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TOXICITY TESTING OF LEACHATE FROM WASTE LANDFILLS USING MEDAKA (*ORYZIAS LATIPES*) FOR MONITORING ENVIRONMENTAL SAFETY

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Abstract. To investigate the environmental safety of waste disposal landfill sites and of land reclaimed from such sites, we evaluated the toxicity of leachate from these sites by a combination of bioassays in the Japanese killifish medaka *Oryzias latipes*. We tested for lethal toxicity in adult and larval medaka and for hatching inhibition of embryos from eggs. As biochemical evidence of the effects of leachate exposure, CYP1A (EROD activity) and vitellogenin (Vtg) were induced. We also bioassayed water-treated leachate and downstream river water. Leachate solution was lethal to larval and adult medaka. Embryo hatchability was inhibited, and abnormal hatching, spinal deformity and anisophthalmia occurred in embryos exposed to leachate solution. CYP1A was induced by exposure to leachate solution diluted to 1.0%, and EROD activity was significantly higher than in control. Vtg and unknown proteins were induced in the sera of male medaka exposed to the diluted leachate solution. Conventional water treatments worked effectively to remove toxic compounds but did not work well to remove element ions, including heavy metals. Treated leachate produced neither lethal toxicity nor hatching abnormalities during the exposure period. Fish toxicity tests for leachate would be useful for monitoring the environmental safety of landfill sites.

Keywords: ecotoxicity, hazardous waste, leachate, lethal toxicity, medaka, waste disposal landfill site

1. Introduction

The manufacture and use of large volumes of industrial products have resulted in the production of equally large amounts of waste. Waste disposal and management are environmentally serious concerns owing to the risk of pollution of ambient environments (soil, air, and surface and ground waters), even after land rehabilitation is finished. The areas available in Japan for use as waste disposal landfill sites are limited by the small size of the country. Landfill sites are, therefore, usually established in secluded mountainous places far from cities, and are classified depending on their waste content into least controlled, controlled, and strictly controlled. They are managed by government. Industrial wastes (plastics, rubbers, and some metals) expected not to include toxic substances are reclaimed at least-controlled landfill site. Organic

and/or inorganic wastes (incinerated residues and ashes from domestic wastes, and sludges from wastewater treatment works) are reclaimed at controlled landfill sites. Other hazardous wastes are reclaimed at strictly controlled landfill sites. At the latter two types of controlled landfill sites, waterproof rubber seepage sheets are laid at the base of the landfill to prevent underground leakage of leachate. Leachate from landfill sites is known to contain numerous types of chemicals (Shiraishi *et al.*, 1999), and some chemicals are present at biologically hazardous levels. Therefore, leachates are collected and treated for detoxification by conventional water-treatment systems, and the treated leachates are then released into the rivers. Regardless of these preventive measures, seepage control does not completely prevent leakage, and although it is not officially reported, opening of the seams in the waterproof sheets and leakage of leachates are known to be frequent occurrences.

Landfill sites in Japan are located in areas where there are watercourses and other natural resources, so leakage of leachate is a serious environmental problem. Furthermore, water treatment may not completely work in removing chemical pollutants, so there can be a high risk of exposure by leakage and/or release of leachates to the ambient environment and its organisms. There is, however, little information available on the integrated toxicity of leachates to environmental organisms. Bisphenol-A (BPA) and organochlorine compounds such as dichlorodiphenyltrichloroethane (DDT) and hexachloro cyclohexane (HCH) have been detected at high frequencies ($>50\%$) in leachates (Shiraishi *et al.*, 1999). These compounds are also known to be endocrine disruptors. To assess leachate toxicity and environmental risk, a multi-criterion approach using biomarkers is needed. We exposed the Japanese freshwater killifish, medaka (*Oryzias latipes*), to leachate or treated leachate from a typical Japanese controlled landfill site, and then measured exposure effects such as lethal toxicity (to assess acute toxicity), embryo hatching inhibition (developmental toxicity), and induction of vitellogenin (Vtg) (endocrine disruption), and cytochrome P450 (CYP) 1A (xenobiotic exposure). Medaka is a remarkable model fish that has been used extensively in aquatic toxicological studies. Most leachates are highly saline (Tian *et al.*, 2005). Although medaka are normally found in freshwater they can tolerate seawater (Inoue and Takei, 2002).

