)
29 March 2001	75.0 (10.7)	883.1 (33.5)	27.5 (23.3)	67.9 (10.1)	136.0 (25.9)	26.8 (2.0)
30 May 2002	61.8 (7.0)	11.0 (25.9)	9.4 (7.5)	2.4 (52.4)	158.0 (14.2)	21.5 (79.0)
31 May 2002	97.0 (9.3)	16.2 (51.1)	5.0 (8.6)	1.6 (41.9)	54.6 (4.9)	7.9 (56.4)
16 July 2004	nd (0)	22.2 (59.0)	36.5 (79.3)	nd (0)	184.3 (82.9)	nd (0)
21 December 2004	49.2 (21.9)	242.1 (92.5)	106.0 (35.3)	nd (0)	51.6 (55.6)	46.0 (63.4)
21 July 2005	173.5 (51.2)	100.7 (44.3)	31.4 (29.3)	nd (0)	98.9 (60.4)	nd (0)
13 October 2005	140.3 (81.0)	102 (104)	12.9 (23.9)	nd (0)	48.4 (43.5)	12.9 (20.1)
Number	11	12	14	13	14	13
Average	88.7 (20.4)	155.1 (41.5)	27.7 (28.7)	26.7 (17.6)	88.3 (27.7)	11.1 (17.9)
Median	75	77.8	17.2	2.1	76.8	7.9
SD	56.1	245.9	30.2	40.6	64.5	14.1
Range	nd–173.5 (0–81.0)	11.0–883.1 (7.8–104)	2.2–106.0 (4.8–79.3)	nd–101.8 (0–52.4)	4.0–200.0 (3.0–82.9)	nd–46.0 (0–63.4)

—: Data are not available.
Figures in parenthesis show the contribution ratio (%) of PBTA-1 or PBTA-2 to total mutagenicity.

1997 and May 2001, and Site Y-2 (Yodo River) in March 2001. The fact that extreme mutagenicity was also detected at the downstream site with a frequency of 9% was a serious problem, because there are several sources for drinking water supply upstream and downstream from Site Y-2.

Maruoka *et al.* (34,35) demonstrated that XAD resin extracts collected in 1982 and 1983 at sites downstream from sewage plants along the Katura River and the Nishitakase River, exhibited consistently strong mutagenic activity to strain TA1538 and TA98 in the presence of S9. Since then, some researchers reported that these tributaries of the Yodo River system were polluted especially with potent frameshift-type indirect-

acting aminoarenes (5,6,12). In conclusion, the results in Table 2 demonstrate that the Yodo River system has been continually and heavily polluted with frameshift-type polycyclic planar mutagens and these mutagens have been released mainly from sewage plants located along the tributaries of the Yodo River system for many years.

Levels of PBTA-1 and PBTA-2 in blue rayon extracts from sewage effluents and river waters collected between 1996 and 2005 (Exp. 1): Table 3 summarizes the levels of PBTA-1 and PBTA-2 in all samples collected at six sites between 1996 and 2005. Determination of PBTA-1 and PBTA-2 was performed with 23 sewage effluent samples, 27 river water samples at sites located below sewage plants, and 26 downstream river water samples. The contributions of PBTA-1 and PBTA-2 to the total mutagenicity of water samples are also shown in parenthesis in Table 3. PBTA-1 was detected in 43% of the samples (33/76) and PBTA-2 was detected in 87% of the samples (66/76), and their levels among water samples fluctuated widely (PBTA-1; nd −268 ng/g BRE, PBTA-2; nd −883.1 ng/g BRE). Average levels of PBTA-1 in effluent samples (U-1 and K-1) were 1.76 and 13.8 ng per g BRE; those in river water samples at sites below sewage plants (N-1 and N-2) were 40.6 and 35.8 ng per g BRE; and those in their downstream river water samples (KA-2 and Y-2) were 1.77 and 0.83 ng per g BRE. On the other hand, average levels of PBTA-2 in sewage effluent samples (U-1 and KA-1) were 88.7 and 155.1 ng per g BRE; those in water samples at sites located below sewage plants (N-1 and N-2) were 26.7 and 88.3 ng per g BRE; and those in their downstream river water (KA-2 and Y-2) were 26.7 and 11.1 ng per g BRE, respectively.

