Study on the Treatment of Endocrine Disrupting Chemicals in Wastewater by Ionizing Radiation

THESIS

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Contents

Chapter I

General Introduction

I-1 Endocrine disrupting chemicals

The earth is facing various problems, including destruction of the ozone layer, global warming, acid rain loss of tropical forests and marine pollution. Water environment on the earth has also been polluted by industrial chemicals. “Silent Spring” reported the environmental pollution by insecticides such as dichlorodiphenyltrichloroethanes (DDTs) in 1962 [I-1]. This book made most of people to have idea of environmental conservation, and has been regarded as a bible on the field of environmental science and technology. Most of toxic industrial chemicals have been detected during these three decades and have been screened by some toxicity tests such as a single dose oral toxicity test, preliminary reproduction toxicity screening test and in vitro chromosomal aberration test [I-2-4]. A part of the chemicals having the unique toxicity, so-called endocrine disrupting chemicals (EDCs), however, could not be evaluated by the conventional toxicity tests.

Hormones are produced by various organs known as endocrine glands, including the testicles, ovaries, pancreas, adrenal glands, thyroid, parathyroid and thymus, and traveled in the bloodstream to cause the biochemical reactions. For example, woman’s ovaries release eggs and natural estrogens such as estrone, estradiol and estriol. These female hormones travel in the bloodstream to the uterus and bind to the estrogen receptors to produce chemical signals, which activates genes in nucleus and trigger growth of the tissue lining the womb in anticipation of a possible pregnancy. The body has hundreds of different kinds of receptors, each one designed for a particular kind of the chemical signals. As shown in Fig. I-1, hormones and their corresponding receptors fit together as a “ lock and key ” mechanism in the endocrine system [I-5]. Estrogen activity is defined as a degree of binding ability of chemicals

with estrogen receptor.

Some chemicals have properties to mimic estrogen or disrupt the endocrine system in living bodies to give ill effects. For example, the male fishes in the Tokyo Bay are exposed to estrogens and estrogenic substances, and the eggs are observed in these male testes as shown in Fig. I-2 [I-6]. Hormone mimic chemicals such as 17 β-estradiol (1,3,5(10)-estratriene-3,17β-diol), alkylphenols, 2,2-bis(4-hydroxyphenyl)propane (Bisphenol A), diethylstilbestrol (DES) and DDTs also bind to the receptor to induce the chemical signal by process (a) as shown in Fig. I-3 [I-5], and their structural formula are shown in Fig. I-4. Hormone blocking chemicals, for example, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) [I-7] and dioxins [I-8], make the hormone not to induce the chemical signal. They bind to another receptors, however, and consequently prevent natural hormones from binding to the hormone receptor as shown by process (b) in Fig. I-3. These hormone mimic and blocking chemicals are so-called EDCs [I-5]. Endocrine disrupting property appears in the range of EDCs concentration from 10^{-9} to 10^{-6} g dm^{-3} [I-9-11].

In 1991, Soto *et al.* [I-12] explained that *p*-nonylphenols trigger mitotic activity in rat endometrium to disrupt the endocrine system. Sumpter *et al.* and Jobling *et al.* [I-13, 14] reported that 17 β-estradiol, DDTs and alkylphenols induce vitellogenin synthesis in male rainbow trout maintained in the effluent of sewage-treatment. In 1996, Colborn *et al.* [I-5] published “Our Stolen Future”, which provided account of emerging scientific research about how a wide range of manmade chemicals disrupt hormone systems. Routledge *et al.* [I-9, 10] and Hu *et al.* [I-11] examined estrogen-inducible screening of some EDCs. In the recent works, natural estrogens such as 17 β-estradiol have much attention since their releasing to the water environment is difficult to be controlled and a part of

them is adsorbed to suspended substances in wastewater [I-15-17]. Monitoring and screening of artificial EDCs such as alkylphenols and bisphenol A have been carried out by GC-MS and LC-MS [I-18, 19]. Endocrine disrupting effects of wastewater containing a variety of EDCs to fishes and mollusks were evaluated by some bioassays [I-19, 20].

Japanese institutions recognized EDCs as chemical pollutants in the latter half of 1990s. Hazardous EDCs of 67 substances were selected by the Ministry of the Environment, Japan in 1998, and were investigated by monitoring of its concentration in the river and by screening tests [I-21]. The project “SPEED 98” was executed for 5 years, and concluded that *p*-nonylphenol, *tert*-octylphenol and bisphenol A affect fishes as endocrine disrupters but not human [I-22]. Other famous EDCs such as organic tins, phthalic acids and benzophenone were also found not to disrupt the endocrine systems of active animals. Therefore in 2005, the Ministry of the Environment, Japan informed that EDCs may be treated as chemicals of the general toxicity [I-23]. Nowadays the natural estrogens such as estrone, estradiol and estriol draw much attention since their releasing to the water environment is difficult to control.

I-2 Water treatment

Water treatment for EDCs is not completely achieved in wastewater treatment plants since there is no efficient technique to remove trace amounts of EDCs at the present stage. Wastewaters containing the harmful chemicals have mostly been treated by activated sludge and the ozone process as shown in Fig. I-5. The activated sludge is, however, often inadequate to absorb trace amounts of biologically harmful substances such as EDCs [I-17]. The ozone process is

based on the direct oxidation by ozone, and has been widely used for treatment of wastewater with its high oxidation reactivity and bactericidal action, although it is difficult to treat some kinds of EDCs by ozolysis. Therefore, development of a new technique or treatment system is required.

Hydroxyl radicals having electrophilicity can decompose for any EDCs which cannot be decomposed by ozone. The treatments of toxic organic compounds by the oxidation with hydroxyl radicals have drawn much attention. A number of papers and patents have reported on treatments with hydroxyl radicals generated by an ozone / UV or hydroxyperoxyl / UV photolysis [I-24-26], Fenton-type oxidation [I-27-29], ionizing radiation [I-30-39] and photocatalyst as a titanium dioxide [I-40-43]. However, the commercial plant based on these methods was not applied since the reactions of hydroxyl radicals with EDCs have not been clearly understood in water. Ionizing radiation can homogeneously produce hydroxyl radicals at the required concentration in water, and the study by use of ionizing radiation would be useful to clarify the decomposition mechanism of EDCs by hydroxyl radicals.

I-3 Radiolysis of water

The basic physical and chemical primary processes of radiolysis of water have been studied [I-44-48]. A series of reactions for radiolysis of water is represented in Fig. I-6 [I-49]. Exposure of water to ionizing radiation produces excited and ionized water molecules and free electrons within approximately 10^{-16} s:

$$H_2O \xrightarrow{\sim} H_2O^* \qquad \text{(I-1)}$$

$$H_2O \xrightarrow{\sim} H_2O^+ + e^- \qquad \text{(I-2)}$$

The ionized water molecules react rapidly to form hydroxyl radicals within 10^{-14} s:

$$H_2O^+ + H_2O \rightarrow OH + H_3O^+ \quad \text{(I-3)}$$

The thermalization and hydration of the free electrons take place within 10^{-13} s:

$$e^- + nH_2O \rightarrow e^-_{aq} \quad \text{(I-4)}$$

Excited water molecules return to the ground state or homogeneously dissociate to produce hydroxyl radicals and hydrogen atoms:

$$H_2O^* \rightarrow H_2O \quad \text{(I-5)}$$

$$H_2O^* \rightarrow OH + H \quad \text{(I-6)}$$

Hydroxyl radicals, hydroxide ions, hydrated electrons and hydrogen atoms are produced within 10^{-12} s. These products of water radiolysis are heterogeneously distributed in the region of several nanometers, which is called “spur”. The spurs are formed by γ-ray and electron beam irradiation at separations of several hundreds of nanometers.

These products of water radiolysis diffuse homogeneously in the spur within 10^{-7} s. Some of the products react with each other in the range of time from 10^{-12} to 10^{-7} s (Spur reaction) [I-49]:

$$e^-_{aq} \rightarrow H + OH^- \quad \text{(I-8)}$$

$$2e^-_{aq} \rightarrow H_2 + 2OH^- \quad \text{(I-9)}$$

$$e^-_{aq} + H + \rightarrow H_2 + OH^- \quad \text{(I-10)}$$

$$e^-_{aq} + OH \rightarrow OH^- \quad \text{(I-11)}$$

$$e^-_{aq} + H_3O^+ \rightarrow H + H_2O \quad \text{(I-12)}$$

$$2H \rightarrow H_2 \quad \text{(I-13)}$$

$$H + OH \rightarrow H_2O \quad \text{(I-14)}$$

$$2OH \rightarrow H_2O_2 \quad \text{(I-15)}$$

$$OH + H_2O_2 \rightarrow H_2O + HO_2 \quad \text{(I-16)}$$

$$2HO_2 \rightarrow H_2O_2 + O_2 \quad \text{(I-17)}$$

$$OH^- + H_3O^+ \rightarrow 2H_2O \quad \text{(I-18)}$$

These products are called “primary products” as listed in Table I-1 [I-49]. Radiation chemical yields have traditionally been described in terms of “*G*-value”, where *G*(X) or *G*(-Y) is the number of molecules of a product X, or of the starting material Y changed, per 100 eV energy absorbed. The SI unit of radiation chemical yields is mol J^{-1} and is related to the *G*-value as follows:

$$G\text{-value } (10^{-2}\ eV^{-1}) = 1.04 \times 10^{-7}\ mol\ J^{-1} \quad \text{(I-19)}$$

The *G*-value may be generally utilized when the number of molecules is in good linear-relationship with absorbed energy.

Destruction kinetics of organic compounds in water by ionizing radiation is, therefore, mainly expressed by the following expression:

$$-dC/dt = k_{e^-}[e_{aq}^-]\,C + k_H[H]\,C + k_{OH}[OH]\,C \quad \text{(I-20)}$$

where C is the concentration of the solute, and k_{e^-}, k_H and k_{OH} are the second-order rate constants of hydrated electrons, hydrogen atoms and hydroxyl radicals, respectively. Hydroxyl radicals rapidly attack organic compounds with rate constants close to the diffusion-controlled limit of water, but reactions of hydrated electrons and hydrogen atoms are comparatively slow as shown in Table I-2 [I-50-65]. The diffusion-controlled limit of water at 293 K is estimated from the equation $k_{diff} = 8000\,RT / 3\eta = 7.4 \times 10^9\ mol^{-1}\ dm^3\ s^{-1}$ [I-66]. Most of EDCs having high estrogen activity are aromatic compounds as shown in Fig. I-4, and would be mainly decomposed by radiation-induced hydroxyl radicals.

I-4 Radiation source

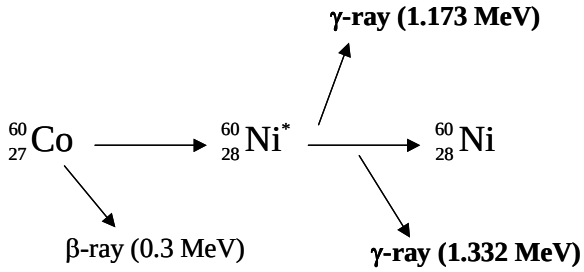
There are several ionizing radiation, such as α-ray, β-ray, γ-ray, electron

beam and heavy ion beam. Alpha particles are the nuclei of helium atoms, and are emitted by radioactive nuclei with discrete energies that are characteristic of the radioisotope decaying. Sources of the α, β and γ radiation are in various forms as shown in Table I-3 [I-46]. Alpha particles lose energy rapidly when passing through liquid or solid materials. Beta particles are fast electrons emitted by radioactive nuclei. In contrast to α particles, β particles are not all emitted from a particular radioactive element with the same energy but with energies ranging from zero up to a maximum value that is characteristic of the element.

Gamma-rays are electromagnetic radiation of nuclear origin with wavelengths in the range from 3×10^{-11} m to 3×10^{-13} m. Cobalt-60 is probably the most widely used source of γ-ray irradiation, and is given by the reaction as follows,

$$^{59}\mathrm{Co} + \mathrm{n} \rightarrow {}^{60}\mathrm{Co} \qquad \text{(I-23)}$$

The γ-rays of 1.173 and 1.332 MeV are released by radioactive decay of the ^{60}Co source at 5.27 years of half-life [I-46];



(I-24)

The half thickness value is used to relate γ-ray transmitted without loss of energy to the thickness of the absorbing material. The half thickness value of ^{60}Co γ ray of energy 1.25 MeV is 11 cm in water [I-46]. While the range of α particles of 4.795 MeV energy emitted by Radium-226 is 3.3×10^{-3} cm in water, and the range of electron of 1.71 MeV emitted by P-32 is 0.79 cm in water [I-46]. The γ-rays are appropriate for the fundamental research, e.g. study

of oxidation mechanism, since the reactive species are produced homogeneously in water and their amount can be easily controlled by changing a dose rate and an irradiation time.

Particle accelerators produce beams of accelerated electron and heavy ion with energies in the keV or MeV range for most of radiation chemical purposes. Several electron accelerator designs have been adapted to give machines suitable for routine irradiation. Compared with γ-ray sources, electron beam machines are suited to the high-speed irradiation of the surface layers of thicker materials, but less adapted to the irradiation of bulky samples. Economical evaluation of treatment of wastewater showed that the electron beam is useful for the industrial purposes.

I-5 Scope of the thesis

The purpose of this study is to confirm whether or not ionizing radiation is available for the treatment of trace amounts of EDCs in water environment. 17 *β*-Estradiol (E2, having the highest estrogen activity) and *p*-nonylphenols (NPs, exhibiting one of the highest estrogen activity among artificial chemicals) were selected as one of the most serious EDCs. Treatment of real wastewaters having estrogen activity was examined by use of ionizing radiation to obtain the fundamental data, i.e. reduction of estrogen activity and product analysis in the real wastewater, for development of the practical water treatment method.

In Chapter II, decomposition of E2 in water was studied by use of ^{60}Co γ-ray irradiation. Elimination of estrogen activity in the E2 solution was examined by enzyme-linked immunosorbent assay (ELISA). In Chapter III, decomposition of NPs was also investigated by γ-ray irradiation, and its

decomposition mechanism was discussed. Degradation of estrogen activity in the NPs solution was evaluated by the yeast two hybrid assay and ELISA. Treatment of real wastewater showing the estrogen activity was investigated in Chapter IV. The treatment of model wastewaters containing E2 or NPs were studied by γ-ray irradiation. Decrease in estrogen activities of the real wastewaters was simulated by use of the results of decomposition of the model wastewater. The cost for the water treatment plant with electron beam was discussed based on the simulation results.

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Table I-1 Primary yields of decomposition of water by ionizing radiation having a variety of linear energy transfer (LET).

	LET	*G*-value (molecules 10^{-2} eV^{-1})					
	(eV nm^{-1})	$-H_2O$	e^-_{aq}	OH	H	H_2	H_2O_2
γ-ray	0.23	4.08	2.63	2.72	0.55	0.45	0.68
18 MeV D^+	12.3	3.46	1.48	1.78	0.62	0.68	0.84
32 MeV He^+	61	3.01	0.72	0.91	0.42	0.96	1.00
12 MeV He^{2+}	108	2.84	0.42	0.54	0.27	1.11	1.08

Table I-2 Rate constants of reactions of radiation-induced reactive species with organic compounds in water.

Solute	Rate constant (mol^{-1} dm^3 s^{-1})		
	k_{OH}	k_H	k_e
Phenol	6.6×10^9	1.7×10^9	3.0×10^7
Toluene	5.1×10^9	2.6×10^9	1.1×10^7
Chlorobenzene	5.6×10^9	1.4×10^9	5.0×10^8
Hexane	6.6×10^9	1.4×10^9	-
Cyclohexane	6.1×10^9	6.0×10^7	-
Methanol	9.7×10^8	2.6×10^6	1.0×10^4
Diethylether	4.2×10^9	4.3×10^7	$<1.0\times10^7$

Table I-3 Sources of α, β, γ-ray irradiation

Isotope	Half-life	Type and energy in MeV of principal radiation emitted
Natural isotopes		
Polonium-210	138 days	α, 5.304(100%) γ, 0.8 (0.0012%)
Radium-226	1620 years	α, 4.777 (94.3%) α, 4.589 (5.7%) γ, 0.188 (~4%)
Radon-222	3.83 years	α, 5.49
Artificial isotopes		
Cerium-137	30 years	β, 1.18 (8%) β, 0.52 (92%) γ, 0.6616 (82%)
Cobalt-60	5.27 years	β, 0.314 γ, 1.332 γ, 1.173

