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Genotoxicity of Water Extracts from Sewage Effluents in the Kanagawa Prefecture, Japan Using the Novel *umu* Tester Strain

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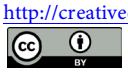

Abstract

The genotoxic activities of effluents from drainage water treatment plants were examined by using the novel *umu* tester strain NM8001, which lacks *MutMst* genes. To enhance the sensitivity of the LacZ assay, a BugBuster mix protein extraction reagent and TokyoGreen-β Gal for a fluorescence-galactosidase substrate were applied. Of the 24 sampling locations present in Kanagawa prefecture, Japan, water extracts from nine sampling points showed apparent genotoxic activities without metabolic activation. In contrast, water extracts from the upper sites of these water treatment plants did not show any significant genotoxic activities. The selected samples with genotoxic activity did not show significant mutagenicity toward Ames strains TA98 and TA100. Genotoxicity was also well correlated with the activity of a classical *umu* strain of TA1535/pSK1002; these findings indicate that the genotoxicity induced by oxidative damage was not a significant component of the genotoxicity.

Keywords

Genotoxic Compounds, Mutagenic Compounds, Surface Water Contamination, *umu* Tester Strain

1. Introduction

The contamination of surface water causes severe public health problems and probable adverse effects on aquatic biota [1] [2] [3]. The presence of contaminants derived from daily human activity in the natural environment, including pharmaceutical and personal care products (PPCPs), microplastics, and endocrine-disrupting materials, is a newly emerged global problem that must be

solved from a holistic perspective [3] [4] [5]. The individual amounts of these materials present in the environment are generally quite small. However, the net adverse effects, especially on aquatic biota, are still unknown. These materials are released into surface water and reach river water via water treatment plants.

In some cases, agricultural and industrial wastewater is treated together in the same sewage treatment plant as commercial chemicals. These circumstances cause effluent chemical compounds to be much more complex following chemical and biological water treatment, resulting in a complicated mixture of chemicals in the drainage water that reaches a river [6] [7]. As the complexity of the chemical matrix of effluent water makes the identification of all chemical compounds a challenge, the estimation of biological activities for the evaluation of the presence of toxic materials is also an effective procedure to ascertain river water quality [8].

Genotoxicity can be used as a biological estimation to understand the adverse effects on river biota affected by drainage water. A previous study demonstrated that some rivers have been highly contaminated with mutagenic/genotoxic compounds [9]. To date, many studies have evaluated the genotoxic and/or mutagenic activity of river water extracts in Japan [10] [11]. Several Japanese rivers, such as the Nishitakase and Asuwa rivers, contain mutagenic phenyl benzotriazole type (PBTA) compounds that can be adsorbed on blue rayon adsorbents [12]. PBTA requires the metabolic activation system of the S9 mix to show mutagenicity. The presence of a novel PCB mutagen has been reported in river locales in Wakayama city [13]. Recent studies have also demonstrated the genotoxic activities of the effluent of water treatment plants by using the *umu* test, which is the conventional genotoxicity assay system that uses Salmonella [14] [15] [16].

The *umu* test is a widely used genotoxicity test because its conventional procedures require a relatively small number of samples [17]. The *umu* strain contains *the umuC-lacZ* fusion gene that utilizes a mechanism based on an SOS system derived from DNA damage, resulting in the formation of LacZ proteins [18]. The genotoxicity assay of the *umu* test is generally used as a counterpart to the Ames assay. Owing to the safe handling and the short assay period, it is possible to treat many samples at once. In the *umu* test, the classical TA1535/pSK1002 strain can detect several direct-acting mutagens, such as nitroarenes. Recently, glutathione *S*-transferase overproducing strains have been detected using mutagens activated by GST and GSH. Some halogen compounds can also be detected by this strain [18].

Recently, the BugBuster Master mix (B.M. mix) of a protein extraction reagent was found to enhance assay activity. This was likely a result of the alternative use of sodium dodecyl sulfate (SDS) to destroy the cell surface and extract LacZ proteins [16]. The presence of genotoxic compounds in the water treatment plant effluent was shown using the B. M. mix. When compared with a water treatment plant that utilizes the ozonation system, the traditional coagulation and filtration system did not sufficiently remove genotoxic compounds [16].