2. Materials and Methods

We used leachate and treated leachate from a controlled landfill site in a city in Okayama Prefecture, located in western Japan. The area of this landfill site is 52 000 m^2 (a typical size for Japan) and it has reclaimed about 300 000 m^3 of solid waste, consisting of incinerated ashes, industrial wastes, and incombustible refuse, over the last 30 years. Most, but not all, of the base of the landfill is seepage controlled. Leachates are collected into a water treatment facility, where the treatment procedure is coagulate-sedimentation followed by activated sludge processing, coagulate-sedimentation, sand filtration, activated carbon adsorption,

and chlorine disinfection. Treatment capacity is 100 m^3/day. The water qualities of the leachate and treated leachate are monitored and are shown in Table I. Analytical data sets of dissolved oxygen concentration (DO), biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS) and dioxins have been made available to the public by the local government body that runs the landfill site. We also referred to other analytical data sets of nonmetal and metal elements, including heavy metals, from our previous study (Yamada *et al.*, 2002). Landfilling

TABLE I
Water qualities of the leachate and treated leachate used in this study (2000–2001), and reported minimum lethal toxic concentrations of ions to adult medaka

Item	Minimum lethal toxic concentration of element ion	Leachate	Treated leachate
Temperature (°C)		24.1 (0.6)	24.1 (0.4)
pH		7.7 (0.2)	7.8 (0.1)
DO		5.9 (0.7)	7.4 (0.5)
BOD		52*	2.6*
COD		79*	7.6*
SS		100*	5.0*
B	n.a.	1.91 (0.42)	1.79 (0.49)
Na	>1000 (NaCl)	648 (136)	666 (240)
Al	200 ($AlCl_3$)	0.0085 (0.011)	0.015 (0.031)
K	520 (KCl)	416 (215)	379 (158)
Ca	n.a.	130 (101)	68.9 (27.5)
Cr	3 ($Cr(NO_3)_2$)	0.0084 (0.0026)	0.0072 (0.0021)
Mn	>400 ($Mn(NO_3)_2$)	0.92 (0.51)	0.31 (0.14)
Fe	75 ($FeSO_4$)	29.6 (84.4)	0.25 (0.14)
Ni	80 ($Ni(NO_3)_2$)	0.011 (0.017)	0.0059 (0.0026)
Cu	1.2 ($CuSO_4$)	0.0040 (0.0027)	0.0046 (0.0028)
Zn	20 ($Zn(NO_3)_2$)	0.028 (0.031)	0.049 (0.039)
Sr	415 ($Sr(NO_3)_2$)	0.47 (0.23)	0.42 (0.13)
Cd	24 ($Cd(NO_3)_2$)	0.0058 (0.0037)	0.0059 (0.0035)
Pb	<30 ($Pd(C_2H_3O_2)_2$)	0.017 (0.0055)	0.0092 (0.0021)
Dioxins		0.015*	0.0083*

Fifty analyses were processed for calculating the mean and standard deviation for each element. *Single data set was available. n.a. means not available. Temperature, pH, and DO values were measured seasonally. Element ion concentrations were measured monthly. Analytical data units are mg/L for elements and pg-TEQ/L for dioxins. Standard deviation is given in parentheses. Analytical data sets of DO, BOD, COD, SS, and dioxins were made available to the public by the government body running the waste landfill site. Analyzed element data sets were also derived from a previous study (Yamada *et al.*, 2002). Minimum lethal toxic concentrations of ions in adult medaka were derived from a paper by Kasai; concentration units are mg/L (Kasai *et al.*, 1970).

has occurred for 30 years but is nearly at an end because the landfill is a nearly full. Therefore, the site is undergoing environmental safety assessment for reuse of the reclaimed land, and this prompted us to choose the site for leachate assessment. The landfill site is located in a hilly rural area, and treated leachate is released into a nearby river, so we also analyzed the downstream river water. The river water was sampled at a point 50 m downstream from the release point of the treated leachate.