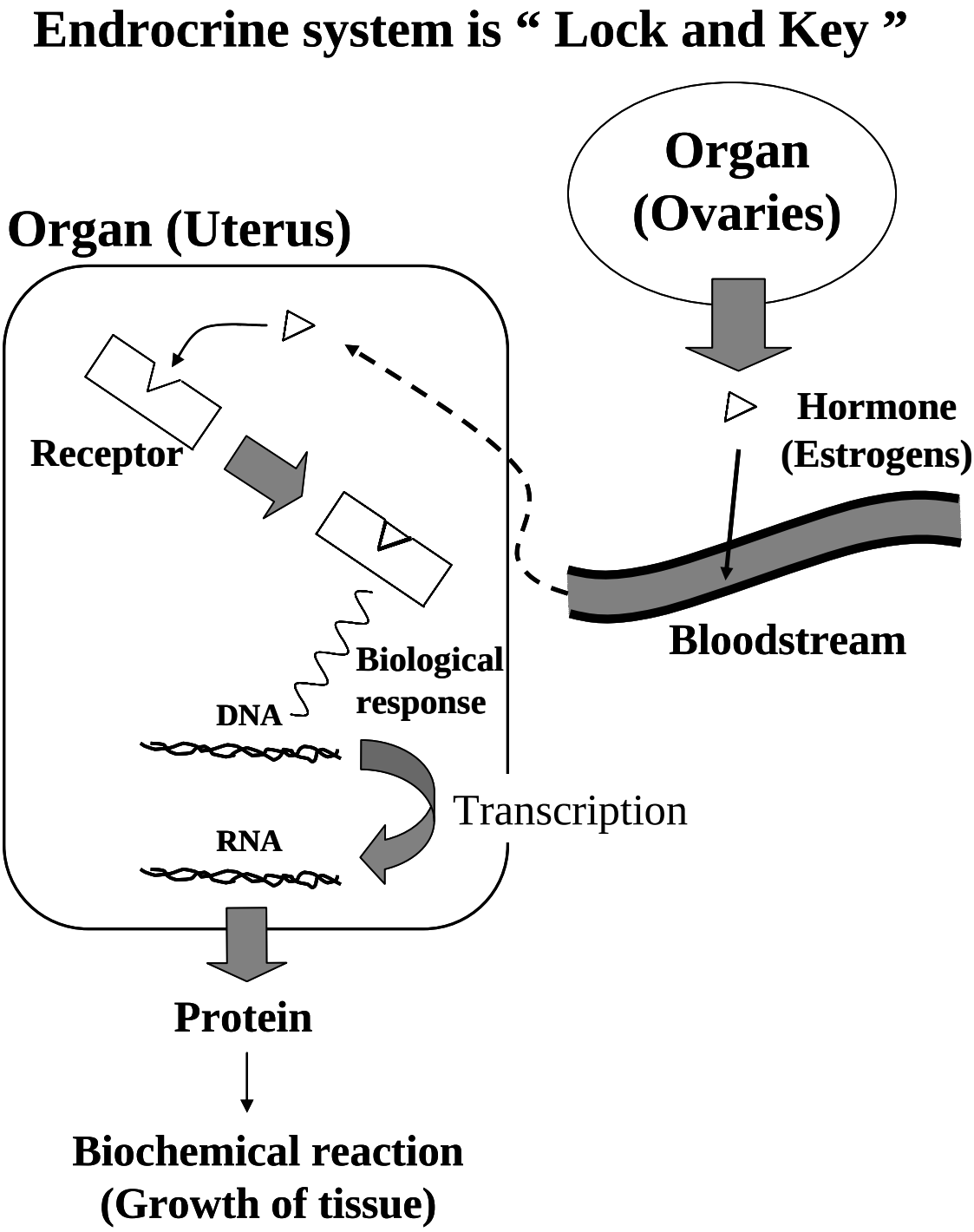
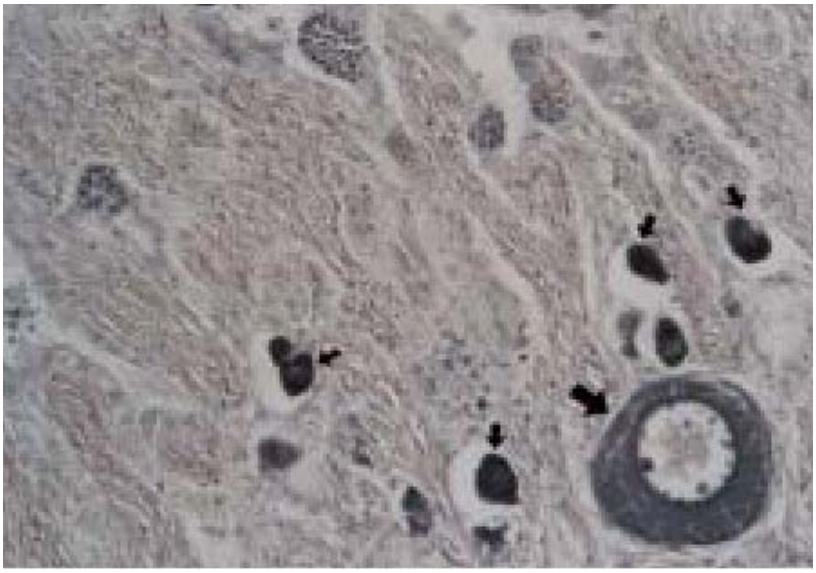


Fig. I-1 Biochemical reaction of hormones and their corresponding receptors in the endocrine system.

(a)



(b)

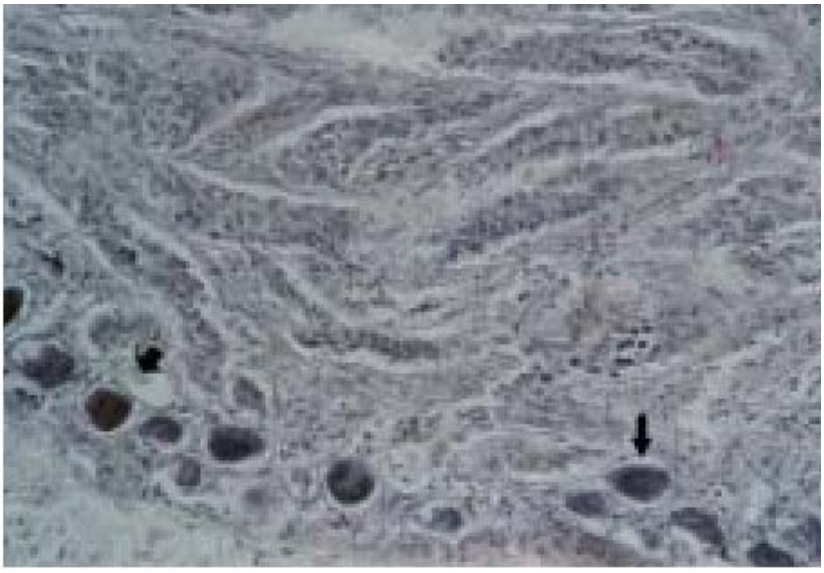


Fig. I-2 Testes of sea bass (a) and conger (b) exposed to estrogens and estrogenic substances in the Tokyo Bay, referred from [I-6].

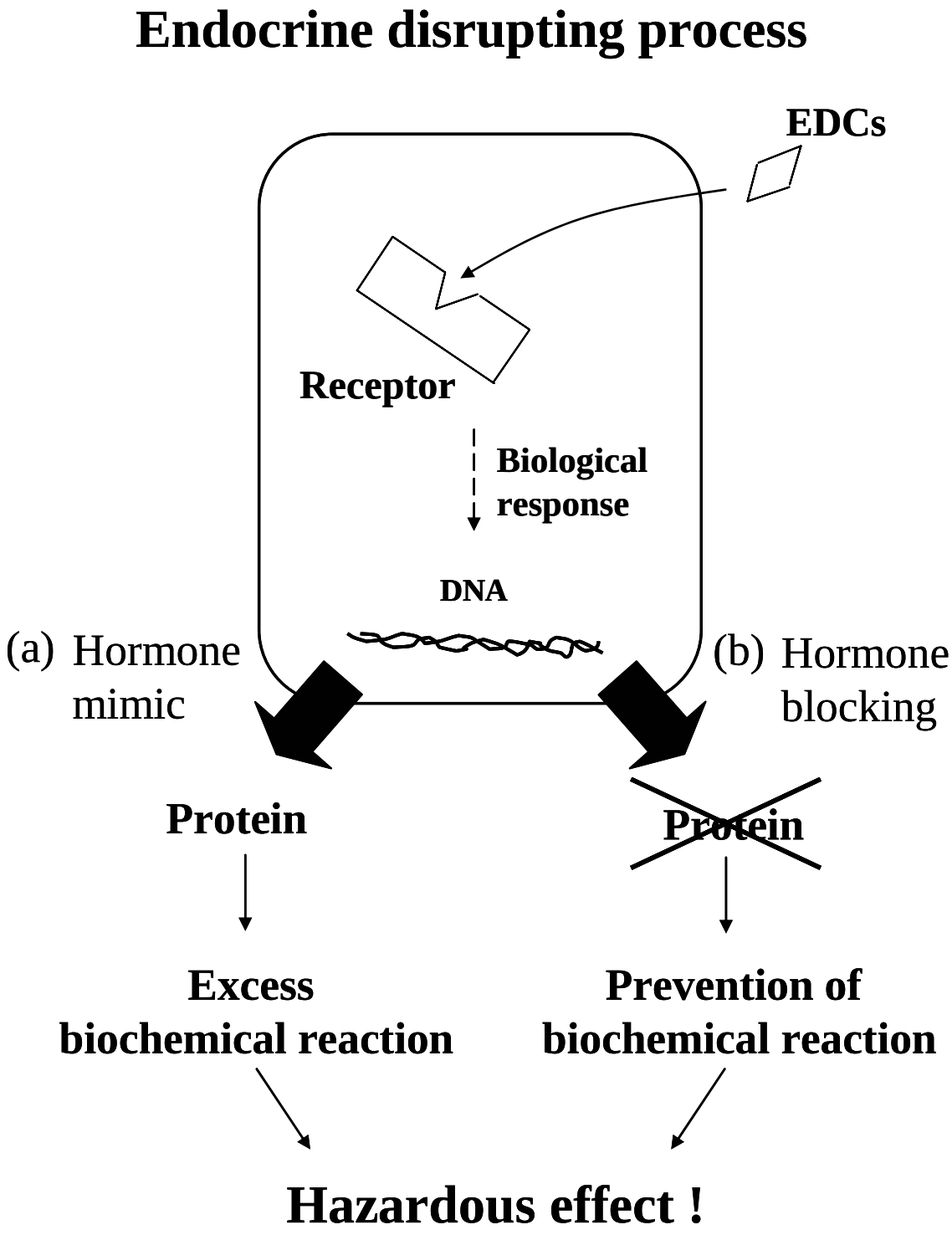
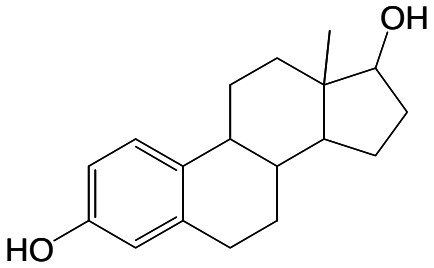


Fig. I-3 Endocrine disrupting processes by mimicking (a) or prevention (b).

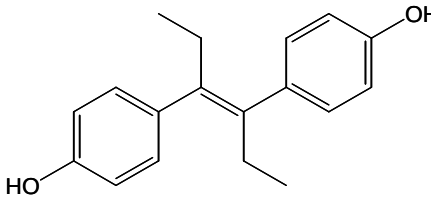
Hormone and hormone mimic chemicals

Natural estrogen



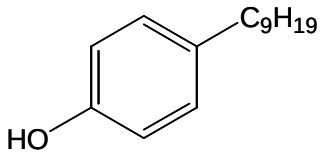
17β-Estradiol (E2)

Synthetic estrogen

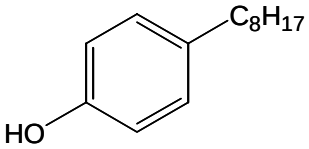


Diethylstylbestrol

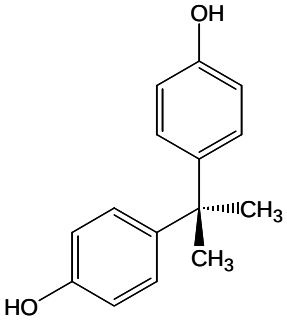
Artificial EDCs



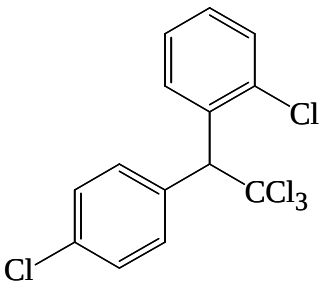
p-Nonylphenol (NPs)



tert-Octylphenol



Bisphenol A



DDTs

Fig. I-4 Structure of hormone mimic chemicals

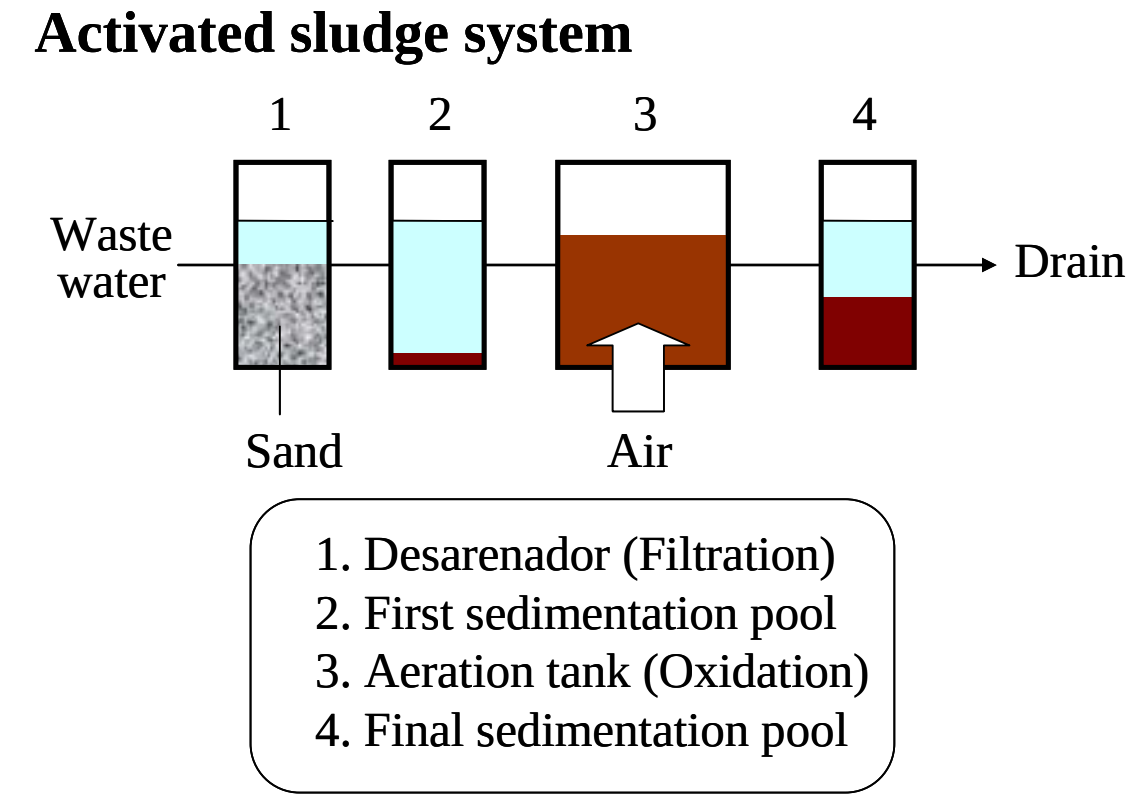


Fig. I-5 Wastewater treatment methods of activated sludge (a) and ozolysis (b).

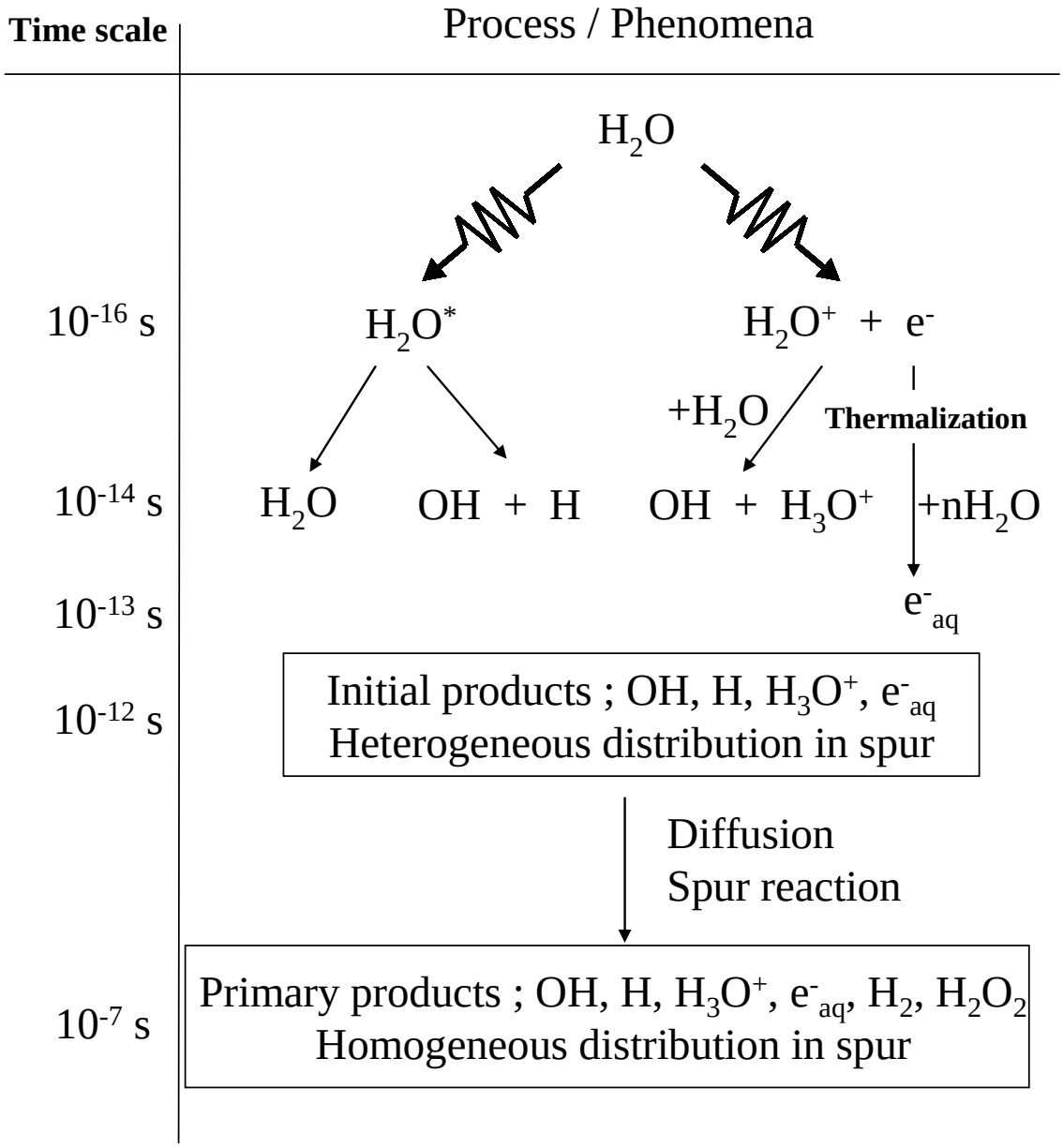


Fig. I-6 Decomposition processes of water by ionizing radiation

Chapter II

Decomposition of 17 β-estradiol by γ-ray irradiation

II-1 Introduction

17 β-estradiol (E2: 1,3,5(10)-estratriene-3,17 β-diol, shown in Fig. I-4, Chapter I) is a steroid hormone produced primarily within the female ovaries and in the male testes. E2 is released into the environmental water from humans and domestic animals. All animals generally produce E2 within their bodies according to their needs and to fulfill their particular purposes. E2 entering into the body from outside, however, interferes with the normal physiological processes and creates many deleterious effects [II-1-2]. Such an external E2 induces serious problems in aquatic organisms and animals by playing as EDCs. These effects appear at concentrations of E2 above about 0.03 nmol dm^{-3} (about 1 ng dm^{-3}) [II-3-4]. Natural hormones such as E2 in the wastewater were incompletely treated by an activated sludge system, and part of them adsorbed to suspended substances was released into the water environment [II-5]. Removal of natural hormones should be a significant matter since their releasing to the water environment is difficult to control.

