

# Biological Evaluation of the Pollution of Rivers Flowing into Tokyo Bay with the 7-Ethoxycoumarin *O*-deethylase (ECOD) Activity Induced by River Sediment Extracts in HepG2 Cells

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The extent of pollution in the rivers flowing into Tokyo Bay was studied comparatively using river sediment extracts in an assay system based on the arylhydrocarbon receptor (AhR)-dependent induction of 7-ethoxycoumarin *O*-deethylase (ECOD) activity in HepG2 cells. The sampling points of river sediment were as follows: Namamugi on the Tsurumi River, Rokugo on the Tama River, Hirai, Senjyu and Funado on the Ara River, Baraki on the Edo River, and Makuhari on the Hanami River (Fig. 1). The Tsurumi River (Namamugi) located in the middle of Keihin Industrial District was considered most polluted, followed by the Ara River (Hirai). The river sediment collected at Baraki on the Edo River was least polluted. The extracts of highly polluted river sediments sampled at Namamugi and Hirai exhibited a reverse U-shaped dose-response curve, for which polycyclic aromatic hydrocarbons (PAHs) might be mainly responsible, in a range of non-toxic concentrations. The pollution of river sediment collected at Namamugi was roughly estimated to be 2 to 20  $\mu\text{g}$  as PAHs/g sediment by consulting the data of Nakama *et al.*<sup>1)</sup> and 0.2 to 2  $\mu\text{g}$  as PAHs/g was assigned to the river sediment of Hirai.

**Key words** — HepG2 cells, 7-ethoxycoumarin *O*-deethylase, river sediment, CYP1A1, AhR

## INTRODUCTION

The various chemicals that are accumulating in the air, water and soil are a threat to public health as well as the sustainable development of terrestrial ecosystems. The pollution of the water system is of particular concern, affecting humans directly, through the water supply and indirectly, in terms of industrial, agricultural and fishery products. Water pollution has been studied extensively both physico-chemically and biologically. Among the biological approaches, the Ames assay for mutagenicity using his(-) mutants of *Salmonella typhimurium*<sup>2,3)</sup> and the sister chromatid exchange assay (SCE) for cytogenotoxicity using mammalian cells<sup>4)</sup> are probably the most popular, although they are not satisfactory in terms of sensitivity.

Many environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs) and polychlo-

rinated dibenzo-*p*-dioxins and -furans (PCDDs/Fs) are known to induce the synthesis of cytochrome P450s represented by CYP1A1 in hepatocytes both *in vivo* and *in vitro*.<sup>5-7)</sup> Such phenomena are mediated by the activation of an aryl hydrocarbon receptor (AhR), which generally disappears in immortalized hepatoma cells. However, the stable expression of AhR was shown in the highly differentiated human hepatoma cell line HepG2, in which the expression of CYP1A1 but not CYP1A2 is inducible by PAHs.<sup>8)</sup> Although the poor sensitivity of the above-described mutagenic and cytogenotoxic assays could be overcome with the receptor-mediated HepG2 assay, the application of CYP induction in HepG2 cells as a bioassay to monitor environmental pollution has not been well studied.<sup>1)</sup>

7-ethoxyresorufin *O*-deethylation specifies the function of CYP1A1. In contrast, 7-ethoxycoumarin *O*-deethylation, more sensitive than 7-ethoxyresorufin *O*-deethylation, is catalyzed by a rather wide range of CYP forms such as CYP1A1, 1A2, 2E1, 2A6 and 2B6. Among hepatic microsomal enzymes, CYP1A2 and 2E1 are the major *O*-