The results showed that levels of PBTA-1 and PBA-2 in sewage effluent samples and river water samples at sites located below sewage plants were much higher than those for their downstream river water samples. PBTA-1 and PBTA-2 accounted for 6% and 26% on average, respectively, of the total mutagenicity in all samples analyzed. High levels of PBTA-1, with more than 100 ng/g BRE were detected in four samples from Site N-1 and N-2 (Nishitakase River) before 2001, but they were not detected at all after 2002 except for Site KA-1. On the other hand, such high levels of PBTA-2 with more than 100 ng/g BRE were detected in ten samples before 2001, and in eight samples after 2002. Furthermore, the average contribution (%) of PBTA-2 in samples

Table 4. Levels of PBTA-type mutagens and their contribution ratio of each PBTA-type mutagen to total mutagenicity of water samples

Sampling point	Sampling date	Levels of PBTA-type mutagens (ng /L)								Mutagenicity* (revertants/L)
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	
Nishitakase River (N-2)	12 November 1999	nd	7.45	1.46	2.55	nd	nd	0.13	0.04	32,300
	17 December 1999	nd	2.11	5.81	5.43	nd	nd	0.44	0.31	35,500
	28 September 2001	5.28	0.54	1.58	0.62	nd	nd	0.19	0.01	19,000
	Average	**1.76**	**3.37**	**2.95**	**2.87**	**0**	**0**	**0.25**	**0.12**	**28,900**
Katsura River (KA-1)	12 November 1999	nd	1.84	3.04	6.55	nd	1.14	0.24	0.2	41,600
	17 December 1999	nd	2.79	1.36	5.29	nd	2.54	0.32	0.18	49,400
	28 September 2001	nd	2.84	6.56	1.66	nd	nd	0.06	0.03	21,000
	Average	**0**	**2.49**	**3.65**	**4.5**	**0**	**1.23**	**0.21**	**0.14**	**37,300**
Uji River (U-1)	12 November 1999	nd	0.8	1	3.4	nd	0.24	0.26	0.26	50,800
	17 December 1999	nd	1.52	4.08	1.47	nd	nd	0.04	0.04	33,300
	28 September 2001	nd	0.57	1.76	1.78	nd	0.52	0.31	0.03	24,300
	Average	**0**	**0.97**	**2.28**	**2.22**	**0**	**0.25**	**0.2**	**0.11**	**36,100**
		Contribution ratio to total mutagenicity (%)*								
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	Total
Nishitakase River (N-2)	12 November 1999	0	18.1	5.8	18.9	0	0	1.2	0.2	44.2
	17 December 1999	0	4.7	20.9	36.6	0	0	3.7	1.7	67.6
	28 September 2001	24.5	2.2	10.6	7.8	0	0	3	0.1	48.2
	Average	**8.2**	**8.3**	**12.4**	**21.1**	**0**	**0**	**2.6**	**0.7**	**53.3**
Katsura River (KA-1)	12 November 1999	0	3.5	9.4	37.7	0	1.3	1.7	0.9	54.5
	17 December 1999	0	4.4	3.5	25.7	0	2.5	1.9	0.7	38.7
	28 September 2001	0	10.6	40	34.6	0	0	0.2	0.1	85.5
	Average	**0**	**6.2**	**17.6**	**32.7**	**0**	**1.3**	**1.3**	**0.6**	**71.4**
Uji River (U-1)	12 November 1999	0	1.2	2.5	10.6	0	0.2	2.8	1	18.3
	17 December 1999	0	3.6	15.7	16	0	0	0.8	0.2	36.3
	28 September 2001	0	2.1	10.7	20.3	0	0.5	4.4	0.3	38.3
	Average	**0**	**2.3**	**9.6**	**15.6**	**0**	**0.2**	**2.7**	**0.5**	**31.0**

*Mutagenicity towards *S. typhimurium* YG1024 with S9 mix.

Table 5. Estimated amounts of PBTA-type mutagens discharged from sewage plants into river waters

Sampling point*	Discharged effluents volume† ($\times 10\ m^3$/day)	Estimated amounts of PBTA-type mutagens discharged from sewage plants (g/year)‡								
		PBTA-1	PBTA-2	PBTA-3	PBTA-4	PBTA-5	PBTA-6	PBTA-7	PBTA-8	Total
Nishitakase River (N-2)	637	409	784	686	667	0	0	58	28	2,632
Katsura River (KA-1)	523	0	475	697	859	0	235	40	27	2,333
Uji River (U-1)	155	0	55	129	126	0	14	11	17	352
Total dischargd volume	1,315§	409	1,314	1,512	1,652	0	249	109	72	5,317

*N-2: The site located below sewage plants SP 1 and SP 2 as shown in Fig. 2.
KA-1: The outlet of SP 2 as shown in Fig. 2.
U-1: The outlet of SP 3 as shown in Fig 2.
†The discharged effluents volume was estimated from the treatment capacity of sewage plants (SP 1: 114, SP 2: 1,047 and SP 3: $155 \times 10^3\ m^3$/day). Discharged volume from SP 2 into KA-1 and N-2 was estimated to be fifty-fifty.
‡Figure is calculated from average levels of PBTA-type mutagens (ng/L) as shown in Table 4 and discharged effluents volume (m^3/day) as shown in this Table.
§This volume occupy more than 70% of sewage treatment capacity of six sewage plants, which their discharges flow into Nishitakase, Katsura and Uji rivers.