In this Chapter, the application of ^{60}Co γ-ray irradiation for the decomposition of E2 was studied at extremely lower concentrations than those of the generally used toxic compounds in water. The reaction rate constant of hydroxyl radicals with E2 was estimated relative to that of hydroxyl radicals with phenol. The decomposition of E2 by γ-ray irradiation was examined by a Liquid Chromatography-Mass Spectrometric (LC-MS) system. Estrogen activity of the aqueous solution was evaluated by enzyme-linked immunosorbent assay (ELISA) before and after γ-ray irradiation.

II-2 Experimental

II-2-1 Reagents and sample preparation

17 β-estradiol (E2) was obtained from Takeda Chemical Industries, Ltd., Japan. 3,4-Dimethylphenol (DMP, GR, Tokyo Chemical Industry) and 5,6,7,8-tetrahydro-2-naphthol (THN, GR, Tokyo Chemical Industry) were used as model chemicals in order to clarify the oxidation mechanism of E2. Phenol (Wako Pure Chemical Industries, Ltd., >99.0%) was used without further purification as a reference material to evaluate the rate constant of the reaction of E2 with hydroxyl radicals. E2 and phenol at 1 μmol dm^{-3} each were dissolved in pure water. Water was purified by the ULTRA-PURE WATER SYSTEM (MILLIPORE) to that of 18.2-MΩ cm^{-1} electric conductivity. Oxygen was purged from the solutions by bubbling with He gas for 30 min.

For the study on the decompositions of E2 by ^{60}Co γ-ray irradiation, aqueous E2 solutions were prepared at the different concentrations of 1.8, 0.74, and 0.18 nmol dm^{-3}. The aqueous E2 solutions at around 5×10^{-3} dm^3 were poured into the glass vessels (volume: ϕ 12 × 104 mm, glass thickness: 1 mm) with screw caps saturated with air, He, O_2, or N_2O to investigate the effect of gas in the solution.

II-2-2 γ-ray irradiation

The γ-ray irradiations were carried out at the different temperatures of 273, 283, 293, 298, 313 and 348 K at Japan Atomic Energy Agency (JAEA), Takasaki to the doses in the range from 1 to 100 Gy at the dose rates (*DR*) ranging from 10 to 150 Gy h^{-1} using ^{60}Co γ-ray plague sources (452.4 × 1755.2 mm, 4 TBq and 452.4 × 1974.6 mm, 2011 TBq). The apparatus of γ-ray irradiation is shown in Fig. II-1. The aqueous E2 solution was set on a parallel to

the radiation source. Dosimetry was carried out with alanine (2-aminopropanoic acid) dosimeter (alanine 70%, polystyrene 30%, volume: ϕ 3 × 30 mm) in plastic container (ϕ 12 × 50 mm, 4 mm in thickness) to determine the absorbed dose. The stable radicals are produced in the alanine crystalline by the following scheme:

$$CH_3CH(NH_2)COOH \xrightarrow{\sim\sim} CH_3\dot{C}HCOOH + \dot{N}H_2 \qquad \text{(II-1)}$$

Electron spin resonance (ESR) spectrum of the radicals was measured with an ESR spectrometer (JES-FR30, JEOL). The peak height of the ESR spectrum was related to the amount of the radicals [II-6].

II-2-3 Evaluation of reactivity of 17 β-estradiol with species in water

In order to evaluate the reactivity of E2 with species produced by irradiation, the aqueous mixed solution of E2 and phenol was analyzed by High Performance Liquid Chromatography (HPLC; Agilent 1100 series) with a reversed phase column (Shodex RSpak DE-613) at 313 K. Water with 70%(v/v) methanol (Wako Pure Chemical Industries, Ltd. >99.7 wt% for HPLC solvent) was used as an eluent, and its flow rate was controlled at 1.0×10^{-3} dm^3 min^{-1}. The absorbance at wavelength of 280 nm was measured for the eluted compounds using a UV/VIS detector (Waters, Programmable Multiwavelength Detector 490), since the absorption spectrum of E2 in water shows the maximum at 281 nm as shown in Fig. II-2.

II-2-4 Measurement with an LC-MS system

Aqueous E2 solution at 1.8-nmol dm^{-3} concentration was analyzed by a Liquid Chromatography-Mass Spectrometric (LC-MS) system (JEOL, JMS-LC

mate) with the column switching method for condensing E2 beyond detection limit of the MS. Fig. II-3 shows the chromatographic conditions for the column switching methodology in detail. The irradiated solutions were injected twice for 5×10^{-4} dm^3 each, and condensed within a reversed phase column (GL Science, Inertsil ODS-2). Purified water was used as an eluent, and its flow rate was 1.0×10^{-3} dm^3 min^{-1}. The six-port valve switched from position 1 to position 2 after the condensation. The eluent was then graded from 0 to 100% methanol at a linear gradient in 60 min. The condensed samples were extracted, and transferred to a reversed phase column (Shodex, RSpak DE-613). All the eluted compounds were allowed to flow into the mass spectrometer through a monitoring cell with the UV/VIS detector. An atmospheric pressure chemical ionization (APCI) ion source was selected for mass-spectrometry, and was used in the negative mode for selected ion mass at 271.4 amu. The following conditions were used after the optimization to the maximum signal intensities; vaporizer temperature, 793 K; orifice temperature, 373 K; and accelerating voltage, 2500 V. Calibration curves of concentration were drawn using standard E2 dissolved in methanol under the same conditions as for the irradiated samples.

Products by the irradiation from E2 at a 5-μmol dm^{-3} initial concentration were also analyzed using a reversed phase column (GL Science, Inertsil C8-3) by HPLC and detected by the mass spectrometer under the same conditions as mentioned above. The eluent used was as follows: 20% (v/v) aqueous methanol solution for the first 1 min, 20-50% methanol at a linear gradient condition for the next 19 min, and 50% methanol for the last 35 min.

II-2-5 Evaluation of 17 β-estradiol-equivalent concentration by ELISA

The estrogen activities of the above-mentioned aqueous E2 solutions before and after γ-ray irradiation were estimated as the E2 equivalent concentration using a 17 β-estradiol ELISA Kit (Takeda Chemical Industries, Ltd.) [II-7]. E2 solution with the initial concentration lower than 0.18 nmol dm^{-3} was condensed for the measurements. The C-18 bonded cartridge was washed with methanol and subsequently with pure water. The irradiated aqueous E2 solution was passed through the cartridge. The cartridge containing E2 was washed with pure water and hexane in turn. E2 was then eluted with 5×10^{-3} dm^3 dichloromethane. The effluents were completely evaporated to dryness with a gentle stream of N_2 gas and dissolved into 1.0×10^{-4} dm^3 of 10% (v/v) aqueous methanol solution.

Fig. II-4 is a schematic representation of the experimental method of ELISA. The first step is the competition reaction. The E2 solutions are mixed with solution of a hapten-protein conjugate. The mixed solution is infused into a cell, which has monoclonal antibodies on inside-wall. The second step is the color reaction. After incubation at room temperature for 1 h, the cell is washed with a washing reagent for three times, and filled with a color reagent (aqueous solution mixed citrate salt, phosphorate salt, hydroxy peroxide and 3,3',5,5'-tetramethylbenzidine). The reaction is stopped after 30 min by a reaction-stop reagent (phosphoric acid). The third step is the evaluation of the E2 equivalent concentration. Absorbance of the E2 solutions after the coloring reaction is measured using a micro plate reader (Bio-Rad Model 550) at 450 nm. Calibration curves of the E2 equivalent concentration against the optical density

are drawn using the standard E2 solutions just before measurement of the irradiated samples.

II-3 Results and Discussion

II-3-1 Estimation of rate constants based on HPLC measurement

Hydroxyl radicals rapidly attack phenol with rate constants of k_{OH} = 6.6 × 10^9 mol^{-1} dm^3 s^{-1} [II-8], while hydrated electrons and hydrogen atoms react slowly with phenol (k_{e^-} = 2 × 10^7 mol^{-1} dm^3 s^{-1} and k_H = 1.7 × 10^9 mol^{-1} dm^3 s^{-1}) [II-9]. Reaction probabilities of radicals with phenol are related to the product of the primary yield and the rate constant:

$$\text{Reaction probability of OH}\,(\%) = 100 \times \frac{k_{OH} * G(OH)}{k_{OH} * G(OH) + k_{e^-} G(e^-) + k_H G(H)} \quad \text{(II-2)}$$

G-values of the primary yields of hydroxyl radicals, hydrated electrons and hydrogen atoms are 2.72, 2.63 and 0.55 as listed in Table I-1, Chapter I. Therefore, more than 94% of phenol molecules were degraded by hydroxyl radicals. In the presence of oxygen, hydrated electrons and hydrogen atoms are converted into superoxide radical anions immediately [II-10], which are very less reactive compared with hydroxyl radicals [II-11]. The reactions of benzene and its derivatives with hydroxyl radicals have been studied and found to involve the addition of a hydroxyl radical to aromatic ring because of its strong electrophilicity [II-8-15].

Both phenol and E2 have a phenyl ring, and hydroxyl radicals would attack mainly the phenyl ring. Thus phenol can be regarded as a standard to estimate the relative rate constant of E2 with hydroxyl radicals.

$$\text{E2} + \text{OH} \xrightarrow{k_{\text{E2}}} \text{products} \qquad \text{(II-3)}$$

$$\text{Phenol} + \text{OH} \xrightarrow{k_{\text{Phenol}}} \text{products} \qquad \text{(II-4)}$$

k_{E2} and k_{phenol} are the reaction rate constants of hydroxyl radicals with E2 and phenol, respectively. The percentage of hydroxyl radicals reacting with E2 among the total hydroxyl radicals should be related to the rate constants with solutes at the same concentration of E2 and phenol. When the dose rate (*DR*) is constant, the decomposition yield of E2 can be expressed by the rate constant and the concentration of hydroxyl radicals:

$$-\frac{\mathrm{d}[\text{E2}]}{\mathrm{d}D} = -\frac{1}{DR}\frac{\mathrm{d}[\text{E2}]}{\mathrm{d}t} = -\frac{1}{DR}k_{\text{E2}}[\text{E2}][\text{OH}] \qquad \text{(II-5)}$$

where *D* and *t* are dose and irradiation time, respectively. In this study, the concentrations of both solutes were 1 μmol dm^{-3}, and the decomposition ratio of E2 to phenol is the ratio of the rate constants under the same dose rate irradiation:

$$-\frac{\mathrm{d}[\text{E2}]}{\mathrm{d}D} \Big/ -\frac{\mathrm{d}[\text{Phenol}]}{\mathrm{d}D} = k_{\text{E2}} / k_{\text{Phenol}} \qquad \text{(II-6)}$$

Fig. II-5 shows the decomposition of E2 and phenol at the 1-μmol dm^{-3} initial concentration by γ-ray irradiation. The decomposition of solutes was controlled not to exceed 20% within 3 Gy. Accordingly, the reactions of hydroxyl radicals with irradiation products from E2 or phenol are negligible to the first approximation. The reduction of concentrations can be adequately fitted by the straight lines. The ratio of the decomposition yield of E2 to that of phenol was determined to be 2.44 from the slope of the fitted lines. The reaction rate constant of E2 with hydroxyl radicals (k_{E2}) was estimated to be 1.6×10^{10} mol^{-1}

$dm^3\ s^{-1}$ since k_{Phenol} is $6.6 \times 10^9\ mol^{-1}\ dm^3\ s^{-1}$ [II-8]. This large rate constant of E2 should be due to an increment of the electron density on the phenyl ring because of an electron-donating property of cycloalkane.

Decomposition of 3,4-Dimethylphenol (DMP) and 5,6,7,8-tetrahydro-2-naphthol (THN) in water were also investigated by γ-ray irradiation to compare the rate constants with that of E2. The reaction rate constants of DMP and THN with hydroxyl radicals were estimated to be 1.5×10^{10} and $1.2 \times 10^{10}\ mol^{-1}\ dm^3\ s^{-1}$, respectively, by using the competition reaction method. The values are close to the rate constant of E2 as represented in Table II-1.

II-3-2 17 β-estradiol decomposition analysis with LC-MS system

A mass spectrum of E2 at 1.8-nmol dm^{-3} concentration was obtained by the LC-MS system in the negative mode of the APCI ion source, and a peak identified as E2 was determined at a mass to charge ratio (*m/z*) of 271.4 amu, which comes from proton-release from E2 (271.4 = 272.4 - 1). Fig. II-6 shows the chromatogram recorded by monitoring the selected ion mass at 271.4 of E2 in water. The largest peak observed at the 58-min retention time was assigned to E2 itself, and the area of this peak was linearly proportional to the concentration of E2. The systematic back-ground peaks were also observed in all time regions.

Concentrations of E2 were estimated from the area of the relevant peaks in the chromatograms recorded before and after irradiation. The concentration of E2 in air-saturated water decreased exponentially with absorbed dose, as shown in Fig. II-7. The concentration of E2 decreased by more than one order of magnitude with the γ-ray irradiation of 10 Gy; the concentration of E2 decreased from 1.8 nmol dm^{-3} to less than 0.05 nmol dm^{-3} at a dose of 10 Gy. This concentration is known as the threshold level below which E2 does not show

any effect on the environmental ecology [II-3, 4].

The number of E2 molecules is 1.1×10^{15} molecules dm^{-3} at 1.8-nmol dm^{-3} concentration. The number of hydroxyl radicals produced is estimated to be 1.75×10^{17} molecules dm^{-3} (290 nmol dm^{-3}) at the dose of 1 Gy assuming *G*-value of 2.8 for hydroxyl radicals [II-15]. Because the number of hydroxyl radicals is sufficiently large compared with that of E2, the concentration of hydroxyl radicals can be regarded as a constant during the irradiation and therefore the reaction is considered to be the pseudo first order reaction: the degradation behavior of E2 should be expressed by an exponential curve as the function of the dose as shown in Fig. II-7.

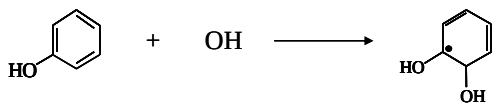
II-3-3 Reduction of 17 β-estradiol-equivalent concentration

Degradation of E2 by γ-ray irradiation was observed on the basis of LC-MS analysis. The decrease in the estrogen activity of the sample solution after the irradiation must be confirmed because the products of the EDCs formed by irradiation would show estrogen activity. The estrogen activities (E2 equivalent concentration) of the sample solution before and after irradiation were evaluated by ELISA. This method does not indicate the real E2 concentration but the total concentration of the chemicals with estrogen activity. Therefore, if the irradiation products have no estrogen activity, the reduction curves of the E2 equivalent concentration must be the same as those of the E2 concentration after the irradiation. The reductions in the E2 equivalent concentration as a function of dose are presented for the samples with the initial E2 concentration of 1.8(△), 0.74(□), and 0.18(○) nmol dm^{-3} in Fig. II-8. The E2 equivalent concentration decreased to almost zero at a dose of 30 Gy. The decrease in the E2 and E2 equivalent concentrations are different as shown in Fig. II-9. A

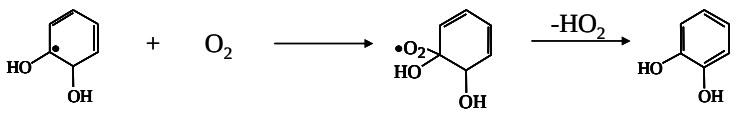
possible explanation of this fact is that the primary products formed from E2 by γ-ray irradiation have the estrogen activity, and these products should be further decomposed by additional irradiations.

Fig. II-10 shows chromatograms of 5-μmol dm^{-3} E2 solution after irradiation observed at *m/z* of 271.4 amu (a) and 287.4 amu (b). This 5-μmol dm^{-3} concentration is so low that interaction may not occur among reaction intermediates produced from E2 molecules. The peak of E2 was observed at 49 min in the chromatogram observed at *m/z* of 271.4 amu. Intense two and weak five peaks in the chromatogram at *m/z* of 287.4 amu are additionally observed at the retention time from 23 to 36 min. These peaks may be assigned to OH substitution products of E2 having the estrogen activity.

Influence of gases saturated in the aqueous solution on the decomposition yields of E2 equivalent concentration was studied, and the relation of decomposition yields and dose are shown in Fig. II-11. Degradation yields of E2 in the air-saturated solution are almost the same as those in the absence of oxygen under saturated helium. The decomposition yield increased slightly under oxygen saturated condition. For phenolic compounds, oxygen attacks cyclohexadienyl-type radicals to give peroxyl radicals, and then HO_2 group is released to give OH substitution products shown in the following equation II-8 [II-15]. E2 may have the same oxidation mechanism as phenolic compounds.



(II-7)



(II-8)

The highest degradation yield of E2 equivalent concentration was obtained in

the presence of N_2O gas in the aqueous solution. It is due to the conversion of the hydrated electrons into hydroxyl radicals by N_2O shown in the following equation II-9 [II-16-18]:

$$N_2O + e^- + H_2O \longrightarrow OH + OH^- + N_2 \quad \text{(II-9)}$$

The yield of hydroxyl radicals in the N_2O-saturated aqueous solution becomes about twice (G (OH) + G (e^-) = 5.3) of that in the He-saturated aqueous solution (G (OH) = 2.7) as shown in Table I-1, Chapter I [II-15]. Therefore, a half dose for the He saturated solution was required at the same decomposition yields of E2 for the N_2O saturated solution, as shown in Fig. II-11. This is obvious evidence for the decomposition of E2 by hydroxyl radicals

Decomposition yield of E2 does not show any temperature dependence ranging from 273 to 348 K during irradiation. The G-value of hydroxyl radicals increases slightly with temperature from 2.8 at 275 K to 3.13 at 338 K [II-15]. As mentioned above, the amount of hydroxyl radicals produced even at G (OH) = 2.7 was about 100 times higher than that of E2 in this experiment, and a small increment of hydroxyl radicals does not influence the E2 decomposition [II-15].