Similar trends were also observed in other studies using the Ames system [19].

Kanagawa prefecture, located in the South Kanto area in Japan, is home to approximately 9 million people. The ratio of urban land use in the prefecture; for example, the proportion of roads and residential spaces in the total prefecture is very high, at approximately 35.7%. On a national basis, this ratio is only approximately 8.8%. Several water treatment plants in Kanagawa are located around relatively small-sized rivers, and this affects the small habitats living in the river.

We evaluated river water extracts with the new NM8001 strain, which has recently been developed for the facile detection of oxidative DNA damage [11]. Our research groups have previously evaluated the effectiveness of this strain for the detection of photo-induced genotoxicity of several compounds. Despite the numerous studies on river water drainage, there have been relatively few reports of the genotoxic effects of river water [20] (Kameya *et al*., 2011). In our study, we evaluated 60 points from 20 rivers located in the Sagami Bay area. The Sagami region contains scattered industrial areas owing to the high accessibility of water sources from nearby small rivers. The main river in the region is the Sagami River, which flows through Sagami Bay.

2. Methods

The *umu* strains of NM8001 were developed by Y. Oda, and TokyoGreen-β Gal was obtained from Goryo-Kayaku (Hokkaido, Japan). The BugBuster protein extraction reagent was purchased from Millipore (MA, USA), and chlorophenol-β-galactopyranoside was purchased from Wako (Osaka, Japan). Other general reagents were of experimental grade and were used without further purification. The pH and conductivity of river water were measured by using Laqua Twin pH <pH-33B> and Cond <EC-33B> (Horiba, Kyoto, Japan), respectively.

2.1. Strains

For the *umu* test, *Salmonella typhimurium* NM8001 and TA1535/pSK1002 were used. NM8001 is a derivative of YG3001 [11]. The latter strain lacks mutMst and is highly sensitive to oxidative damage [21]. In the Ames assay, classical *S. typhimurium* TA98 and TA100 were used. The TA98 and TA100 strains were kindly provided by Dr. Nohmi at the National Institute of Health Science, Japan.

2.2. Sampling

The sampling locations are illustrated in **Figure 1**. The sampling points were selected at the upstream and downstream sites of the presumed emission points of sewage effluents from the drainage of water treatment plants. We selected these effluent plants for easy accessibility to the sampling points in the Kanagawa Prefecture.

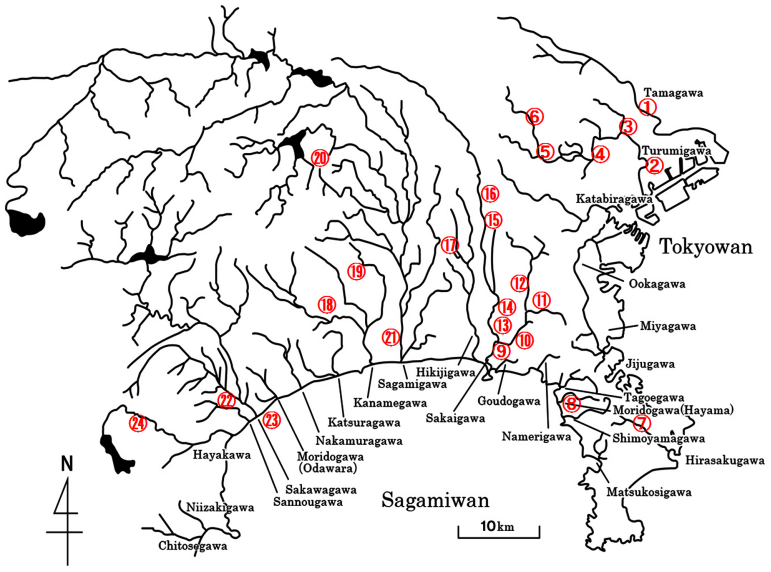

Figure 1. Sampling location in Kanagawa, Japan.