Adults, larvae, and embryos of the orange-red variety of medaka were used. Adults medaka were purchased and some males were separated from the females for vitellogenin assay. First, the medaka were pre-cultured for 72 h in Elbaziu solution (Ueno Fine Chemicals Industry Ltd., Osaka, Japan) to destroy microorganisms at room temperature (25 ± 1 °C) under conditions of 16:8 h light:dark; the solution was changed every 24 h, without feeding of the fish. After this disinfection process, the medaka were transferred into a 60-L glass aquarium containing 40 L of fresh water and kept at room temperature (25 ± 1 °C) under conditions of 16:8 h light:dark. Tetra killifish food (Tetra, Melle, Germany) was fed twice a day. The density of medaka was 1.0 individuals/L and the freshwater used was dechlorinated tap water passed through a combination column of glass wool and activated carbon. The medaka were kept under the conditions mentioned above for at least 2 weeks to minimize any potential chemical effects from the environment. Body length and weight were in the range of 2.5 to 3.5 cm and approximately 0.3 g, respectively. To obtain medaka eggs, 10 males and 30 females were kept together at a water temperature of 27 ± 1 °C under conditions of 16:8 h light:dark and fed four or five times a day. Harvested egg embryos were separated from clusters and immediately used for hatching inhibition tests or were incubated to generate larvae. Egg embryos were incubated at room temperature (25 ± 1 °C) under conditions of 16:8 h light:dark. Hatched larvae were also immediately used for lethal toxicity testing.

Lethal toxicity and hatching inhibition tests were carried out as follows. In triplicate, 15 adults medaka were exposed together to 1000 ml of leachate or treated leachate solution, each diluted to 20%, 40%, 60%, 80%, or 100% with dechlorinated aerated tap water. Exposure was carried out at room temperature of 25 ± 1 °C for 72 h under conditions of 16:8 h light:dark. The number of dead medaka was recorded every 24 h, and then half the test water was replaced with new water in accordance with OECD Test Guidelines for Testing Chemicals #203. In triplicate, 15 medaka larvae were also exposed to 100 ml of leachate or treated leachate solutions under the same conditions as for the adult exposure. The concentrations of the leachate and treated leachate that were lethal to 50% of the exposed medaka fish or larvae (LC_{50}) were calculated by a computer program (Eco-Tox-Statics, Yoshimasa Yoshioka PhD, 1998, distributed by the Japanese Society of Environmental Toxicology). Leachates generally are highly saline, possibly because of the presence of reclaimed incinerated ashes. The leachate that we used had mean ionic compositions of 764 mg/L sodium ion, 77 mg/L calcium ion, and 563 mg/L potassium ion, and an osmotic pressure of 42 mOsm/L. Because this osmotic pressure is much lower than that of sea water (about 1000 mOsm/L), osmotic pressure from

these ions would seem not to be a contributor to leachate toxicity. However, to make sure that this was the case we tested the lethal toxicity to adult medaka of an equivalent saline solution composed of NaCl, $CaCl_2$, and KCl under the same conditions as mentioned above. In triplicate, 15 fertilized egg embryos were exposed together to 30 ml of leachate or treated leachate solution diluted to 20%, 40%, 60%, 80%, or 100% with egg-rearing solution composed of 200 μM $MgSO_4 \cdot 7H_2O$, 30 μM KCl, 300 μM $NaHCO_3$, and 800 μM $CaCl_2 \cdot 2H_2O$ (Yamamoto, 1939). These reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Exposed medaka embryos were incubated until hatching or death under the same incubation conditions as used for the toxicity tests in adults and larvae mentioned above, and then observed under a dissecting microscope every day. Test water samples were totally replaced every 48 h. Statistical analyses of data were done using one-way analysis of variance (ANOVA).