II-4 Conclusion

Trace amounts of E2 in water was degraded almost completely by ^{60}Co γ-ray irradiations up to 10 Gy. The estrogen activity of the E2 solution, however, still remained after the 10-Gy irradiation. Further irradiations up to 30 Gy are needed to decrease the estrogen activity to the level lower than the threshold of contamination to induce some estrogenic effects on the environmental ecology. A half dose for the He saturated solution was required at the same

decomposition yields of E2 for the N_2O saturated solution, this is obvious evidence for the decomposition of E2 by hydroxyl radicals.

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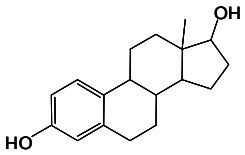
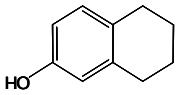
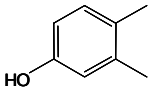
Table II-1 Reaction rate constants of E2 and its model chemicals with hydroxyl radicals

			
	E2	**THN**	**DMP**
Ratio of the decomposition yield of sample to that of phenol	2.44	2.21	1.85
Reaction rate constant of sample with OH (mol^{-1} dm^3 s^{-1})	1.6×10^{10}	1.5×10^{10}	1.2×10^{10}

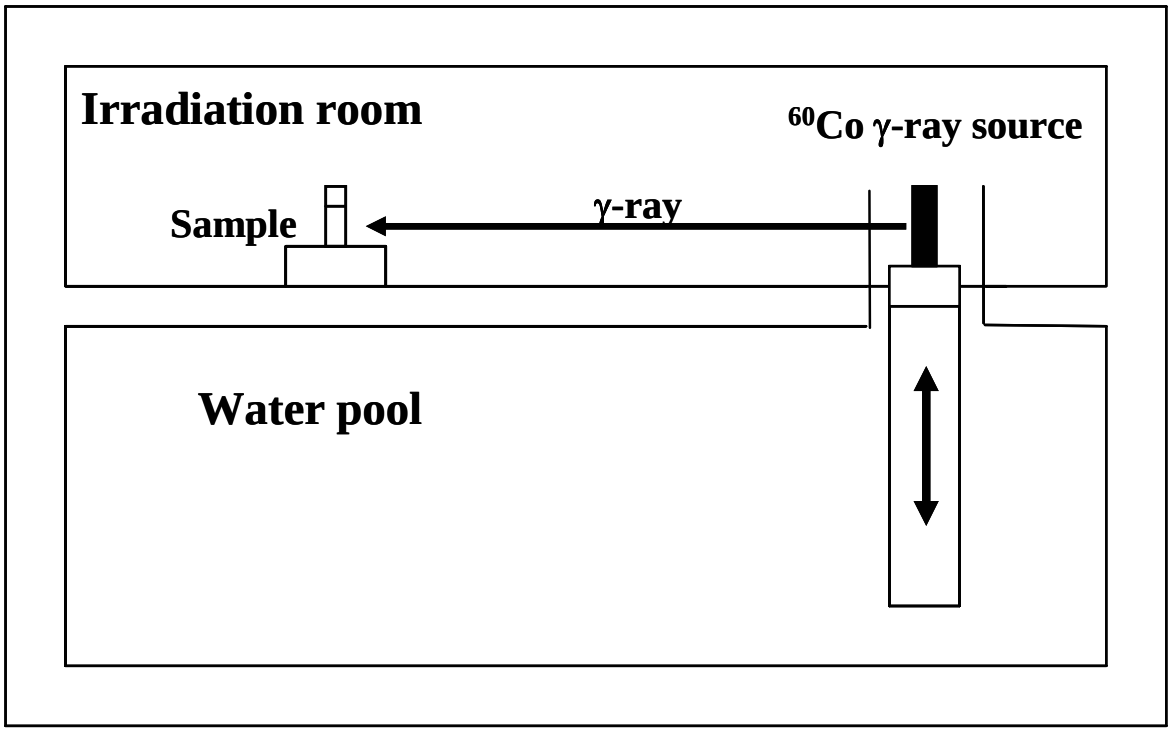


Fig. II-1 Apparatus of ^{60}Co γ-ray irradiation facility at Japan Atomic Energy Agency.

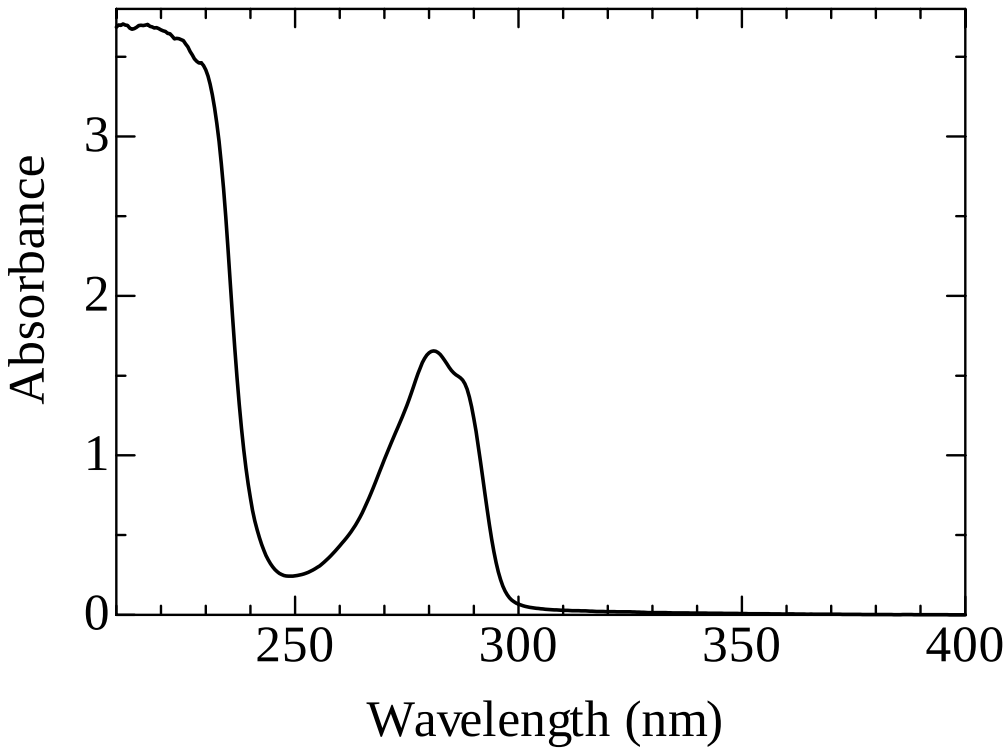
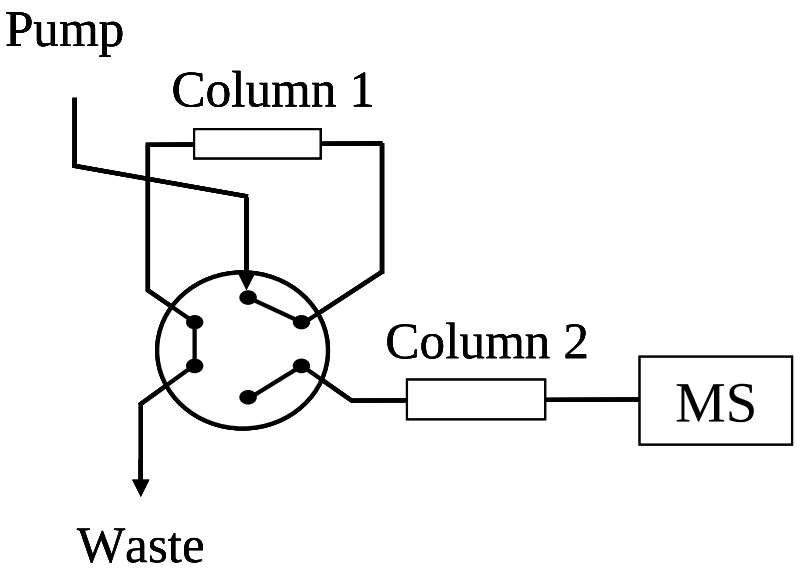
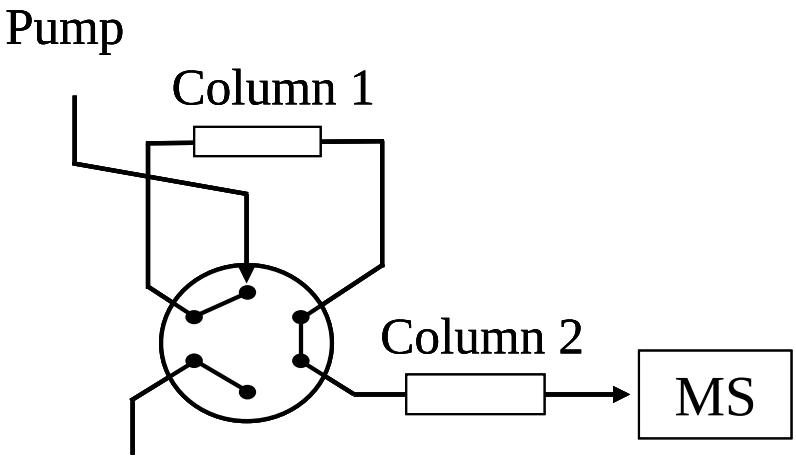


Fig. II-2 Absorption spectrum of 17 β-estradiol in water.



Position 1.



Position 2.

Fig. II-3 Schematic diagram of the column-switching Liquid Chromatography-Mass Spectrometric system.

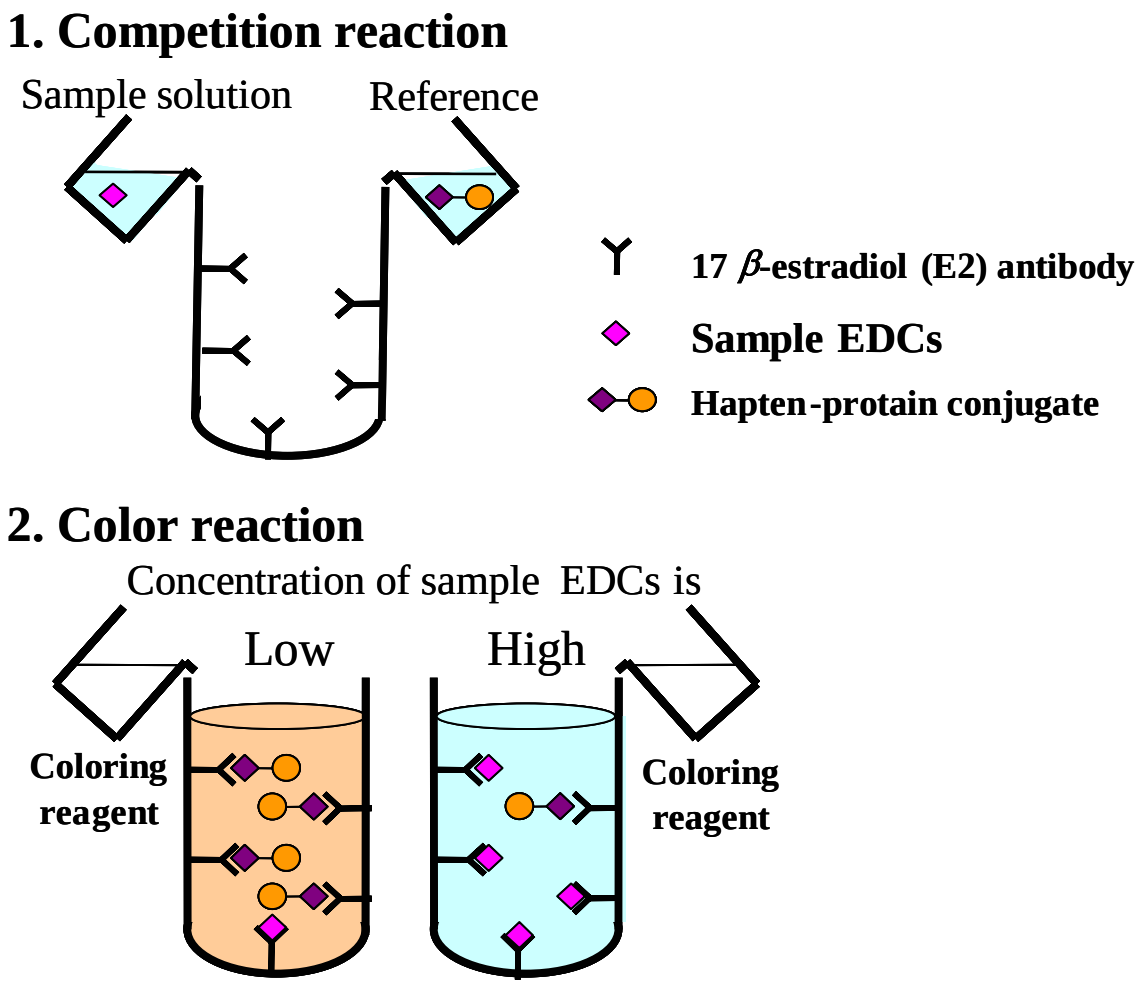


Fig. II-4 Experimental method of enzyme-linked immunosorbent assay (ELISA).

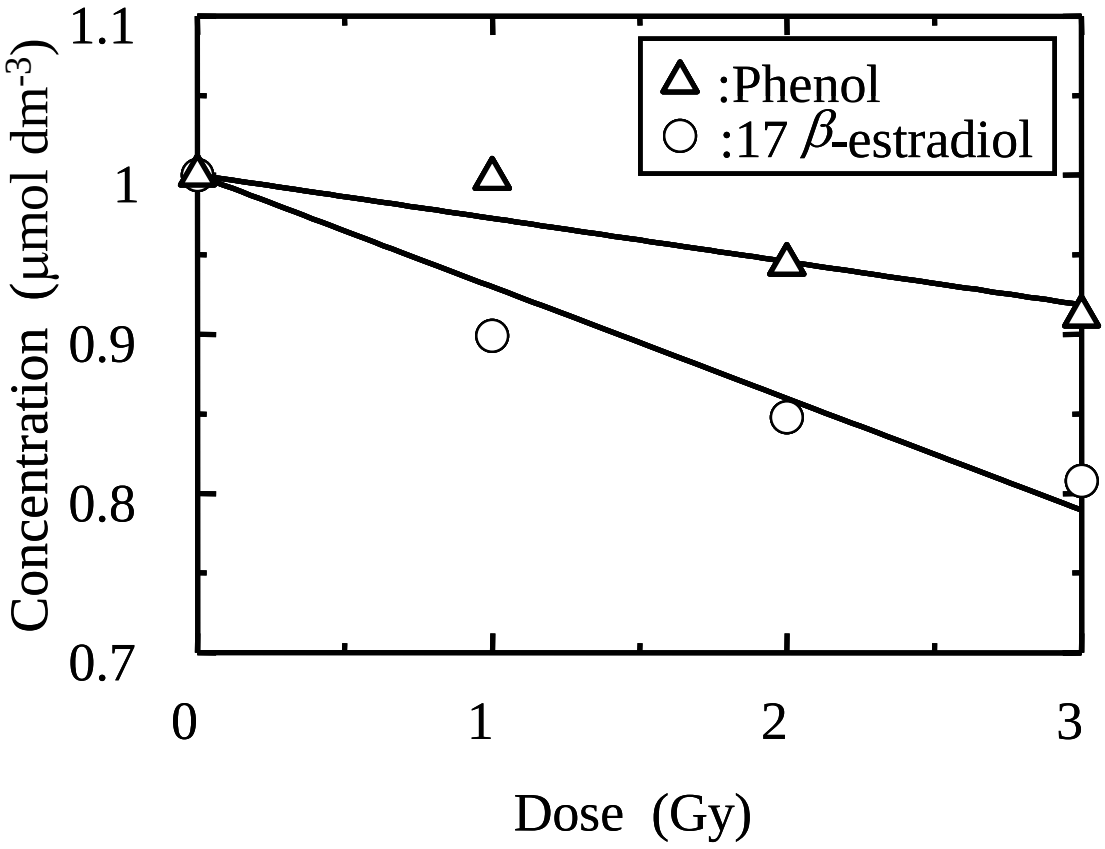


Fig. II-5 Decomposition of 17 β-estradiol and phenol in He-saturated water by ^{60}Co γ-ray irradiation at the initial concentration of 1 μmol dm^{-3} each.

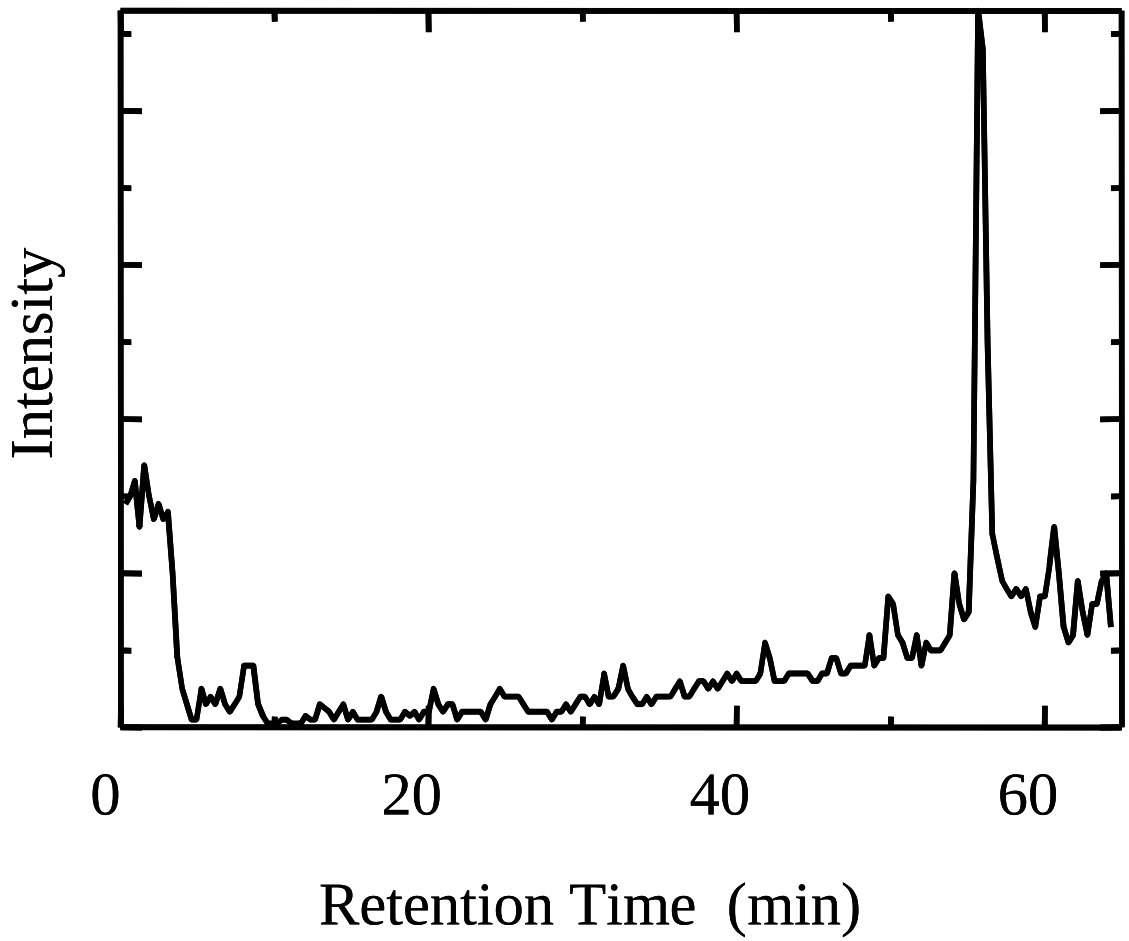


Fig. II-6 Mass chromatogram at a mass to charge ratio (*m/z*) of 271.4 amu of 17 β-estradiol in water at 1.8-nmol dm^{-3} concentration.