2.3. Extraction of River Water

Water samples (2 L) were collected using plastic bottles at the above sampling points from August 2018 to January 2019, and returned to the laboratory within the same day. The samples were filtered through a PES membrane filter (0.45 μM, Millipore Express Plus) and the filtered samples were then passed through an OASIS HLB column (500 mg). The retained materials were recovered with methanol (6 mL) and the solvent was evaporated to dryness. Each sample was then dissolved in 100 μL of DMSO.

2.4. *umu* Test

The *umu* test was performed based on a modified microtiter plate method [17]. In total, 80 μL of the overnight culture solution in LB media was added to 8 mL of TGA medium and incubated for 2 h while stirring at 120 rpm using a seesaw shaking water bath. When the OD_{600} value was confirmed to be equal to 0.2, 98 μL of the incubated bacterial medium was placed into each well of the 96-well titer plate and combined with 2 μL of water extract in DMSO that had previously been diluted with DMSO. The 96-well plate was then incubated at 37˚C with shaking. After 2 h, the OD_{595} value was measured by using a microplate reader (Tecan Infinite 200). A 10 μL aliquot of the Salmonella solution in the well was then transferred to another 96-well plate that had been pre-loaded with 90 μL of Z-buffer, 50 μL of 10% SDS solution or 50 μL of BugBuster reagents, and 10 μL of *β*-galactosidase detection reagents for CPRG (4 mg/mL in phosphate buffer at pH 6.8) or TokyoGreen-*β*-Gal (10 mM in phosphate buffer at pH 7.4). The mixed solution was incubated for 20 min at 37˚C, and the reaction was stopped by the addition of 1 M Na_2CO_3 solution (100 μL). In the case where CPRG was used as a coloring reagent, the OD_{570} value was measured. When using Tokyo-Green reagents, the emission wavelength fluorescence at 535 nm with an excitation

wavelength of 485 nm was used. The LacZ unit was obtained using Equation (1), and the "fold induction" was calculated as the ratio of LacZ of the sample divided by the value of the blank sample,

$$\text{lacZ unit} = 1000 \times \frac{\text{OD}_{570} \text{ or F535}}{t \times v \times \text{OD}_{595\text{nm}}} \quad (1)$$

where t was the indicated incubation time of 0.5 h, and v was the ratio of bacterial solution to the whole reaction volume. OD_{570} was the absorbance value at 570 nm for CPRG, and F535 is the fluorescence intensity obtained from TokyoGreen. The fold induction ratio was obtained by dividing the value of a known sample by the value of a blank sample [17].

2.5. 4-NQO Equivalent

To compare genotoxicity strength, 4-NQO sample equivalents were calculated using the 4-NQO-LacZ dose-response curves. As the typical S-type curve of the 4-NQO-LacZ dose-response curve had been previously obtained, we used Hill's equation to obtain the approximate straight-line dose-response curve.

$$A = \alpha / (\alpha_{\max} - \alpha) \quad (2)$$

where α is the LacZ unit obtained from a selected concentration of extracted river samples, and $\alpha_{\max}$ is the maximum LacZ unit obtained from the 4-NQO treatment.

$$\log A = n \log S + Ks \quad (3)$$

where S is the concentration of 4-NQO (4-NQO equivalents obtained from the A values of the extracted sample), and Ks is the equation constant.

3. Results and Discussion

3.1. TokyoGreen and Bug Buster Mix Treatments

A novel extraction procedure using the B. M. mix, a useful protein extraction detergent, was recently introduced by Tian *et al.* [16]. This procedure proposes a sufficient elevation of the LacZ value at lower concentrations of genotoxicant when compared with the SDS extraction used for the classical extraction method. The efficiency of the novel procedure in our lab was initially checked and compared with the classical SDS extraction method. As the B. M. mix treatment time was 20 min (in accordance with our reagent suppliers' instructions), the incubation time of 1 h for SDS treatment in an original manner was also changed to 20 min. We also observed similar trends of higher LacZ activity with the B. M. mix, especially at lower concentrations. This was likely due to the need to maintain the stability of β-galactosidase. TokyoGreen-β Gal is also known to be a cell penetration reagent, as it can penetrate the inside of mammalian cells; galactosidase activity can be measured without the use of protein extraction reagents. Although the ability for TokyoGreen-β Gal to permeate cell membranes is well established, this is not the case for bacterial cell surfaces. We observed insufficient LacZ elevation when only TokyoGreen-β Gal reagent was used without

protein extraction reagents. However, the addition of TokyoGreen-β Gal following B. M. mix treatment resulted in the highest β-galactosidase activity among the procedures used in our study (Figure 2).