To measure CYP1A activity, ethoxyresorufin-*O*-deethylase (EROD) activity was measured in liver microsomes from exposed adult medaka (Tatarazako *et al.*, 2002). A water flow-through system with a flow rate of 0.69 ml/min was used to expose male medaka and to obtain a constant leachate concentration (Kashiwada *et al.*, in press). Medaka fish were exposed to each leachate solution, which was diluted to 1.0% with dechlorinated tap water. Treated leachate released into the river was expected to be diluted to 1:100 from water volumes of treated leachate discharged and of river to which was discharged, so 1.0% diluted leachate was chosen for the exposure concentration. This was the same rationale behind the concentrations chosen for the Vtg assay. In triplicate, 15 male medaka fish were exposed together to 1000 ml of diluted solutions of leachate or treated leachate, or to river water, for 10 days. They were fed twice a day. Leachates before and after chlorine treatment were used for exposure to treated leachate solution. After exposure, the livers were taken from medaka anesthetized in ice-cold water and immediately frozen in liquid nitrogen and stored at –80 °C until use. Liver homogenates and microsomes were prepared as previously described (Osaki *et al.*, 2003). The microsomal protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) with a standard protein (bovine serum albumin). Twenty microliters of a microsomal solution was added to 340 μl of reaction mixture, which consisted of 60 mM phosphate buffer (pH 7.7), 6 mM $MgCl_2$, 0.6 mM β-NADP, 8.0 nano-unit glucose-6-phosphate dehydrogenase, and 2.4 μM 7-ethoxyresorufin. This was followed by the addition of 40 μl of 100 mM glucose-6-phosphate. These reagents were purchased from Sigma Chemical (St. Louis, MO, USA). This reaction mixture was incubated at 25 °C for 20 min. The reaction was stopped by incubation at 85 °C in a water bath for 5 min, and the mixture was then stored on ice for 5 min. According to Hanioka *et al.* (2000), EROD activity remains linear for 30 min at most, so we terminated the reactions at 20 min. Concentrations of resorufin, a product of 7-ethoxyresorufin, were determined by HPLC with a fluorescence detector. The HPLC system consisted of an LC-10AD pump, RF-10A fluorescence detector, C-R5A chromatopac integrator, and SIL-$10A_{XL}$ auto injector with a 50-μl sample

loop (Shimadzu Co., Kyoto, Japan). A Symmetry C18 column (15 cm, particle size 5 μm, Nihon Waters Ltd., Tokyo, Japan) was used. The mobile phase was acetate buffer (0.001 M, pH 5.7) and methanol, controlled by a gradient program, starting at 60/40 (v/v) and reaching 40/60 in 5 min. The flow rate was 1.0 ml/min. Retention time of resorufin was 4.2 min. Detection wavelengths were 550 nm for excitation and 585 nm for emission. The detection limit for resorufin was 1.0 nM. Statistical analyses of data were done by a one-way ANOVA.

The estrogenic activity of the leachate was tested follows. The flow-through system was used for Vtg assay as well as EROD assay. In triplicate, 15 male medaka fish were continuously exposed for 10 days to leachate diluted to 1.0% and fed twice a day. Leachate was diluted with dechlorinated tap water. On days 4 and 10 of exposure, five medaka fish were sampled and anesthetized in ice-cold water. The tail was cut off and escaping blood was drawn into a glass capillary tube. The serum (about 2 or 3 μl) was collected as a supernatant of centrifuged blood (10000 $\times$ g for 1 min). Ten microliters of a stock buffer (20 mM phosphate buffer [pH 6.8], 50 mM EDTA, 1 mM phenylmethylsulfonylfluoride was added to each serum sample and kept at –20 °C until use. Protein concentrations of serum solutions were measured by BioRad protein assay (Bio-Rad Laboratories, Tokyo, Japan). All serum samples (15 μg protein per well) were separated by electrophoresis through two 12% polyacrylamide gels with a 3% to 10% stacking gel gradient (Atto Co., Tokyo, Japan) using a Tris/glycine buffer (25 mM Tris, 192 mM glycine [pH 8.3]) (Bio-Rad Laboratories) at 16 A per gel. One gel was then stained with Coomassie Brilliant Blue (CBB) dye (Coomassie Stain Solution, Bio-Rad Laboratories, Tokyo, Japan) and another gel was subjected to Western blot analysis. Western blot analysis was carried out using a polyvinylidene fluoride membrane (Atto Co.) at 2.0 mA per square centimeter for 30 min. The protein-blotted membrane was incubated with a primary polyclonal antibody to medaka Vtg, which was an antibody made from rabbit immunization with column-purified Vtg (MW 420 kDa) obtained from the ascitic fluid of male medaka exposed to 0.5 mg/L of 17β-estradiol for 3 days, in a 1% (w/v) non-fat powdered milk in Tris-buffered saline solution. It was then incubated with anti-rabbit secondary antibody conjugated with alkaline phosphate from an Alkaline Phosphate Conjugate Substrate Kit (Bio-Rad Laboratories) and then protein targets were visualized.