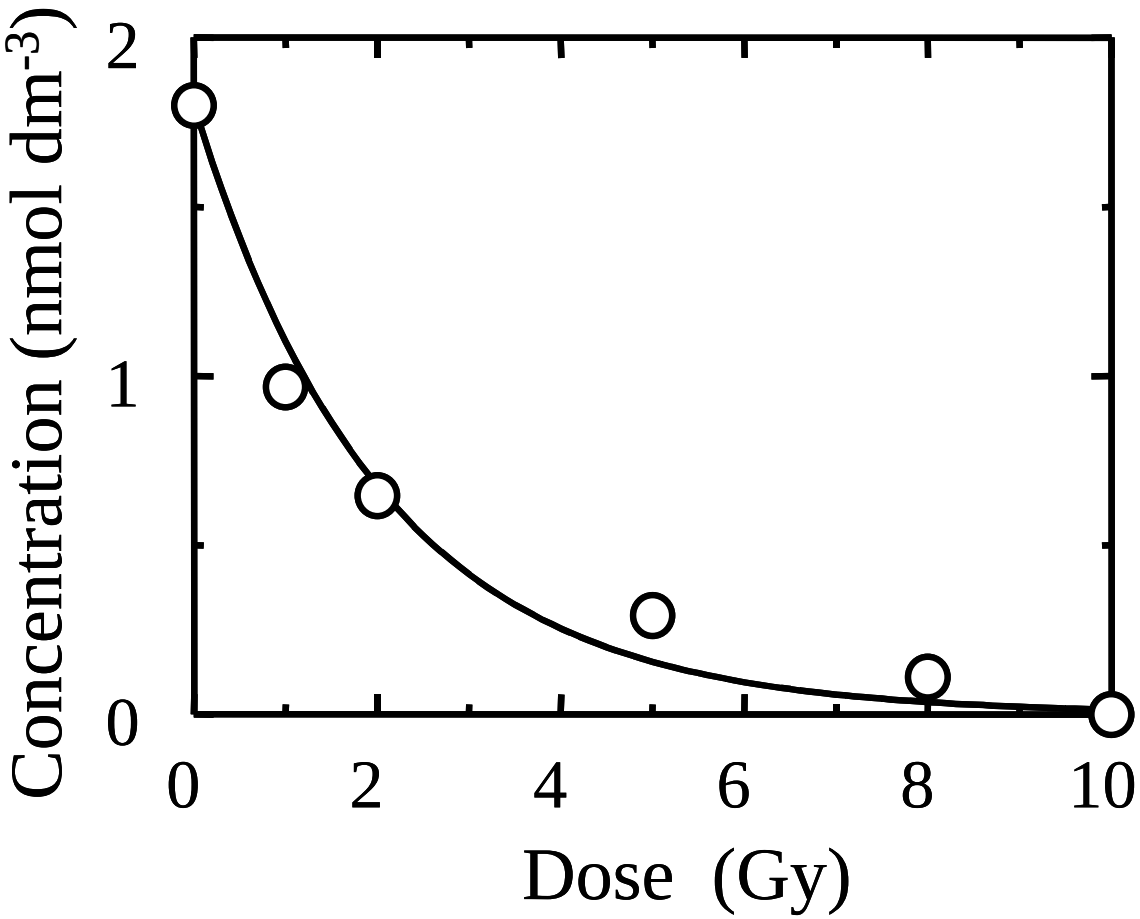


Fig. II-7 Decomposition of 17 β-estradiol in water by γ-ray irradiation measured by using the column-switching Liquid Chromatography-Mass Spectrometric system.

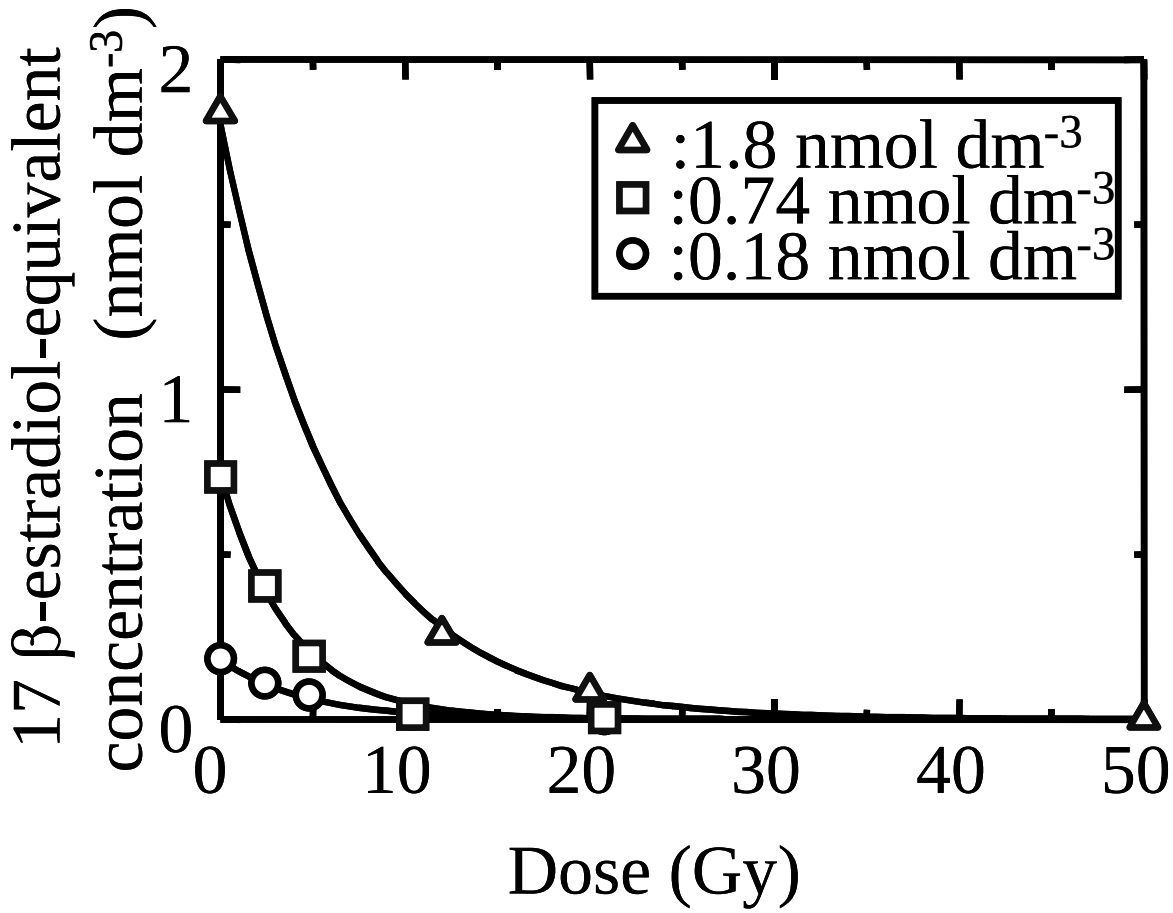


Fig. II-8 Decrease in 17 β-estradiol-equivalent concentration at 1.8(△), 0.74(□), and 0.18(○) nmol dm^{-3} measured by the enzyme-linked immuno-sorbent assay (ELISA) after γ-ray irradiation.

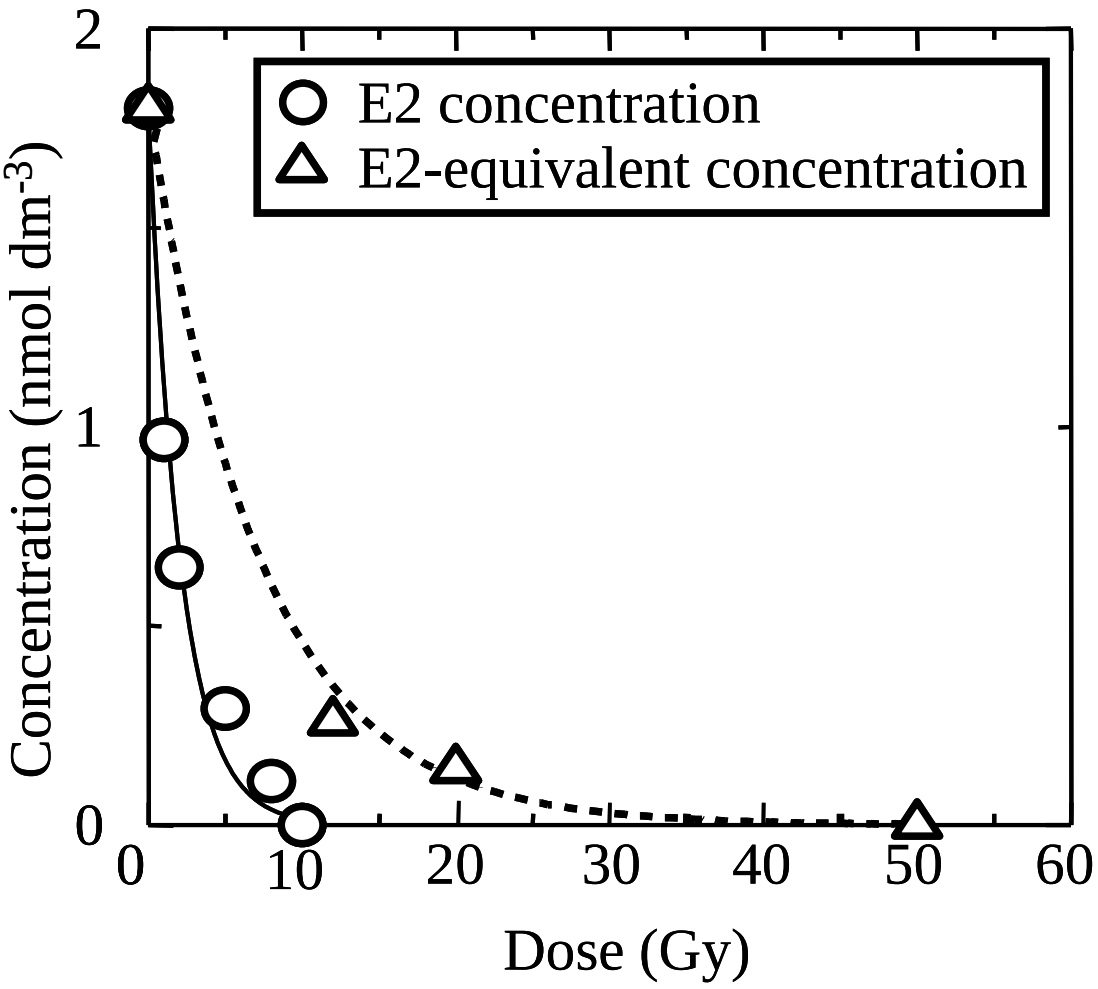


Fig. II-9 17 β-estradiol (△) and 17β-estradiol equivalent concentrations (□) as a function of dose under the air saturated condition.

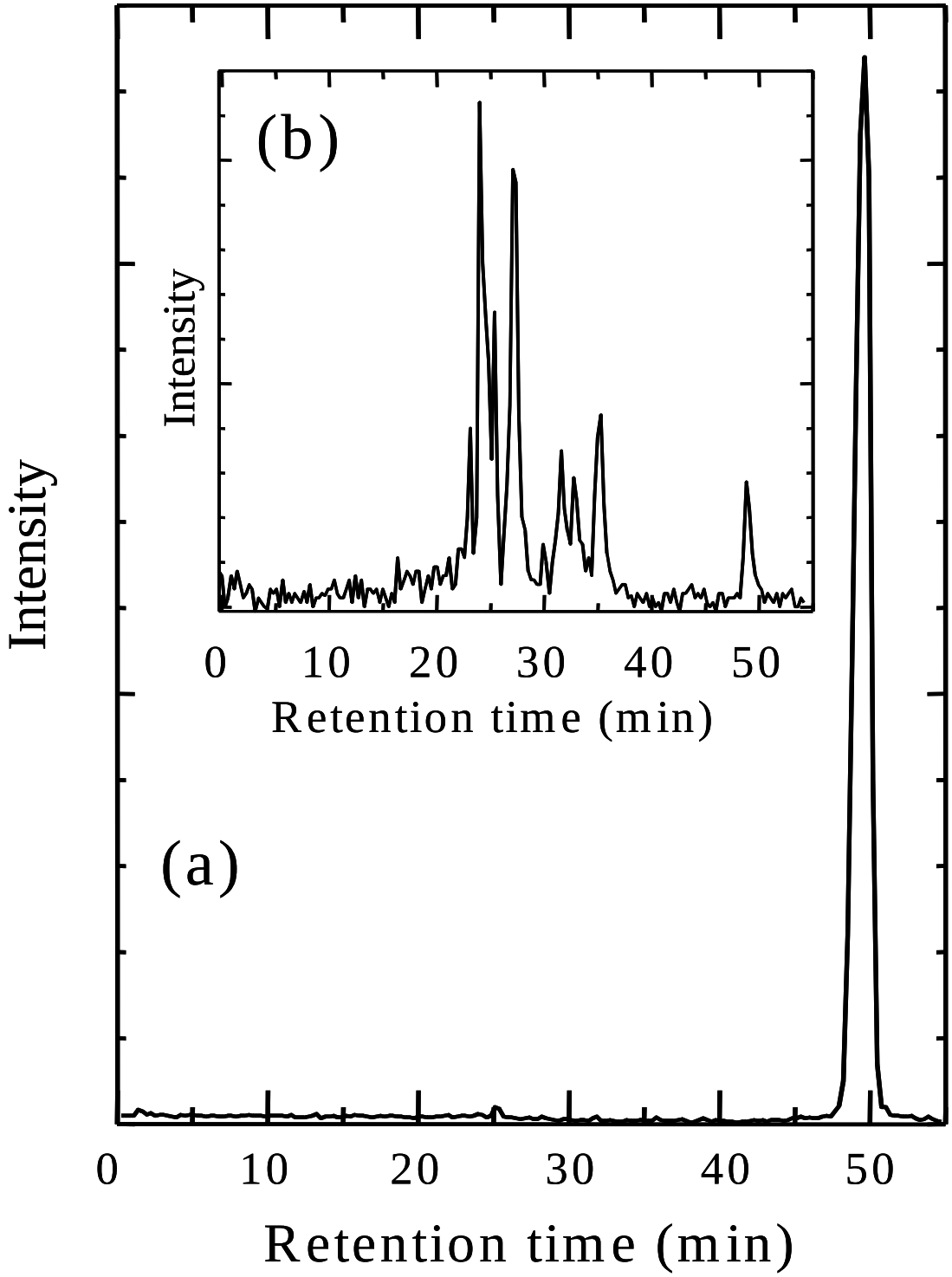


Fig. II-10 Mass chromatogram at mass to charge ratio of 271.4 amu (a) and 287.4 amu (b) of 17 β-estradiol in water after γ-ray irradiation. The initial concentration of 17 β-estradiol is 5 μmol dm^{-3}.

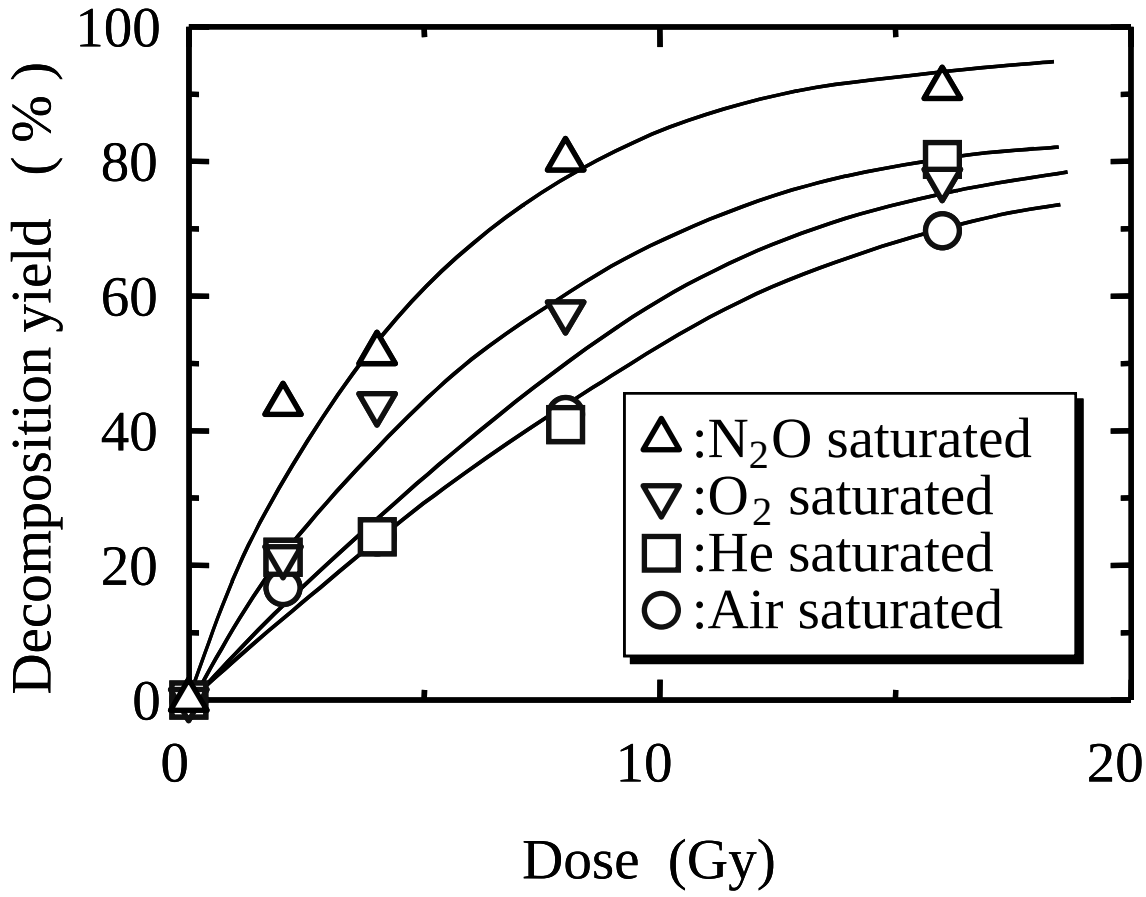


Fig. II-11 Decomposition yields of 17 β-estradiol-activity at the 1.8-nmol dm^{-3} initial concentration in aqueous solution saturated with nitrous oxide (△), oxygen (▽), helium (□) and air (○) by γ-ray irradiation.