Table 6. Mutagenicity of river sediments in the Yodo River system

Sampling site	Sampling date	Extraction solvent	YG1024, +S9 mix (revertants/g dry basis)
U-1	31 May 2002	Methanol	943*
		Methanol:benzene (3:1)	1217
	6 Sep 2002	Methanol	1505
		Methanol:benzene (3:1)	1323
Y-2	31 May 2002	Methanol	233*
		Methanol:benzene (3:1)	111
	6 Sep 2002	Methanol	110
		Methanol:benzene (3:1)	110

*The sample was used for the determination of PBTA-type mutagens.

collected between 2002 and 2005 to the total mutagenicity was ~36%, which was higher than that the 16% between 1996 and 2001. This result demonstrates that PBTA-2 accounts for a large amount of the total mutagenicity of the Yodo River system during recent years.

Estimation of PBTA-type mutagens amounts discharged from sewage plants into river waters (Exp. 2): We estimated amounts of eight kinds of PBTA-type mutagens discharged from sewage plants into the Yodo River system. Water samples were collected at two outlets of sewage plants (U-1 and KA-1) and the site located below two sewage plants (N-2) on 1999 and 2001. Table 4 summarizes the levels of eight kinds of PBTA-type mutagens obtained by the blue rayon column method. All values were corrected for compound recoveries (56, 56, 42, 40, 50, 50, 40 and 46%, respectively for PBTA-1, PBTA-2, PBTA-3, PBTA-4, PBTA-5, PBTA-6, PBTA-7, and PBTA-8). As shown in Table 4, PBTA-2, PBTA-3, PBTA-4, PBTA-6, PBTA-7, and PBTA-8 were detected in most of the tested samples. Their average concentrations were 2.20, 2.96, 3.53, 0.49, 0.22 and 0.12 ng/L. PBTA-1 was detected only in one sample. PBTA-5 was below the detection limit in all samples analyzed. Salmonella mutagenicity assay results for all samples are also shown in Table 4, with the relative contributions of PBTA-type mutagens to total mutagenicity. All samples showed significant potency in strain YG1024 with S9 mix. Seven kinds of PBTA-type mutagens except for PBTA-5 accounted for 31–71% of the total mutagenicity, with an average contribution of 52%. The compounds that made large contributions to the total sample mutagenicity were PBTA-4, PBTA-3 and PBTA-2, accounting for 23, 13 and 6%, respectively. In addition, we estimated the amount of PBTA-type mutagens discharged into the river waters from three sewage plants according to data on average levels of PBTA-type mutagens and discharged effluents volume from three sewage plants (SP 1, SP 2 and SP 3). Table 5 shows estimated amounts of PBTA-type mutagens discharged from sewage plants. In conclusion, ~5.0 kg of seven kinds of PBTA-type mutagens were discharged from sewage plants into the rivers studied here. In addition, the major part of the mutagenicity of river water in the Yodo River system was due to PBTA-type mutagens formed by chemical

modification of industrial discharges in sewage plants, although the compounds in these discharges were diluted or decomposed while moving down the Yodo River system.