3. Results and Discussion

The qualities of leachate and treated leachate were surveyed three times a month (about every 10 days) through 2000 and 2001. There were no significant differences in water temperature and pH (Table I). Both values were also similar to those of downstream river water (data not shown). The mean DO value of leachate was raised from 5.9 to 7.4 by treatment, and the latter value was equal to that in the river water (data not shown). The values of BOD, COD, and SS were reduced

to less than 10% of those of the leachate after treatment. We expected that the dioxin concentration of the leachate would be high, because reclaimed ash from incinerators is a major content of the landfill and is generally known to contain dioxins at high concentrations (Kim *et al.*, 2005); however, the concentration was much lower than that we expected. Furthermore, the treatments worked to halve the dioxin concentration of the leachate. Dioxins may be adsorbed to SS in the leachate. Water treatment worked to remove organic SS but seemed not to work for elemental ions. This may explain why comparatively high salinity remains in treated leachate. The concentrations of ions were all less than the reported minimum lethal concentrations to adult medaka (Kasai *et al.*, 1970).

Adults and larvae medaka were exposed to solutions of leachate or treated leachate for 72 h. The leachate solutions showed high lethal toxicity to adults and larvae (Figure 1). Exposure to solutions containing leachate at more than 60% or more and 20% or more resulted in high lethal toxicity to adults and larvae,