Chapter III

Decomposition of *p*-nonylphenols by γ-ray irradiation

III-1 Introduction

p-Nonylphenols (NPs), one of the persistent and toxic artificial chemicals, are generated from a dissolution of additives used as plastic flexibilizers or biodegradation of alkylphenol polyethoxylates (APE) as non-ionic surfactants [III-1-3]. NPs have the highest estrogen activity among artificial chemicals, and show the estrogen activity at the concentration above about 1 μmol dm^{-3} [III-4-6]. NPs and APE are produced about 17,000 and 47,000 tons $year^{-1}$ in Japan, and about 40,000 tons $year^{-1}$ of them are totally released into the water environment. Some artificial chemicals having endocrine disrupting property was investigated by the Ministry of the Environment, Japan in 1998 [III-2]. It was concluded that NPs, *tert*-octylphenol and bisphenol A disrupted the endocrine system of fishes. There is, however, no legal regulation on drain of them into the water environment in Japan. In this Chapter, the reduction of decomposition of aqueous NPs solution was studied by means of ^{60}Co γ-ray irradiation.

III-2 Experimental

III-2-1 Sample preparation and γ-ray irradiation

p-Nonylphenol mixture consisting of isomers with branched side chains (Tokyo Chemical Industry) was used as commercially available *p*-nonylphenol reagent. *p*-Cresol (GR, Tokyo Chemical Industry) and 4-ethylphenol (EP, Tokyo Chemical Industry) were used as model chemicals in order to clarify the oxidation mechanism of NPs. Phenol (99.0%, Wako) was used as a standard for the competition reaction method. Ultra-pure water (Total organic carbon : 4 ppb, electric resistance : 18.2 MΩ cm^{-1}) was supplied from a Milli-Q system

(MILLIPORE). Methanol (for HPLC Analysis, Wako) was used as organic eluent for HPLC analyses. Ferrous sulfate heptahydrate (GR, Wako) and hydrogen peroxide (GR, MITSUBISHI GAS CHEMICAL CO., INC.) were used for the decomposition of NPs by the Fenton reaction to prepare the reference decomposition products.

NPs sample solutions at the concentration of 0.045 to 20 μmol dm^{-3} were saturated with each of air, He and O_2, and full-filled into glass vessels (volume: ϕ12 × 104 mm, glass thickness: 1 mm) with screw caps. Concentration of dissolved oxygen in the NPs solution was measured using a pH / DO meter (D-25, HORIBA) within the error range about ±3 μmol dm^{-3}. The γ-ray irradiations were carried out at 298 K using three different ^{60}Co γ-ray sources (4 TBq, 2011 TBq and 10567 TBq) at JAEA, Takasaki, in the range of the doses from 1 to 5,000 Gy at dose rates ranging from 5 to 2,000 Gy h^{-1}.

III-2-2 Analysis

An HPLC (Agilent 1100 series) with a reversed phase column (Shodex RSpak DE-613 and Inertsil C8-3 GL Sciences) at 313 K was used for analyses of NPs solutions before and after γ-ray irradiation. Flow rate of 80 volume percent (vol %) methanol as an eluent was 1.0×10^{-3} dm^3 min^{-1}, and the sample solutions were injected at 5.0×10^{-4} dm^3 in the HPLC. Fig. III-1 shows absorption (a), emission (full line of b) and excitation (broken line of b) spectra of NPs in water. The absorption maximum was observed at 275 nm in water, and the emission spectrum was recorded on the excitation at 280 nm, and excitation spectrum was monitored at 310 nm in water. For HPLC analysis, absorbance was monitored at 280 nm using a UV/VIS detector (Waters 2487 Dual λ Absorbance Detector) and the emission was detected with a fluorescence

detector (Waters 2475 Multi λ Fluorescence Detector) at 310 nm by the 280-nm light excitation.

Mass spectrometer (JEOL, LC-mate) connected with the HPLC was used to determine molecular weights of NPs and their irradiation products. Atmospheric pressure chemical ionization in the negative mode was selected, so that the molecular weights of NPs and their irradiation products were measured as the negative ions with molecular weight of solute minus one (MW-1).

Amounts of the total organic carbon (TOC) in the NPs solutions were measured by a TOC analyzer (Shimadzu, TOC-Vwp) before and after γ-ray irradiation. Concentrations of organic acids obtained as irradiation products were evaluated by ion chromatograph (Metrohm 761, Compact IC) with an anion suppressor. Sample solutions were separated by a column (IC SI-90 4E Shodex) for the anion analysis. An aqueous solution composed of 1.7 mmol dm^{-3} sodium hydrogen carbonate (Cica-Reagent, KANTO CHEMICAL CO., INC.), 1.8 mmol dm^{-3} sodium carbonate (Cica-Reagent, KANTO CHEMICAL CO., INC.) and 5% acetonitrile (for HPLC Analysis, Wako), was used as an eluent at a flow rate of 1.2×10^{-3} dm^3 min^{-1}.

The estrogen activities of NPs solution were measured by the yeast two-hybrid assay using yeast cells (*Saccharomyces cervisiae* Y190) into which the human estrogen receptor ERα and the coactivator TIF2 had been introduced [III-7]. Aqueous NPs solutions were used after dilution so as not to change the characteristics of the modified Sabouraud's dextrose (SD) medium (lacking tryptophan and leucine). Aliquots of test solutions were incubated (303 K, 4 h) with the yeast cells that had been preincubated (303 K, overnight) in the modified SD medium. A mixed solution inducing a chemiluminescence (AURORA™ GAL-XE, MP Biomedicals), an enzymatic digestion (Zymolyase

20T, SEIKAGAKU CORPORATION), and a light emission accelerator (AURORA™ GAL-XE, MP Biomedicals) were added in this order to the test solutions. The chemiluminescence of β-galactosidase produced by binding of sample with estrogen receptor was measured with a luminometer (LUMINESCENCE READER BLR-301, Aloka).

NPs concentrations before and after γ-ray irradiation were evaluated by the enzyme-linked immunosorbent assay (ELISA) using AP ELISA Kits (ECOLOGIENA) [III-8]. Sample preparation and measurement were the same as mentioned in section II-2-5, Chapter II.

III-3 Results and Discussion

III-3-1 Decomposition of *p*-nonylphenol

A fluorescence detector and an absorbance detector are available for the liquid chromatograms of NPs at initial concentrations ranging from 0.045 to 20 μmol dm^{-3} and 1 to 20 μmol dm^{-3} were recorded by HPLC, respectively. At first the initial concentration of NPs was set at 1 μmol dm^{-3}, which was a threshold concentration for an appearance of the estrogen activity to the aquatic animals [III-3, 5, 6]. Chromatogram of NPs at the 1 μmol dm^{-3} before irradiation is shown in Fig. III-2. A negative singal at the retention time of 5 min resulted from the system of HPLC. Weak peaks at retention times of 9.6, 12.2 and 13.5 min could be due to impurities of NPs. An intense peak of NPs was observed at the retention time of 18 min. The peak seems to be broad because NPs are mixture of isomers of NPs. The concentration of NPs was quantitated with the peak area integrated from 15 to 25 min.

Generally, hydroxyl radicals are main reactive species for radiolysis of

organic compounds in water [III-9-10]. In aqueous phenol solution, the decomposition yield of phenol by hydroxyl radicals was determined to be 94% as mentioned in II-3-1, Chapter II [III-11]. Other phenolic compounds such as alkylphenols will be also mainly decomposed by hydroxyl radicals. In order to determine main reactive species responsible for the decomposition of NPs, aqueous NPs solutions were saturated with He or N_2O. Fig. III-3 shows the decomposition curves of NPs in water saturated with N_2O and He. The decomposition efficiencies were estimated to be 2.8×10^{-7} and 1.5×10^{-7} μmol dm^{-3} Gy^{-1} by fitting the decomposition curve with a straight line, respectively. The decomposition efficiency in solutions saturated with N_2O was about two times higher than that with He. N_2O reacts with hydrated electrons to form hydroxyl radicals, hydroxide ions and nitrogen in water as mentioned in section II-3-3, Chapter II [III-12]. According to Table I-1 in Chapter I, *G*-values of hydrated electrons and hydroxyl radicals are 2.6 and 2.7, respectively. *G*-value of production of hydroxyl radicals under the N_2O saturated condition is about twice (G (OH) + G (e^-) = 5.3) of that under the He saturated condition. Therefore, it can be concluded that hydroxyl radicals are responsible for the decomposition of NPs. This conclusion is supported by the fact that the decomposition of NPs in solution was suppressed in the presence of hydroxyl radical scavengers such as phenol.

The reaction rate constant of NPs with hydroxyl radicals was estimated by using the competition reaction method. Phenol is regarded as a suitable compound for the estimation of the rate constant of NPs with hydroxyl radicals since hydroxyl radicals attack mainly the carbon atoms of the phenyl ring. When the concentrations of both solutes are equal to each other, the ratio of decomposition efficiencies of NPs and phenol is equal to the ratio of the rate

constants under a constant dose rate irradiation:

$$-\frac{\mathrm{d[NPs]}}{\mathrm{d}D} / -\frac{\mathrm{d[Phenol]}}{\mathrm{d}D} = k_{\mathrm{NPs}}/k_{\mathrm{Phenol}} \qquad \text{(III-1)}$$

Fig. III-4 shows decomposition of NPs and phenol by γ-ray irradiation under the deoxygenated condition. The ratio of the decomposition efficiency of NPs to that of phenol was determined to be 4.0 from the initial slope of the decomposition curves. The rate constant of NPs was estimated to be 2.6×10^{10} $\mathrm{mol^{-1}\ dm^3\ s^{-1}}$ by using the reported rate constant of phenol ($k_{\mathrm{Phenol}} = 6.6 \times 10^9$ $\mathrm{mol^{-1}\ dm^3\ s^{-1}}$ [III-13]).

Decomposition curves of NPs at the initial concentration of 1 μmol $\mathrm{dm^{-3}}$ by γ-ray irradiation under O_2, air or He saturated conditions are shown in Fig. III-5. The concentration of oxygen dissolved in the solution saturated with O_2, air and He were determined by a pH/DO meter to be 1125, 220 and almost 0 μmol $\mathrm{dm^{-3}}$, respectively. Decomposition curves of NPs determined under these saturated conditions are essentially the same. Therefore, dissolved oxygen seems to be ineffective on the decomposition. Oxidation of the phenolic compounds, e.g. phenol, by hydroxyl radicals is enhanced by oxygen [III-14-16]. Hydroxyl radicals attack phenol to produce dihydroxycyclohexadienyl radicals, which react with oxygen to produce peroxyl radicals as mentioned in section II-3-3, Chapter II. The peroxyl radicals release hydroxyperoxide radical to produce OH substitution products (catechol, hydroquinone and resorcinol). Production of the peroxyl radicals does not occur under the deoxygenated condition, and the dihydroxycyclohexadienyl radicals may revert to phenol. Accordingly, the oxidation of phenolic compounds by hydroxyl radicals is enhanced by dissolved oxygen. Phenolic compounds including NPs are considered to be oxidized with hydroxyl radicals by the same reaction. Since concentrations of NPs in this

study are very low, even a trace amount of dissolved oxygen below the error level of the pH / DO meter used, about 3 μmol dm^{-3}, would enhance the oxidation of NPs. The pH is an essential factor for the decomposition of organic compounds in water by γ-ray irradiation because reactive species produced by γ-ray irradiation react with oxonium ion or hydroxide ion [III-17].

$$H^+ + e^-_{aq} \longrightarrow H \quad \text{(III-2)}$$

$$OH^- + H \longrightarrow e^-_{aq} \quad \text{(III-3)}$$

Effect of pH on the decomposition of NPs by γ-ray irradiation under the air saturated condition was investigated, and the decomposition efficiency of NPs was found to be constant in the range of pH from 2 to 8.

NPs at different concentrations from 0.045 to 20 μmol dm^{-3} were irradiated to doses from 0 to 5,000 Gy. The dose required for the decomposition of NPs to 50% of the initial concentration, D_{50}, was plotted against the initial concentration to investigate the initial concentration dependence of D_{50} for the decomposition of NPs as shown in Fig. III-6. The D_{50} linearly increased against the initial concentration, indicating that secondary reactions are less important.

III-3-2 Irradiation products

Peak areas monitored at the mass to charge ratio (*m/z*) of 235 amu for irradiation products of NPs at the initial concentration of 1 μmol dm^{-3} as a function of dose by LC-MS analyses are shown in Fig. III-7. The selected ion chromatogram (SIC) of NPs at 1 μmol dm^{-3} after the 12.5-Gy irradiation is shown as an inset. Two products of NPs were detected at retention times of 6.3 and 8.5 min by γ-ray irradiation. Both peak areas increased with dose and reached the maximum at the dose of 5 Gy, and then decreased. Weak signals due

to other products were detected by HPLC with a fluorescence detector, although their molecular weight could not be determined by LC-MS analyses because of absence of any characteristic peak in the mass spectra.

Organic acids were not identified for the decomposition of NPs at the 1-μmol dm^{-3} initial concentration since the concentrations were lower than the detection level (~ 1 μmol dm^{-3}) of the ion chromatograph. Some peaks of organic acids, however, were detected for the decomposition of NPs at the 20-μmol dm^{-3} initial concentration. Two of them were identified as formic acid and oxalic acid, and their concentrations at a dose of 1,000 Gy were estimated to be about 10 and 3 μmol dm^{-3}, respectively.

For the investigation of a degree of oxidation in NPs solution by γ-ray irradiation, amounts of TOC and inorganic carbon were estimated as a function of dose as shown in Fig. III-8. Amount of inorganic carbon increased with dose, and as a result TOC in the solution decreased. Inorganic carbon (IC) is sum of amount of carbon dioxide and carbonate ions as final products of oxidation, and the amount of inorganic carbon proportionally increased with dose at an initial irradiation stage. The yield of inorganic carbon against the initial TOC concentration, i.e. the degree of oxidation in NPs solution, was estimated to be 17% at a dose of 37.5 Gy.

III-3-3 Decomposition of alkylphenols

III-3-3-1 *p*-Cresol

Oxidation of *p*-cresol was investigated by HPLC. Fig. III-9 shows chromatograms of aqueous *p*-cresol solution at the 1-mmol dm^{-3} initial concentration before and after the 1500-Gy irradiation under the air saturated condition. A peak at the retention time of 67 min is assigned to *p*-cresol. Peaks

observed at retention times of 12, 20, 35, 43 and 45 min in Fig. III-9 (b) are assigned to 3,4-dihydroxybenzyl alcohol, 4-hydroxybenzyl alcohol (4HBA), 3,4-dihydroxybenzaldehyde, 4-hydroxybenzaldehyde and 4-methylcatechol (4MC), respectively, by comparing with standard samples. 4HBA and 4MC are considered to be primary products of the reaction of *p*-cresol with hydroxyl radicals, and the other products are secondary products. Formation of organic acids from *p*-cresol was investigated by ion chromatography. Formation of formic acid was confirmed as a peak at the retention time of 3.67 min on the chromatogram as shown in Fig. III-10. A negative signal at the retention time of 2 min was the system signal accompanied with the sample injection, and the other peaks were due to unknown ionic products such as organic acids.

Concentration of *p*-cresol decreased rapidly with dose up to 1000 Gy, and then gradually decreased as shown in Fig. III-11. Amount of IC increased linearly at doses lower than 1500 Gy accompanied with the decrease in the amount of TOC. The concentrations of the primary products such as 4HBA and 4MC, the secondary products and formic acid depend on dose as shown in Fig. III-12. The concentration of 4MC increased with dose up to 4000 Gy, and then decreased. The concentration of 4HBA increased gradually up to 1000 Gy. Dissolved oxygen in the solution was consumed at dose of about 1000 Gy. These results suggest that dissolved oxygen enhance the formation of 4MC and 4HBA, and effect of dissolved oxygen was investigated. *G*-values of 4MC and 4HBA formation from *p*-cresol depend on dissolved oxygen as shown in Fig. III-13. *G*-value of 4MC formation increased with increase of the dissolved oxygen concentration, in contrast to the decrease of 4HBA formation; the production of 4MC was enhanced by oxygen, while that of 4HBA was inhibited.

G-values of 4MC and 4HBA formation increase with the initial

concentration of *p*-cresol. The value of *G*(products) / *G*(- *p*-cresol) is plotted against the initial concentration of *p*-cresol in Fig. III-14. The value of *G*(4MC) / *G*(- *p*-cresol) was constant (□), so that formation mechanism of 4MC would be the same in the range of these initial concentrations. While, the value of *G*(4HBA) / *G*(- *p*-cresol) (△) increased with increase of the initial concentration of *p*-cresol. 4HBA may be formed through bimolecular reaction of reactive intermediates with itself or *p*-cresol.