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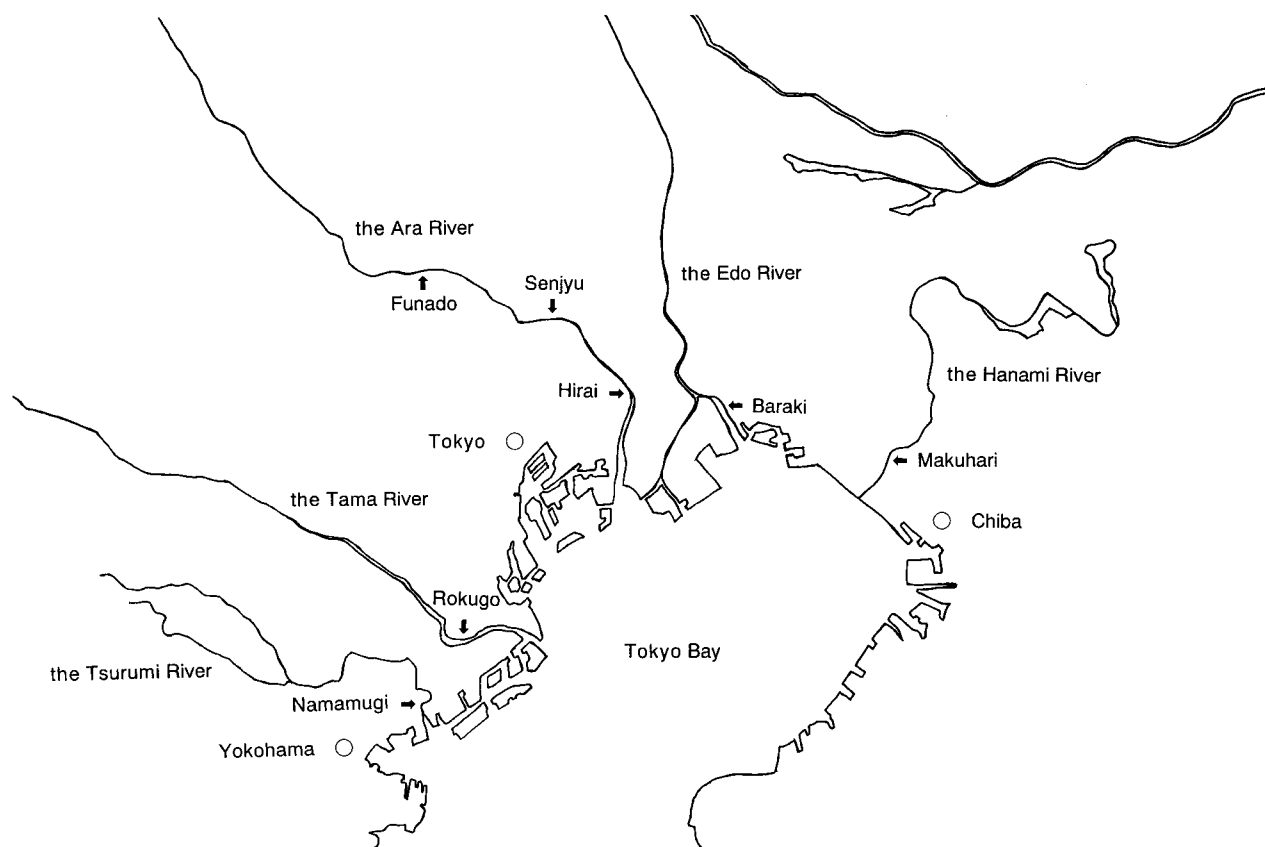


Fig. 1. The Location of River Sediment Sampling Points

deethylation enzymes of 7-ethoxycoumarin because the expression of CYP1A1 is suppressed in hepatocytes until induced.<sup>3)</sup> The contributions of CYP2A6 and 2B6 are minimal due to their poor expression in hepatocytes. In human hepatoma HepG2 cells, however, both CYP1A2 and 2E1 are hardly detected even when the cells are treated with their specific inducers. Thus, induction-dependent CYP1A1 might be mainly responsible for the 7-ethoxycoumarin *O*-deethylase (ECOD) activity in HepG2 cells.

In the present study, the HepG2 assay was successfully applied to a comparative study on the pollution of rivers flowing into Tokyo Bay. The ECOD activity in intact HepG2 cells was measured after being induced by the river sediment extracts.

## MATERIALS AND METHODS

**Sediment Samples** — Surface sediment samples were collected from the lower reaches of the Tsurumi, Tama, Ara, Edo and Hanami Rivers flowing into Tokyo Bay, central Japan, at the sampling points indicated in Fig. 1. Sampling times for indi-

vidual rivers were as follows: the Tsurumi River (Namamugi) and the Tama River (Rokugo), Mar. 2000; the Ara River (Hirai) and the Edo River (Baraki), Oct. 1999; the Ara River (Senjyu and Funado), Feb. 2000; the Hanami River (Makuhari), Oct. 1998.