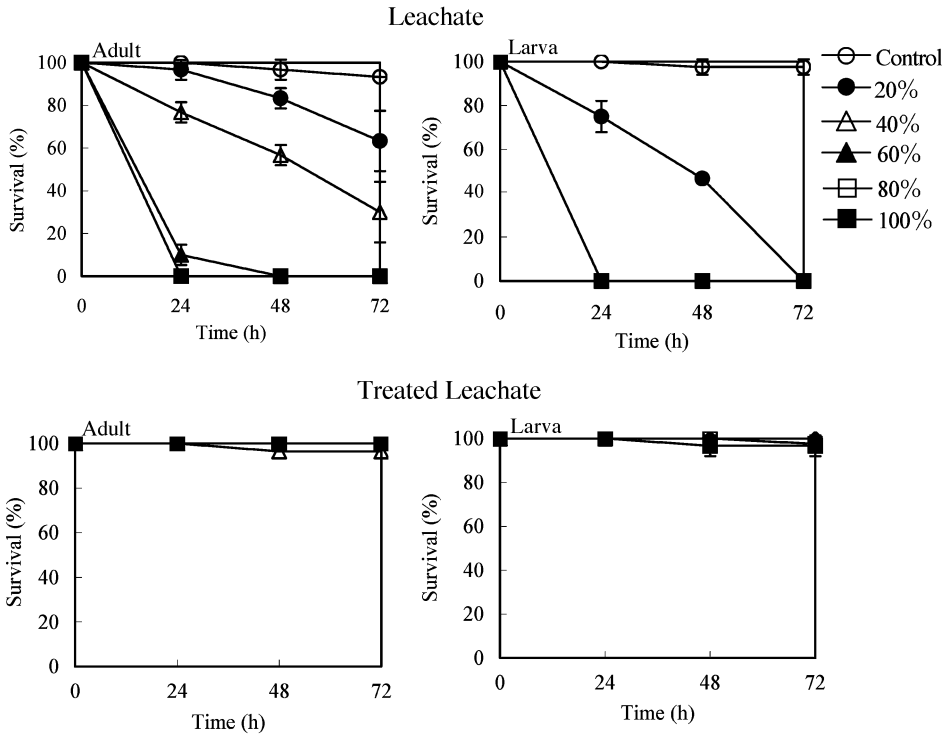

Figure 1. Lethal toxicity of leachate and treated leachate at various concentrations in adults and larvae of medaka. Adult or larval medaka were exposed to solutions of leachate or treated leachate. The leachate showed exposure-time-dependent lethal toxicity, but no lethal toxicity was seen with treated leachate during the exposure period. The LC_{50} values of the leachate concentration were calculated as 53.0% for adults (95% coefficient limit, 47.7–62.4%) and 19.2% for larvae (95% coefficient limit, 18.0–20.3%).

respectively, within 72 h. This meant that it was not always appropriate to calculate LC_{50} values for 72 h, so for the adult and larval toxicity tests, four and two data sets only, respectively, were available for 72 h. Therefore, 48-h toxicity data sets were used to calculate LC_{50} values. With 48 h of exposure, the LC_{50} leachate values were calculated as 53.0% and 19.2% for adults and larvae, respectively (Figure 1). Fish larvae usually have high sensitivity to chemicals (Kashiwada *et al.*, 2002), and our lethal toxicity test also showed higher sensitivity of larval medaka than adult medaka to leachate. All larvae exposed to leachate solution died within 72 h (Figure 1). On the other hand, treated leachate solutions exhibited no lethal toxicity to either stage of medaka (Figure 1). Exposure to leachate solutions reduced the hatchability of medaka embryos in proportion to the leachate concentration, and there was significantly higher inhibition with 100% leachate solution than with 100% treated leachate (Figure 2). In hatched embryos exposed to leachate solutions, we saw *abnormal hatch*, assessed by the portion of the embryo presenting first (normal hatching in medaka occurs with the tail emerging first; abnormal hatching often included head-first hatching, which caused death by strangulation); *spinal deformities*, including abnormal curvatures in the vertebral column; and *anisophthalmia*, including different sizes and position formation of both eyes (Villalobos *et al.*, 2000). However, embryos exposed to treated leachate solutions hatched normally. Embryos have a chorion envelope that helps to protect them physically. We previously reported that medaka embryos have higher tolerance to chemical exposure than do larval and adult medaka (Kashiwada *et al.*, 2002). Also, Villalobos

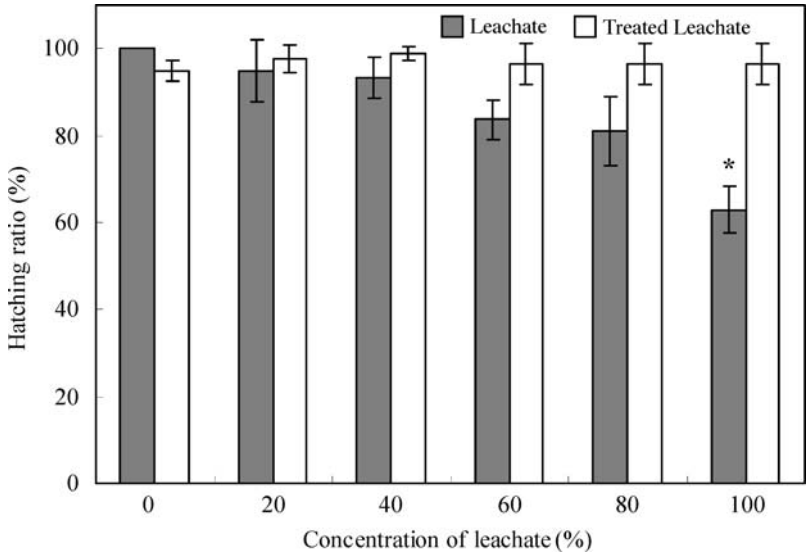

Figure 2. Hatching rate of embryos from medaka eggs exposed to untreated or treated leachate. Untreated leachate exhibited gradual hatching inhibition with increasing concentration. In contrast, there was no hatching inhibition in treated leachate. ANOVA: $^{*}P < 0.05$.