Salmonella mutagenicity and levels of PBTA-type mutagens in river sediment and drinking water (Exp. 3 and 4): In order to evaluate the genotoxic burden of river sediments, we performed the determination of mutagenicity and levels of PBTA-type mutagens in sediment samples collected at two sites. The extracts of sediments collected at Site U-1 located below the sewage plant showed mutagenicity towards YG1024 with S9 mix, and the extract possessed the same frameshift-type mutagenicity as the sewage effluents and river waters (Table 6). The revertants produced with the extracts at Site U-1 were about ten times greater, on average, than those from its downstream Site Y-2. However, the potency of the sediment sample from Site U-1 was not unexpectedly so high considering the result mentioned above showing water samples from Site U-1 had ''extreme mutagenicity'' in the highest frequency as shown in Table 2 and PBTA-type mutagens were discharged from sewage plant (SP 3) as shown in Table 5, although they were higher compared with the study of the Po River, Italy (50–660 revertants/g, YG1024, +S9 mix) (36), and they were much lower compared with the Cristais River, Brazil (37,000 revertants/g, YG1041, +S9 mix) (11). Furthermore, only trace levels of PBTA-2 and PBTA-3 were detected in sample at Site U-1 (PBTA-2; 0.08 ng and PBTA-3; 0.02 ng, respectively, per g) and they accounted for less than 10% of the total mutagenicity. It was suggested that PBTA-type mutagens in water might not easily accumulate in the river sediment, though aromatic compounds with three or more fused rings are known to be adsorbed to particle matter or bottom sediments in aquatic environments (28,36-38).

The Yodo River system flows through the prefectures of Kyoto and Osaka and it is a source of drinking water for more than seventeen million people living in the downstream area. There are several sources for drinking water that are located downstream and upstream from Site Y-2 shown in Fig. 2. We tried to analyze the mutagenicity and the level of PBTA-type mutagens in drinking water that comes from the source located below Site Y-2. Result showed that no significant mutagenicity was detected (<400 revertants/L), and the levels of PBTA-type mutagens were also less than the detection limit (<0.02 ng/L) in these drinking water samples (Data are not shown). The present result showed the absence of mutagenic polycyclic aromatic compounds involving PBTA-type mutagens in drinking water.

Mutagenicity monitoring of the Yodo River system using Sep-Pak C18 column and Blue-Chitin column

Table 7. Mutagenicity monitoring of the Yodo River system using Sep-Pak C18 cartridge and Blue-Chitin column methods

Column	Mutagenicity in YG1024 with S9 mix (revertants/L)											
	Uji River		Katsura River		Nishitakase River		Kizu River	Yodo River				
	U-1	U-2	KA-1	KA-2	N-1	N-2	KI-1	Y-1	Y-2	Y-3	Y-4	Y-5
Sep-Pak C18	26,200±9,500	5,200±4,100	40,200±36,800	12,700±6,200	1,500±800	15,600±7,800	800±600	5,600±3,400	3,000±2,100	2,200±2,200	1,600±1,300	1,600±1,100
Blue-Chitin	11,400±7,400	2,600±1,400	21,100±16,000	6,400±4,100	1,900±500	11,600±6,400	1,500±1,500	4,100±2,300	2,100±1,400	1,200±1,300	2,000±1,300	1,900±1,900

Figure means average±SD (n=7, 4 at N-1).

method (Exp. 5): Water samples were collected at 12 sites 7 times along the Yodo River system as shown in Fig. 2 between June 2004 and December 2005 (4 times at N-1). The Yodo River system is composed mainly of the Uji River, Katsura River, Kizu River, Nishitakase River, and the rest. Large volumes of effluents from many sewage plants, treating industrial, agricultural and domestic wastes, located along the Yodo River system are released into these rivers. Seventy-two samples among 81 (89%) were mutagenic using the Sep-Pak C18 cartridge column method, and 75 samples among 81 (93%) were mutagenic using the Blue-Chitin column method. The average mutagenicity value determined by the Sep-Pak C18 cartridge method was 10,000 revertants/L; it was 5,800 revertants/L for the Blue-Chitin column method (Table 7). For the highly contaminated sampling sites mentioned above (U-1, KA-1, N-1 and N-2), the average mutagenicity values were 23,200 and 12,600 revertants/L by the Sep-Pak C18 and Blue-Chitin methods, respectively. In less contaminated sites (U-2, KA-2, KI-1, Y-1, Y-2, Y-3, Y-4 and Y-5), these values were 4,100 and 2,700 revertants/L, respectively. In this mutagenicity monitoring, the levels of PBTA-type mutagens were not analyzed. To clarify the actual contribution ratio against the total mutagenicity should be clarified in future work. But, there was a significant correlation between Sep-Pak C18 cartridge column method and Blue-Chitin column method ($n = 81$, $r^2 = 0.81$, $p < 0.001$). This result shows that there exists not only planar polycyclic aromatic mutagens with more than three rings, but also a wide range of chemical mutagens including polar and apolar mutagens in the Yodo River system. Furthermore, these chemicals are released mainly from sewage plants into the Yodo River system. More studies will be needed to elucidate the unknown mutagens beyond the PBTA-type mutagens and to determine of the sources of such mutagens. Rigid regulation also should be made to reduce the levels of mutagenicity.

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