**Reagent** — Dichloromethane and ethylacetate of a grade used for the test of pesticides, acetone, dimethylsulfoxide (DMSO) and 3-methylcholanthrene (3-MC) of guaranteed reagent grade, and  $\beta$ -glucuronidase of the grade suitable for biochemistry were obtained from WAKO Pure Chemical Industries, Ltd. Dulbecco's modified Eagle's medium (DMEM) and phenol red-free Eagle's minimum essential medium (MEM) were obtained from Gibco BRL. Cytotoxicity Detection Kit was purchased from Roche.

**Apparatus** — A Type F-3000 fluorescence spectrophotometer (Hitachi) was used for spectrofluorometry.

**Cell Cultures** — HepG2 cells were obtained from Cell Resource Center for Biomedical Research, Tohoku University. The cells were grown in DMEM supplemented with 15% fetal bovine serum and subcultured every 7–10 days at a 1 : 3 split ratio.

**Extraction** — Sediment samples were dried at room temperature and passed through a 10-mesh sieve to remove gravel. Then, 20 g of the sieved fraction was extracted with dichloromethane using Soxhlet extractor for 16 hr. Successively, the extracts were concentrated to approximately 5 ml with a Kuderna–Danish concentrator, transferred to a 10-ml conical tube, and evaporated to dryness under a N<sub>2</sub> gas stream. The residues were dissolved in 400  $\mu$ l of DMSO and further diluted 10, 100 and 1000 times; each solution of 12  $\mu$ l corresponds to 600, 60, 6 or 0.6 mg of sediment.

**Determination of ECOD Activity** — The induction and determination of ECOD activity were performed as described by Nakama *et al.*<sup>9)</sup> except that DMEM supplemented with 15% fetal bovine serum was used instead of serum-free medium. HepG2 cells were seeded on 60-mm type I collagen-coated dishes at  $1 \times 10^6$  cells/dish in 6 ml of culture medium. When the cultures reached confluence, the medium was replaced with 6 ml of fresh medium containing 12  $\mu$ l of DMSO solution of individual sample diluents or 3-MC (0.5 or 2.5  $\mu$ M, final concentration). The ECOD induction was carried out at 37°C for 16 hr. The cells were washed once with phosphate buffered saline (PBS), scraped off the dishes in 6 ml of phenol red-free MEM supplemented with 1 mM 7-ethoxycoumarin, 30 mM D-glucose and 12 mM HEPES, and transferred to 50-ml Erlenmeyer flasks. The whole was incubated at 37°C for 3 hr before the reaction was terminated by the addition of 3 ml of ice-cold acetone. The content was transferred to a 10-ml conical tube and centrifuged at 1000 rpm for 10 min. The precipitate was used for the determination of protein by Lowry's method, while the supernatant was adjusted to a pH of 4.8 with sodium acetate buffer after deacetonization under a N<sub>2</sub> gas stream and incubated for another 16 hr after  $\beta$ -D-glucuronidase (700 U/ml at final) had been added to hydrolyze the  $\beta$ -D-glucuronate conjugate of 7-hydroxycoumarin. The 7-hydroxycoumarin that formed was extracted three times with 3 ml of ethylacetate. The pooled organic phases were evaporated and residues were dissolved in an appropriate volume of glycine-sodium hydroxide buffer (pH 10.4) and analyzed by spectrofluorometry ( $\lambda_{ex} = 370$  nm,  $\lambda_{em} = 454$  nm).