TABLE II
EROD activity in livers of medaka exposed to 1.0% leachate, treated leachate (before/after chlorine treatment) solution, or downstream river water

Sample	EROD activity (SD) (pmol min^{-1} mg^{-1} protein)
Leachate	18.9 (3.1)**
Treated leachate (before chlorine treatment)	7.7 (5.5)*
Treated leachate (after chlorine treatment)	4.5 (3.8)
Downstream river water	3.5 (2.4)
Negative control (dechlorinated tap water)	3.0 (1.3)
Positive control (0.1 mg/L of 3-methylcholanthorene)	135.2 (32.0)

ANOVA: $^{*}P < 0.1$, $^{**}P < 0.01$.

et al. (2000) have reported that dechorionated medaka embryos are more sensitive to chemicals. Induction and activity of CYP1A were estimated as EROD activity in liver microsomes prepared from exposed medaka. EROD activity in medaka exposed to 1.0% leachate solution and 1.0% treated leachate solution was 6 and 2.5 times higher, respectively, than in the negative controls (Table II). This treated leachate had not been given chlorine treatment. On the other hand, only about 1.5 times the negative control was seen with exposure to 1.0% treated leachate solution after chlorine treatment, and only slight EROD activity was induced upon exposure to downstream river water. Blood serum was analyzed by polyacrylamide gel electrophoresis (PAGE) and stained by CBB or subjected to Western blotting using Vtg antibody. For Vtg detection, an antibody against medaka Vtg was obtained by purification of antisera of rabbits immunized with purified medaka Vtg. This antibody specifically detected Vtg in the sera of mature female medaka used as positive controls and did not detect any proteins in the sera of control male medaka (Figure 3A). Medaka Vtg is known to have a molecular weight of 420 kDa (Hamazaki *et al.*, 1987). We detected a strong single Vtg band (arrowhead in Figure 3A) between markers at 232 kDa and 440 kDa in the control female serum, although the band was smeared owing to the native PAGE. Several bands were seen above the Vtg band. These probably represented preformative or combined Vtg (Tabata *et al.*, 2000). In serum analyses of male medaka exposed to leachate, analysis on days 4 and 10 of exposure revealed that Vtg was induced by exposure to 1.0% leachate solution (Figure 3A). Vtg induction was observed in all triplicates male serum. Fifteen days after exposed medaka had been moved to fresh and clean water for 15 days, Vtg induction was not observed in Western blot analysis of their sera (Figure 3A). Other unknown proteins were found to be induced on the CBB staining image (Figure 3B). We expected these proteins to be P450 proteins, heat shock proteins and/or other stress proteins; however, further protein analysis did not demonstrate these proteins.