Fig. III-15 shows the chromatogram of *p*-cresol at the 1-mmol dm^{-3} initial concentration recorded on HPLC and LC-MS after the 1500-Gy irradiation. The peak at the retention time of 18.5 min is assigned to *p*-cresol, and some peaks observed at retention time from 5 to 17 min are ascribable to OH substitution products, e.g. 4MC and 4HBA, and non-aromatic compounds, e.g. formic acid and so on. Peaks at retention times from 40 to 45 min were only detected for the samples with initial concentrations of *p*-cresol higher than 250 μmol dm^{-3}. These peaks are considered to be due to hydrophobic compounds since ODS column retains organic compounds of low solubility in water. Since molecular weight of *p*-cresol is 108, peaks observed at retention times from 32 to 42 min on SIC for *m/z* of 213 amu shown in Fig III-15(b) must be due to dimers of molecular weight of 214 (MW = 213 + 1). Three peaks observed at the retention time of 24, 32 and 35 min and the broad peak from 37 to 48 min on SIC for *m/z* of 229 amu shown in Fig III-15(c) are considered to be due to OH substitution products of dimers of molecular weight of 230 (MW = 229 + 1). Dose dependences of peak areas of the products are shown in Fig. III-16. Peak area of the products having molecular weight of 214 (◎) increased with doses over 500 Gy, and that of molecular weight of 230 (◈) increased with doses over 1000 Gy. Since dissolved oxygen in *p*-cresol solution was consumed at about 1000 Gy as

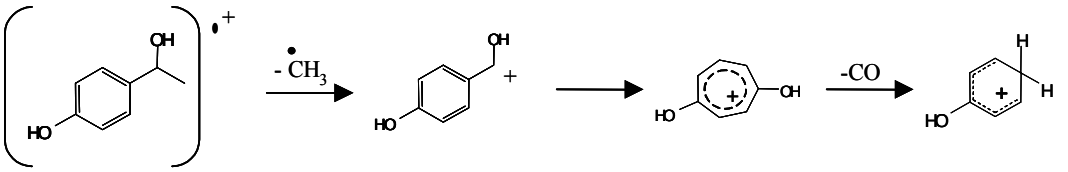
mentioned above, the bimolecular reaction would preferentially occur to produce dimers at low concentrations of dissolved oxygen. The dimers possibly react with hydroxyl radicals to produce OH substitution products. The oxidation mechanism of *p*-cresol is represented in Scheme III-1.

III-3-3-2 4-Ethylphenol

Liquid chromatograms of aqueous 4-ethylphenol solution at the 1-mmol dm^{-3} initial concentration were recorded under the air or He saturated conditions after the 1500-Gy irradiation as shown in Fig. III-17. Peaks observed at the retention time of 33, 46 and 81 min in Fig. III-17 (a) are assigned to 4-hydroxyacetophenone, 4-ethylcatechol (4EC) and 4-ethylphenol, respectively, by comparing with standard samples. A sharp peak at the retention time of 22 min was outstanding in other peaks of the products under the He saturated condition as shown in Fig. III-17 (b). Fig. III-18 (a) shows enlarged chromatogram at around 22 min shown in Fig. III-17 (b). Here, 4-hydroxyphenylethanol is one of the candidates of primary products from 4-ethylphenol by the reaction with hydroxyl radicals. Fig. III-18 (b) shows the chromatogram of aqueous 4-hydroxyphenylethanol solution under the same condition as that of Fig. III-18 (a). The sharp peak at the retention time of 21.8 min cannot be assigned to 4-hydroxyphenylethanol because of the difference in the peak position from that of 22.3 min in Fig. III-18 (a).

The product at the retention time around 22 min was collected by the fraction collector. The product was extracted with ethylacetate, and then the extracted solution was concentrated into 1.0×10^{-2} dm^3. The concentrated solution was analyzed by GC-MS. Fig. III-19 shows mass spectrum of the product. The fragment ion peaks were mainly detected at *m/z* of 77, 95, 123 and

138 amu. This fragment ion pattern is similar to that of benzyl alcohols and 4-ethylphenol [III-18]. 1-(*p*-hydroxyphenyl)-1-ethanol (pHP1E, MW=138) are ionized by electron impact to produce characteristic fragment ions. The parent ion releases methyl radical to produce *p*-hydroxybenzyl alcohol cation (138-15). This cation isomerizes to give dihydroxycycloheptatrienyl cation (123), which releases carbon monoxide to produce hydroxycyclohexadienyl cation (123-28).



(III-4)

MW = 138 MW = 123 MW = 95

The product at the retention time of 22.3 min is assigned to pHP1E by GC-MS since the mass spectrum is well explained by the characteristic fragment patterns of benzyl alcohol and 4-ethylphenol as mentioned above. As a result, it is understood that hydroxyl radicals do not attack β-carbon of 4-ethylphenol.

Concentration of 4-ethylphenol, and amounts of TOC and inorganic carbon changed upon the irradiation as shown in Fig. III-20. Dose dependences of concentrations of OH substitution products and formic acid are the same as those in the case of *p*-cresol as shown in Fig. III-21. As shown in Fig. III-11, 12, 20 and 21, the decomposition curve of 4-ethylphenol, and the production curves of 4EC and pHP1E are similar to that of *p*-cresol, and those of 4MC and 4HBA, respectively. The oxidation mechanism of 4-ethylphenol is considered to be the same as that of *p*-cresol.

III-3-4 Decomposition mechanism of *p*-nonylphenol

The oxidation mechanism of NPs is discussed based on the results obtained by fundamental study of γ-radiolysis of *p*-cresol and 4-ethylphenol in water as mentioned in the previous section. Both *p*-cresol and 4-ethylphenol are decomposed by γ-ray irradiation to form two OH substitution products which are formed by their oxidation at *ortho*-position of phenyl ring or α-carbon of alkyl group. In the case of 4-ethylphenol, hydroxyl radicals attack α-carbon but not β-carbon. Therefore, the alkyl group of alkylphenols would be oxidized at α- or *ortho*-position.

On the basis of the oxidation mechanisms of *p*-cresol and 4-ethylphenol, the OH substitution products are considered to be formed from NPs. The OH substitution products having the *m/z* of 235 amu would be assigned to 4-nonylcatechols and 1-(*p*-hydroxyphenyl)-1-nonanols. Scheme III-2 shows the oxidation mechanism of NPs by hydroxyl radicals in water. Dihydroxycyclohexadienyl-type radicals would be produced by the reaction of NPs with hydroxyl radicals, and would combine with oxygen to produce *p*-nonylcatechols and non-aromatic compounds. Dehydration of the dihydroxycyclohexadienyl-type radicals and abstraction of a hydrogen atom on α-position of NPs would produce benzyl-type radicals [III-14, 15, 19, 20]. The benzyl-type radicals would be oxidized to produce 1-(*p*-hydroxyphenyl)-1-nonanols. These OH substitution products would be decomposed to produce non-aromatic compounds such as formic acid and oxalic acid which have no estrogen activity, and are ultimately oxidized to form inorganic carbon by further irradiation.

In order to support the above discussion on OH substitution products of NPs, NPs were decomposed by hydroxyl radicals produced upon the Fenton

reaction. Aqueous NPs solution at 20 μmol dm^{-3} containing ferrous sulfate at 100 μmol dm^{-3} (total volume of 1×10^{-3} dm^3) was added into 1.0×10^{-3} dm^3 of hydroxyperoxide at 100 μmol dm^{-3}, and the solution was analyzed by HPLC and LC-MS. Some products were detected by HPLC with an absorption detector monitored at 280 nm. The products at the retention time of 6.3 and 8.5 min have molecular weight of 236, and are considered to be due to the OH substitution products of NPs produced by γ-ray irradiation.

III-3-5 Elimination of estrogen activity

The initial concentration of NPs was set at 20 μmol dm^{-3} for the evaluation of estrogen activity of the NPs solution after irradiation. The concentration of NPs at 1 μmol dm^{-3} was a threshold concentration (about 1 μmol dm^{-3}) for an appearance of the estrogen activity to the aquatic animals. This concentration, however, was below detection limit of the yeast two-hybrid assay. No condensation of the NPs solution was carried out because irradiation products of NPs are not always extracted at high yields. Evaluation of estrogen activity for NPs at the 20-μmol dm^{-3} initial concentration is valid for that below the 20-μmol dm^{-3} initial concentration since the decomposition efficiency of NPs is proportional to the initial concentration in the range from 0.045 to 20 μmol dm^{-3} as shown in Fig. III-6. Decreases in concentration of NPs and the estrogen activity of aqueous NPs solution against dose are shown in Fig. III-22 (a). Inset in Fig. III-22 (a) represents the log-log plot of intensity of luminescence for the yeast two-hybrid assay against NPs concentration, and the plots were analyzed by a linear function of concentration in the range from 0.025 to 5 μmol dm^{-3}. Both NPs concentration and estrogen activity of aqueous NPs solution decreased with dose and were completely eliminated at 5000 Gy, although the degradation

curves were different from each other. If the irradiation products from NPs do not have estrogen activity, degradation curve of the estrogen activity must be the same as that of the NPs concentration. By comparing the curve of the NPs concentration with that of the estrogen activity, the decay of the estrogen activity was slower than that of the NPs concentration. A possible explanation of this fact is that products from NPs by γ-ray irradiation have also estrogen activity. Formation of irradiation products having molecular weight of 236 can be quantified by the peak area of LC-MS. The peak areas are plotted against dose in Fig. III-22 (b). The peak area of the products increased up to 100 Gy, and then decreased and disappeared at 1000 Gy. Formation of the products is responsible for the gradual decrease in estrogen activity of the solution. However, all irradiation products having estrogen activity were essentially decomposed at the dose of 5000 Gy.

These irradiation products were separated with HPLC and collected using a fraction collector. The estrogen activities of fractions from NPs solution were estimated by the yeast two-hybrid assay. The NPs solution was analyzed at 100 Gy when formation of the irradiation products with retention times of 6.3 and 8.5 min reached the maximum as seen in Fig. III-22 (b). The fractions except for NPs and products at the retention times of 6.3 and 8.5 min did not show any estrogen activity, and therefore, the products at the retention time of 6.3 and 8.5 min mostly have estrogen activity in the irradiated NPs solution. Sum of the estrogen activities of these products were found to be about 2 times higher than that of NPs.

ELISA is one of simple methods for the estimation of concentration of NPs in environmental water, and was used for the estimation of NPs concentration in this study. The NPs equivalent concentration of aqueous NPs solution measured

by ELISA decreased with absorbed dose as shown in Fig. III-23. The NPs equivalent concentration was higher than NPs concentration in the all dose region. Decrease in NPs equivalent concentration estimated by ELISA is very similar to that of estrogen activity of NPs solution evaluated by the yeast two-hybrid assay. It is concluded that not only NPs but also irradiation products bind to alkylphenol antibody since the irradiation products have a molecular formula similar to NPs. Thus, ELISA is not suitable for the estimation of the decrease in NPs concentration by hydroxyl radicals.

III-4 Conclusion

Trace amount of NPs in water was mainly decomposed by radiation-induced hydroxyl radicals. Decomposition mechanism of NPs was almost the same in the range of the initial concentration from 0.045 to 20 μmol dm^{-3}. Decomposition yield of NPs apparently did not depend on the concentration of dissolved oxygen. Formation of formic acid, oxalic acid, inorganic carbon and two different products having molecular weight of 236 were identified with HPLC, LC-MS, ion chromatograph and TOC analyzer. The last two products are ascribable to *p*-nonylcatechol and 1-(*p*-hydroxyphenyl)-1-nonanol based on the oxidation mechanism of *p*-cresol and 4-ethylphenol, and have high estrogen activity as evaluated by the yeast two-hybrid assay. NPs and two products were decomposed completely, and estrogen activities in NPs solution were also eliminated at 5000 Gy.

III-5 References

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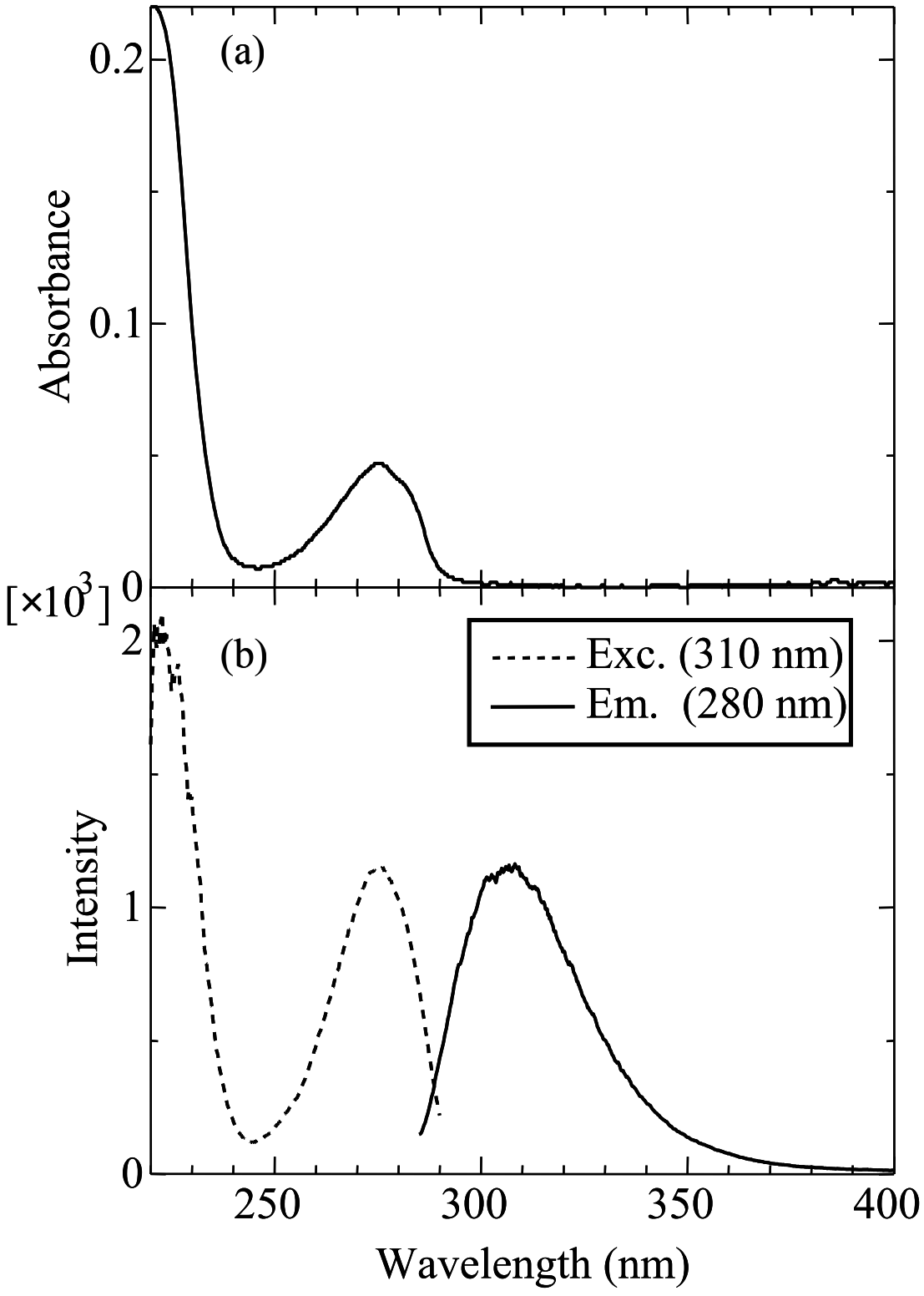


Fig. III-1 Absorption (a), emission and excitation (b) spectra of *p*-nonylphenols in water.

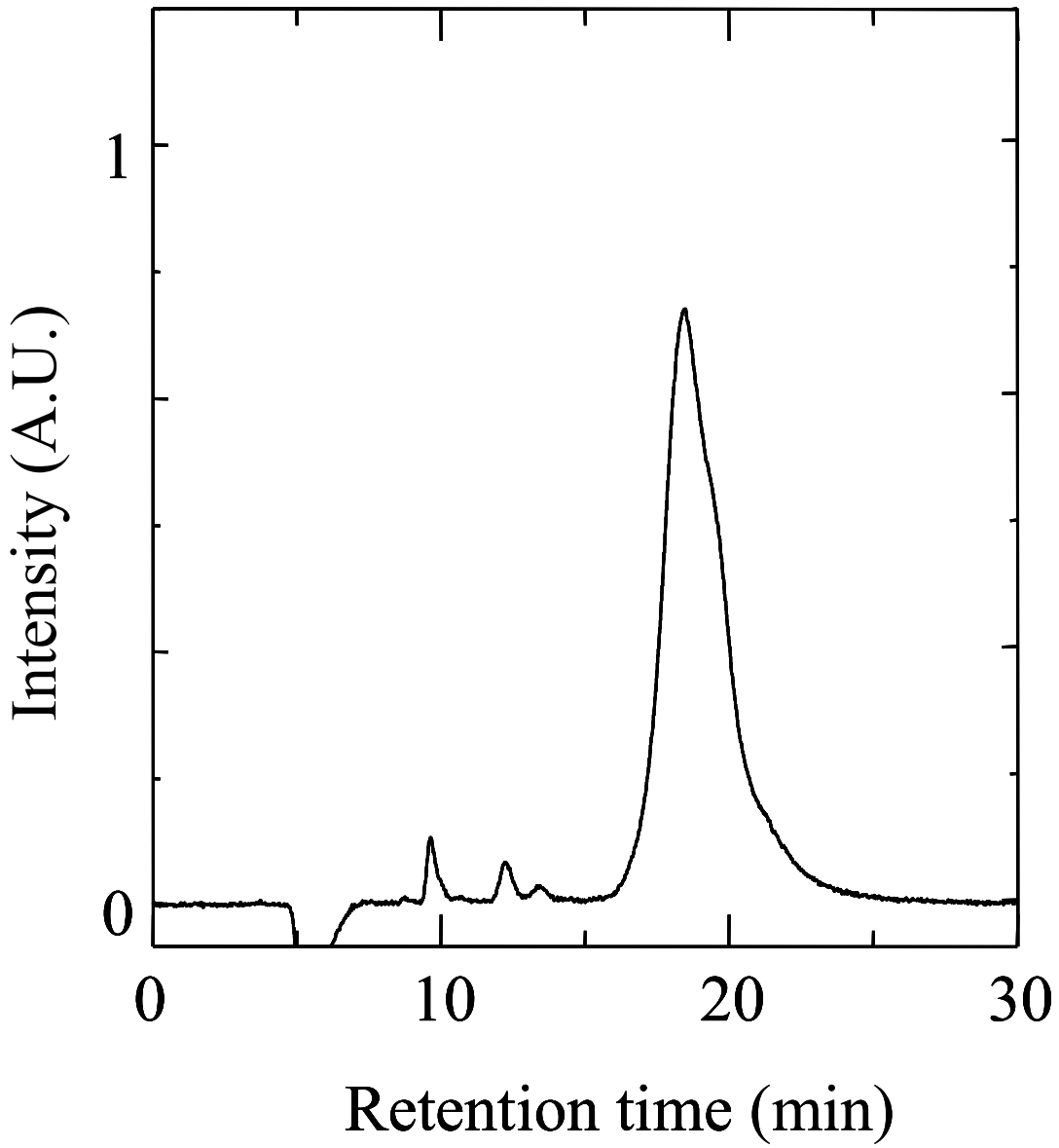


Fig. III-2 Chromatogram of *p*-nonylphenols at 1 μmol dm^{-3} before irradiation. Excitation and monitor wavelengths were set at 280 and 310 nm, respectively.