**Determination of Cytotoxicity** — Subconfluent HepG2 cells were cultured for 24 hr with serially 10-fold diluted sediment extracts of the Tsurumi River (Namamugi) and the Ara River (Hirai) at final concentrations equivalent to those employed for

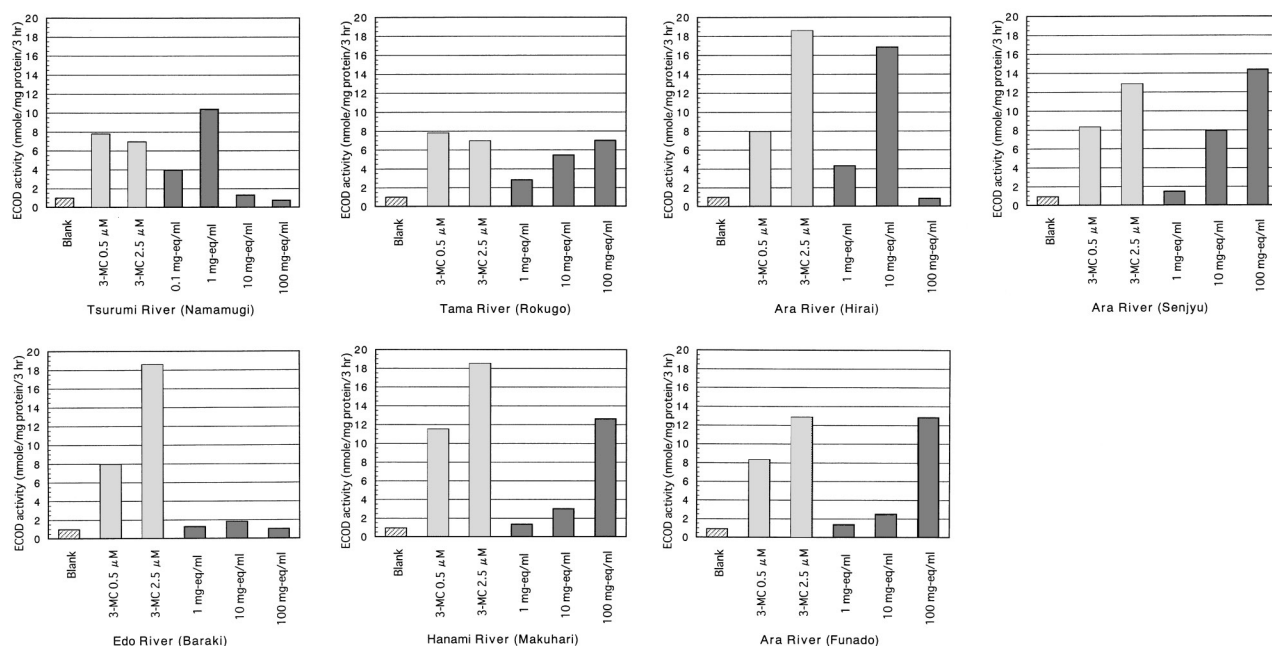
the ECOD induction. Lactate dehydrogenase (LDH) activity was measured by Cytotoxicity Detection Kit.

## RESULTS AND DISCUSSION

The results for the induction of ECOD activity in HepG2 cells are summarized in Fig. 2. With all the samples tested except for the one obtained at Baraki on the Edo River, the ECOD activities were enhanced to different extents. The Tsurumi River (Namamugi) sample was considered most polluted, with a definitive effect being observed at as low as 0.1 mg sediment-equivalent/ml (hereafter referred to as mg-eq/ml) and a peak response of 1 mg-eq/ml. However, at much higher concentrations, the ECOD activity fell to near the control level. As a whole, the sediment extract of Tsurumi River (Namamugi) exhibited a reverse U-shaped curve. Sampling point Namamugi is located in the middle of the Keihin Industrial District, and the results seemed to reflect long-term accumulation of pollutants on the river bed. On the assumption that PAHs were totally responsible for the ECOD activity, the pollution of river sediment at this sampling point was estimated to be 2 to 20  $\mu$ g PAHs/g sediment by consulting Nakama *et al.*<sup>1)</sup> in which 2.5  $\mu$ M 3-MC was equivalent to 10–100 ng/dish of PAHs in terms of ECOD induction potential.

The second most polluted point was Hirai on the Ara River, with a distinct effect on the ECOD activity being observed at 1 mg-eq/ml and a peak value of 10 mg-eq/ml. Compared with the results for the Tsurumi River (Namamugi) sample, the extent of sediment pollution was estimated to be 10-times lower. As in the case of the Tsurumi River (Namamugi) sample, a reverse U-shaped dose-response curve was obtained. The pollution could be from the drainage of factories located along the lower reaches of the river.