3.2. River Water Sampling

River water was collected from the indicated sampling sites shown in Figure 1 between 2018/8/6 and 2019/1/7. The pH and conductivity data, along with water temperature, are shown in Table 1. The pH range was from 5.9 to 8.3. Almost all samples had a pH of approximately 7.4. The temperature of the upper site was lower than that of the downstream site by approximately 0.2˚C - 1.5˚C. The conductivity of the river sample was approximately 300 - 800 μS/cm; however, in some cases much higher conductivities were observed (primarily due to seawater mixing effects).

3.3. *umu* Test of River Water Extract

We examined the genotoxic activities of the water extract from 24 drainage water treatment plants in Kanagawa prefecture. River water was collected from the upstream and downstream sites of drainage water treatment plants. At five of these plants, only the water at downstream sites was collected owing to technical difficulties with the collection of upstream water. Two liters of water samples were collected at each site, and the sample was treated with the Oasis HLB cartridge column as an organic compound adsorbent. Methanol was used as the eluent, and the adsorbed materials were dissolved in 100 μL of DMSO. The typical dose-response curves of the river water extracts are shown in Figure 3. The maximum dose of the extracted sample in one well of the 96-well titer plate was equivalent to 40 mL of river water. At sampling points 12 and 16, the water extracts at the downstream site exhibited a typical dose-dependent increase in LacZ. Although the obtained LacZ value was lower at sampling point 14, a dose-dependent increase in LacZ was also observed. When compared with similar trends of LacZ increases at the downstream sampling positions, the activity at the upstream sites of the sampling points was negligible among the doses tested in our study. All assay data obtained from the 24 sites are shown in Figure 5. We calculated 4-NQO concentration equivalents from the LacZ values of the maximum dose of 40 mL river water equivalent. Lac Z values of 1.25 μg/mL 4-NQO

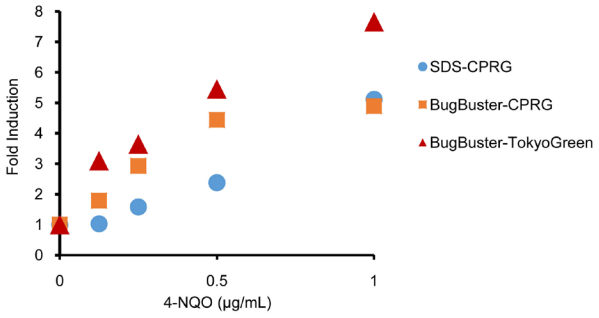

Figure 2. Comparison of several β-galactosidase extraction reagents.

Table 1. Sampling points and water properties.