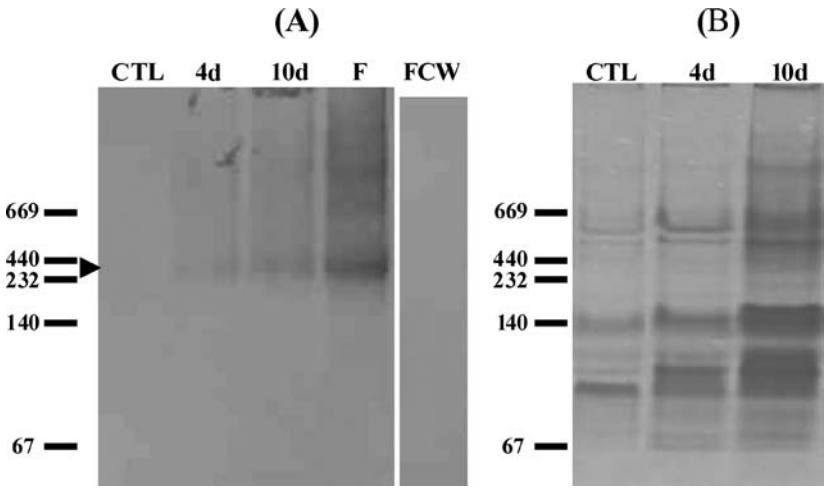

Figure 3. Images from Western blotting (A) and Coomassie Brilliant Blue staining (B) in polyacrylamide gel electrophoresis of medaka serum. CTL: Sera of male medaka not exposed to leachate. 4d, 10d: Sera of male medaka exposed to 1.0% leachate solution and then killed on days 4 or 10. Serum Vtg was induced during the exposure (arrowhead). Expression of other proteins was also enhanced. F: Sera of female medaka as positive controls to demonstrate Vtg expression. FCW: Sera of male medaka subjected to 10 days' exposure and then kept for 15 days in fresh and clean water.

The leachate that we used had a mean sodium ion concentration of 764 mg/L, calcium ions 77 mg/L, and potassium ions 563 mg/L. The annual concentrations of these ions were high (Table I). The concentrations were not changed significantly by the by the series of water treatments, and the concentrations of sodium and potassium ions were less than the reported minimum lethal toxic concentrations in adult medaka (>1000 mg/L as NaCl and 520 mg/L as KCl (Kasai *et al.*, 1970)). A saline solution equal to the concentrations of NaCl, $CaCl_2$, and KCl as the actual leachate showed no toxicity to adults (data not shown). Untreated leachate exhibited lethal toxicity to adult and larval medaka and inhibited hatching; however, treated leachate had no lethal effect and no inhibition of hatching. These results suggest that elemental ions, including those of heavy metals, were unlikely to be involved in toxicity, and that other lethal toxic compounds that were removable by the series of water treatments were present in the leachate. EROD activity following exposure to 1% untreated leachate solution was significantly enhanced; however, EROD activity was reduced by the series of water treatments (Table II). This result indicates that although xenobiotics exist in the leachate and are responsible for the induction of EROD activity, they are effectively removed by water treatment. The chlorine treatment was an effective procedure for removing chemicals that induce EROD activity. This was supported by the fact that untreated leachate showed high toxicity and treated leachate showed no toxicity in the tests for lethal toxicity and egg hatch inhibition. Vtg induction in male medaka exposed to the leachate solution was

dependent upon the number of exposure days, but disappeared following incubation in clean water for 15 days (Figure 3A). This strongly suggests that xenoestrogenic compounds contaminated the leachate. The expression of many proteins other than Vtg was also enhanced in the sera of male medaka exposed to the leachate solution. We did not further analyze these proteins.

Leachates of 24 major Japanese landfill sites have been analyzed for xenoestrogen contamination, revealing that BPA is one of the compounds most frequently (66.7%) detected, in the range of 0.149 to 2980 μg/L, with an average of 256 μg/L (Shiraishi *et al.*, 1999). We have demonstrated that the minimum concentration of BPA required to induce serum Vtg in male medaka is 10 μg/L, and that required to induce spinal deformity in medaka embryos is 100 μg/L (Kashiwada *et al.*, 2001). BPA can therefore be considered one of the candidate compounds for the induction of Vtg and embryo deformity in this study, although BPA was not analyzed here.

We used medaka to evaluate the integrated environmental toxicities of leachate from a waste disposal landfill site that has been reclaiming wastes for 30 years. Leachate is a contaminated mixture consisting of uncountable numbers of chemicals. If leachate were to leak out from a landfill site poorly regulated by seepage control, there would be a high risk of leachate contamination of the ambient environment. We found that the leachate we studied was contaminated by xenobiotics that exhibited lethal toxicity, inhibited embryo hatching and development, and induced CYP1A and Vtg; however, the contaminants were apparently removed when the leachate was treated by conventional water treatments. The fact that induction of Vtg was seen suggests that xenoestrogens can contaminate leachates. Landfilling of the landfill site studied is coming to end, and there is an ongoing reuse plan for the reclaimed land. Our study revealed that the leachate has high aquatic toxicity potential, but that this potential is dramatically reduced by water treatment. For reuse of the landfill site, well-controlled environmental monitoring and chemical risk management will be necessary. Fish toxicity testing of leachates will be useful for monitoring the environmental safety of landfill sites.

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