For the extract of river sediments sampled at Senjyu and Funado, both located up stream from Hirai on the Ara River, the ECOD activity increased in a dose-dependent manner up to the highest concentration of 100 mg-eq/ml; in other words, these points are considered to be less polluted than Hirai located at the lower reaches of the same river. The same dose-response profiles were obtained with the extracts from the river sediments sampled at Rokugo and Makuhari on the Tama and Hanami Rivers, respectively. The induction of ECOD activity by the extract from the river sediment sampled at Baraki

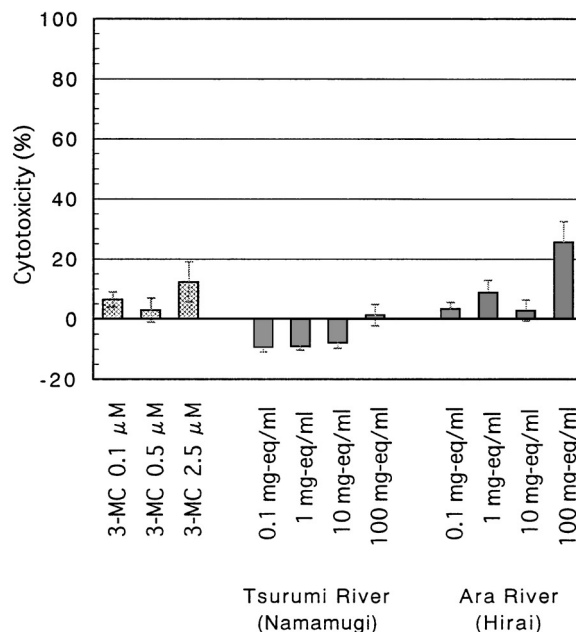


**Fig. 2.** 7-ethoxycoumarin *O*-deethylase Activity Induced by River Sediment Extracts

The ECOD activity was induced in duplicate at 37°C for 16 hr with sediment extracts at final concentrations of 0.1 to 100 mg-sediment equivalent/ml (shown as mg-eq/ml in short) or 3-MC at 0.5 and 2.5  $\mu$ M.

on the Edo River was almost negligible, suggesting that the pollution at this sampling point is no more than the detection limit of the HepG2 assay.

The ECOD induction profiles of the sediment extracts of Tsurumi River (Namamugi) and Ara River (Hirai) showed a reverse U-shape. To see if the decrease in the ECOD activity at the higher concentrations of river sediment extracts was attributable to a cytotoxic effect on the host HepG2 cells, both samples were tested for their cytotoxicities in the same range of concentrations as those used to induce the ECOD activity by measuring the LDH activity released from the fragile cells. As can be seen in Fig. 3, this hypothesis could be excluded, because only a marginal decrease in cell viability was observed even at the highest concentration (100 mg-eq/ml) of the sediment extract of Edo River (Hirai). Consistent with our results, Delescluse *et al.*<sup>8)</sup> reported that the induction of ethoxyresorufin *O*-deethylase (EROD) activity by 3-MC in primary rat hepatocytes and HepG2 cells increased in a dose-dependent manner and decreased after reaching the peak value. However, the same authors observed no decrease in the CYP1A1 mRNA level, implying that the down-regulation of EROD activity in primary and immortalized cells by higher concentrations of 3-MC worked post-transcriptionally. In a mouse hepatoma cell line Hepa-1, PAHs inhibited CYP1A1 induction at higher concentrations, whereas poly-



**Fig. 3.** Cytotoxicity of River Sediment Extract in HepG2 Cells

Measured was the LDH activity released from HepG2 cells after 24-hr treatment with river sediment extracts or 3-MC in the same range of concentrations as those used for the ECOD induction assay. Vertical bars represent  $\pm$  S.E.

chlorinated dibenzo-*p*-dioxins and dibenzofurans (PCDDs/Fs) and polychlorinated biphenyls (PCBs) did not.<sup>6)</sup> Taking into consideration the fact that PAHs represented by 3-MC are metabolically activated to become toxic intermediates by CYP1A1, the down-

regulation of the enzyme activity by PAHs would be part of the cellular homeostatic response. The molecular basis of this phenomenon is now under investigation.

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