Sampling point	Longitude/ latitude	Location relative to drainage water plants	Sampling date	pH	Temperature (˚C)	Conductivity (μg/mL)
1	35.5908/139.6460	Upper stream	20181205	8.0	18.8	390
		Down stream	20181205	8.3	20.0	420
2	35.5187/139.6797	Upper stream	20181205	7.4	18.8	8600
		Down stream	20181205	7.4	19.0	10600
3	35.5214/139.6192	Upper stream	20181205	7.5	20.4	370
		Down stream	20181205	7.4	20.6	410
4	35.5205/139.6193	Upper stream	20181205	7.3	19.3	340
		Down stream	20181205	7.3	19.7	380
5	35.5436/139.6569	Upper stream	20181205	7.7	17.5	16700
		Down stream	20181205	7.1	21.4	8400
6	35.5840/139.4956	Upper stream	20181213	7.6	13.4	320
		Down stream	20181213	7.1	16.3	380
7	35.2523/139.6762	Upper stream	20181121	8.1	15.9	480
		Down stream	20181121	7.3	18.8	1550
8	35.2887/139.602	Down stream	20181121	7.4	20.8	470
9	35.3121/139.5131	Down stream	20181109	7.2	20.3	540
10	35,3457/139.5241	Upper stream	20181109	7.5	20.2	480
		Down stream	20181109	7.5	20.6	460
11	35.3688/139.5483	Upper stream	20181109	7.5	18.8	680
		Down stream	20181109	7.6	20.7	620
12	35.3809/139.5336	Upper stream	20181109	7.3	18.2	510
		Down stream	20181109	7.3	20.2	440
13	35.3566/139.4876	Upper stream	20181003	7.2	23.1	350
		Down stream	20181029	7.4	22.7	350
14	35.3603/139.4894	Upper stream	20181003	7.5	21.6	310
15	35.4598/139.4713	Upper stream	20180913	6.0		350
		Down stream	20180913	5.9		440
		Down stream	20181029	7.1	23.4	420
16	35.4942/139.4673	Upper stream	20180913	6.4		400
		Down stream	20180913	6.3		360
17	35.4310/139.4469	Upper stream	20181130	7.0	17.7	430
		Down stream	20181130	7.2	20.0	520
18	35.3644/139.2446	Down stream	20181130	6.6	22.4	830
19	35.3862/139.2960	Upper stream	20190107	8.3	13.7	260
		Down stream	20190107	7.8	15.5	320

Continued

20	35.4839/139.2861	Upper stream	20190107	7.6	11.5	240
		Down stream	20190107	7.3	16.0	390
21	35.3183/139.3767	Down stream	20181130	6.9	13.5	210
22	35.2690/139.1828	Upper stream	20180806			
		Down stream	20180806			
23	35.2787/139.1563	Down stream	20180806			
24	35.2540/139.0483	Upper stream	20181213	7.5	14.0	370
		Down stream	20181213	7.8	12.3	330

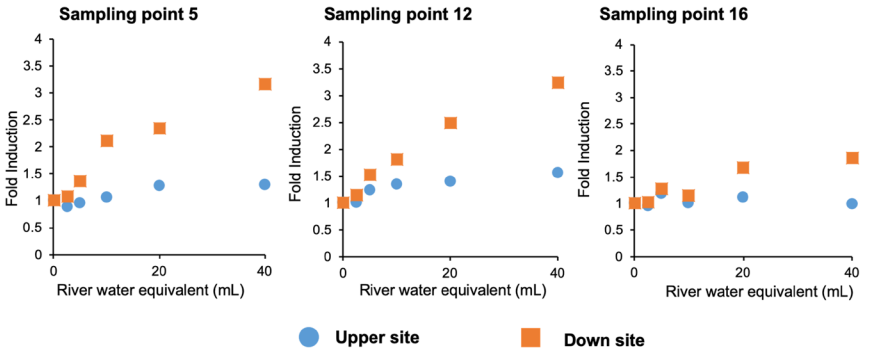

Figure 3. Typical LacZ enhancement at sampling points. Comparison of the data from the upper and lower effluent.

were approximately twice as high as that of the background level. The dashed line related to a 4-NQO concentration of 1.25 μg/mL is also shown in the same figure. As indicated in **Figure 4**, 4-NQO equivalent values of river water (40 mL) downstream of the treatment plants tended to display higher values when compared with those of the upper plant sites, except for sampling points 2, 10, and 24. Specifically, nine downstream sampling points (5, 6, 10, 11, 13, 15, 16, 18, and 19) showed values above 0.5 mg/L 4-NQO equivalents. Among the upstream sites, only sampling point 10 had a value above 0.5 mg/L 4-NQO equivalents. This was likely because the upper side of sampling point 10 flowed into the drainage water treated by sampling point 12. Sampling point 10 allowed us to observe the effects of the upper site on the sampling site.

3.4. Comparison of the Ames Test and Classical *umu* Tester Strain

As the Ames test includes a conventional assay to detect mutagens, we also examined the mutagenic activity of the selected water extracts for comparison with *umu* test data. Sampling points 11, 13, and 18 were also collected, and the same sample extracts were assayed with both the Ames and the *umu* test. Every resampling point that had shown positive *umu* responses in **Figure 5** had positive responses to LacZ production, indicating continuous contamination of genotoxic compounds around these sampling areas. However, TA98 and TA100 mutagenic activities were found to be quite low in the same sample. Only a slight

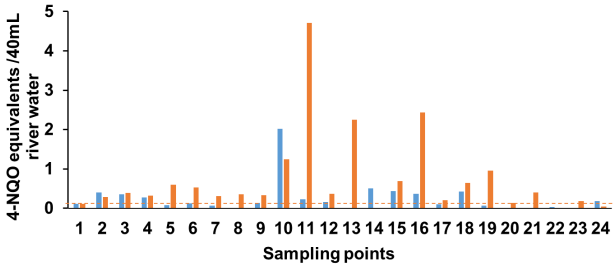

Figure 4. 4-NQO equivalents at each sampling point. Blue bar: upper site; orange bar: downstream site.

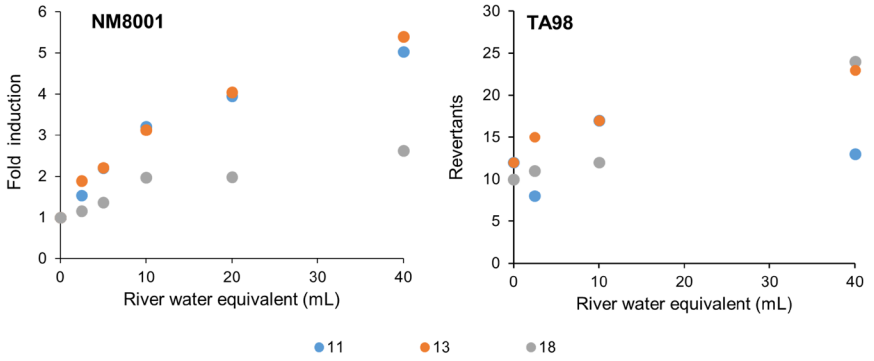

Figure 5. Comparison of genotoxic activities by the *umu* test and mutagenicity by the Ames test (TA98).

increase in dose-dependent revertants was observed. The same sample was also checked by YG3001 and YG1024, but the mutagenicity was quite low.

We also examined genotoxic activities using TA1535/pSK1002 as a classical strain, and a slight enhancement of LacZ induced by NM8001 when compared with TA1535/pSK1002 (**Figure 6**). Therefore, the *umu* test was sensitive for the detection of genotoxicants in the drainage water without the S9 mix, and also sensitive when compared with the Ames assay using TA98. Oxidative damage was not significant for the samples. These results indicated that the compounds in the wastewater contained genotoxic but not potent mutagenic compounds. We have shown that direct-acting genotoxicants were present in the local drainage water effluent, and that this can be adequately tested by the new *umu* tester strain NM8001 and the classical TA1535, but not by Ames tester strains.

We sought to elucidate genotoxic compounds during the course of our experiment. We have previously mentioned blue rayon extracts; however, our data showed that genotoxicants were not adsorbed explicitly on blue rayon [10]. MX, a well-known chlorination product derived from water treatment, is known to be highly mutagenic to TA98 [21]. Considering the low mutagenicity of water samples available in this study, the contribution of MX to genotoxic activities may be negligible. In laboratory-scale experiments, formaldehyde, furfural, carvone, glyoxal, and acrolein were produced as byproducts of ozonation or chlorination [22]. It is plausible that these short-lived genotoxic compounds were present in the effluent extract.

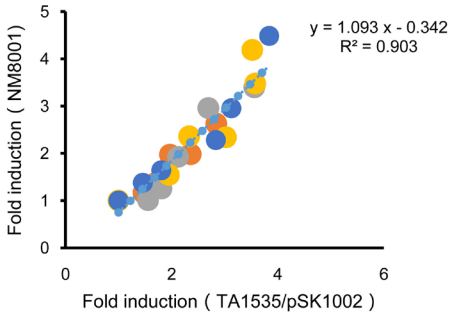

Figure 6. Comparison of lacZ units of NM8001 and TA1535/pSK1002.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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