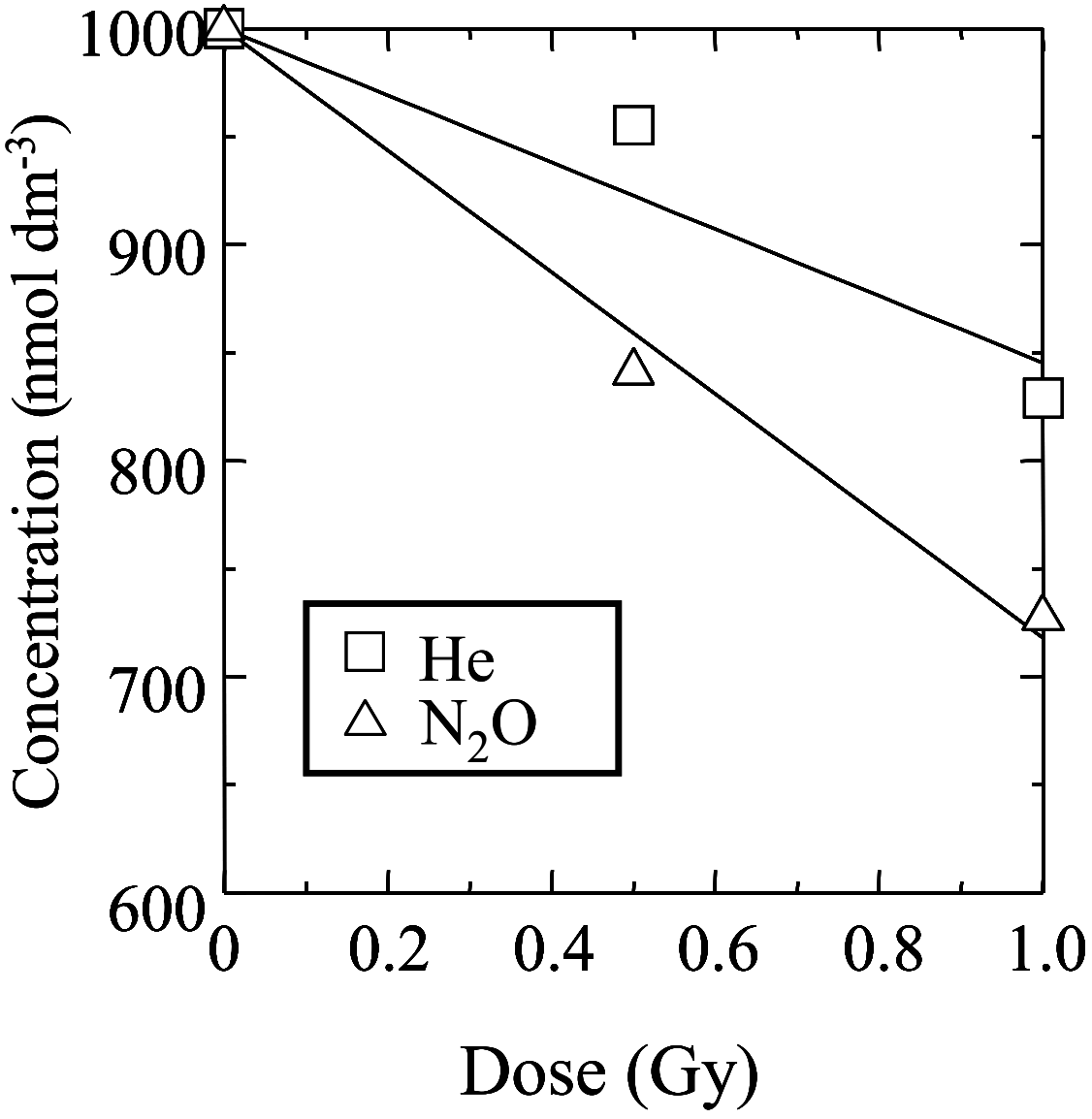


Fig. III-3 Concentration of *p*-nonylphenols in water saturated with helium (□) or nitrous oxide (△) as a function of dose.

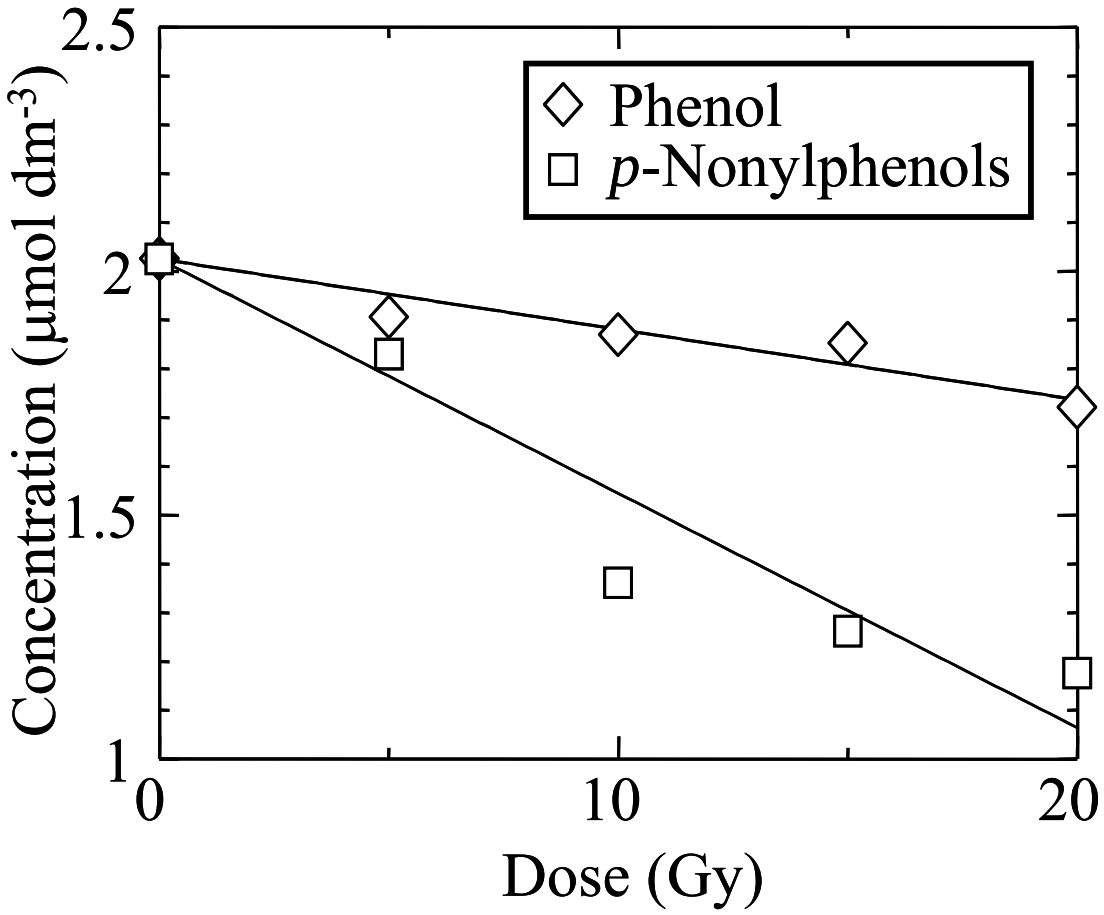


Fig. III-4 Decomposition of *p*-nonylphenols and phenol in water by γ-ray irradiation under the deoxygenated condition.

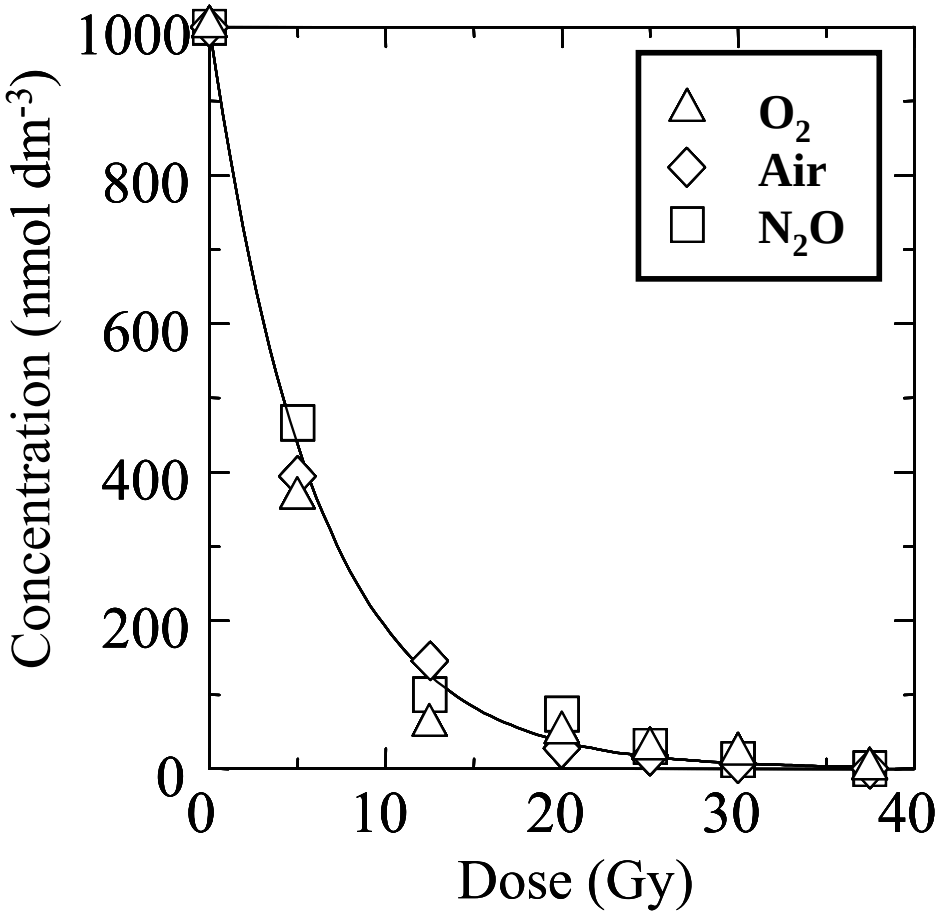


Fig. III-5 Concentration of *p*-nonylphenols in water saturated with oxygen (△), air (◇) or helium (□) as a function of dose.

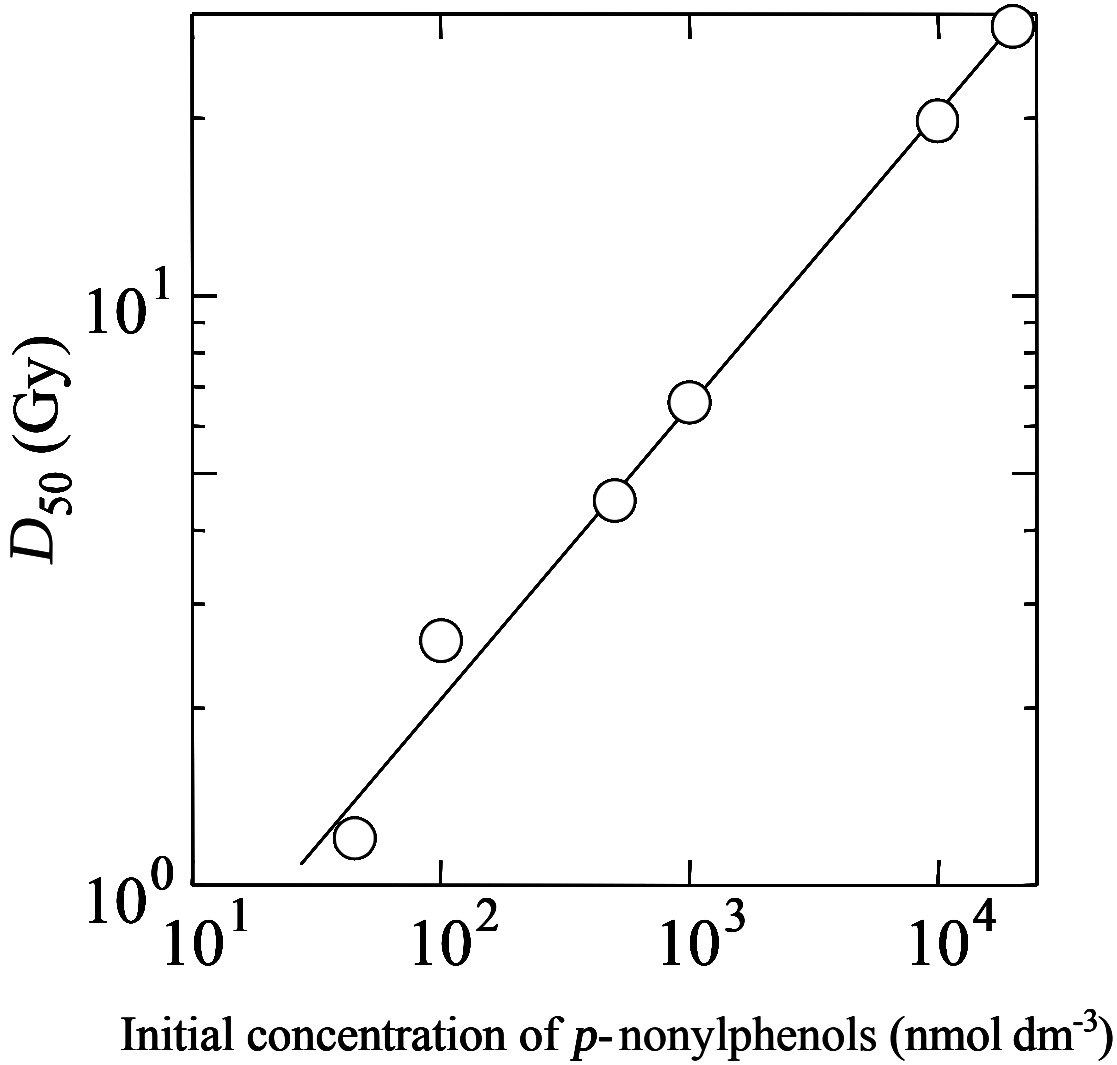


Fig. III-6 Initial concentration dependence of D_{50} in the decomposition of *p*-nonylphenols under the air saturated condition. D_{50} represents the dose required for decomposition of *p*-nonylphenols to 50% of the initial concentration.

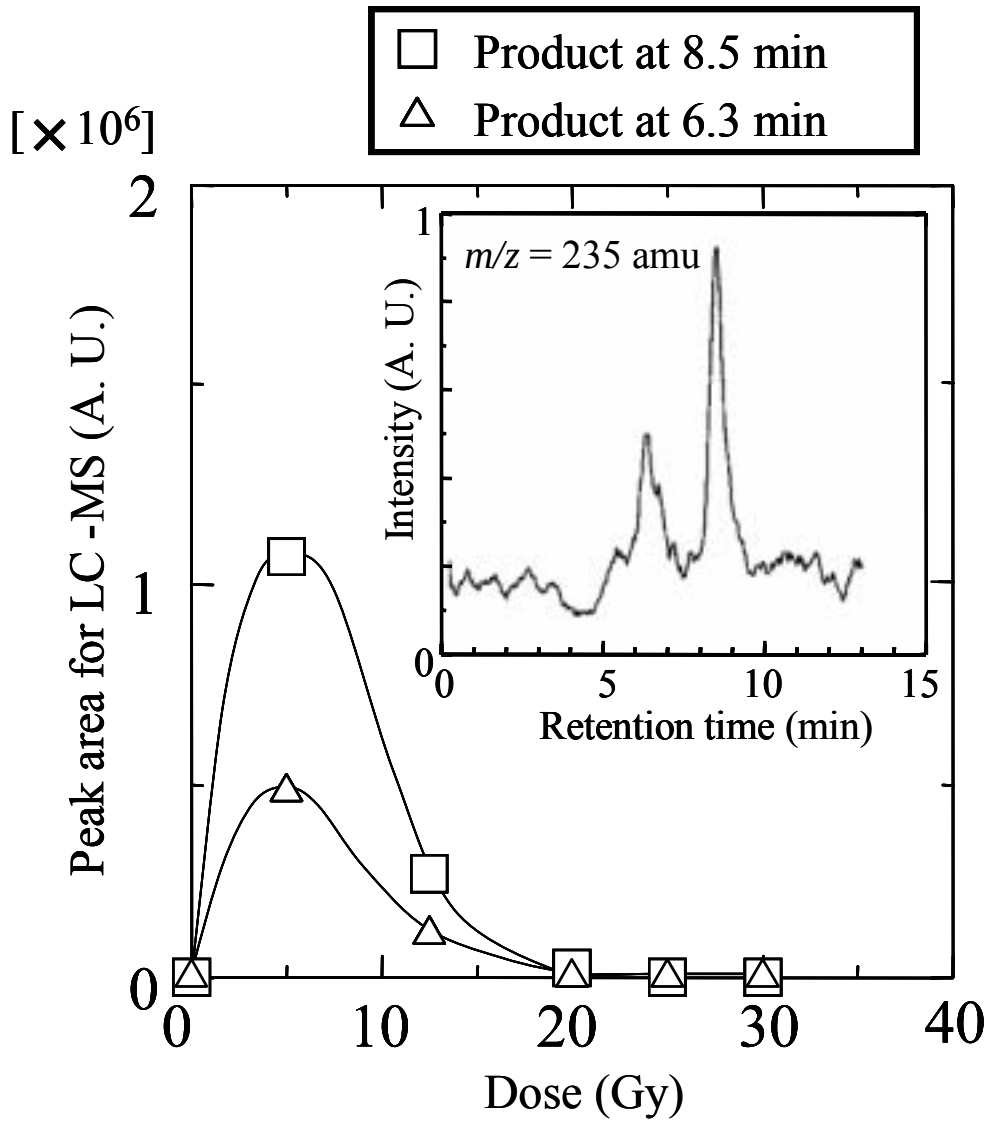


Fig. III-7 Peak areas monitored at the mass to charge ratio (*m/z*) of 235 amu for irradiation products of *p*-nonylphenols at the initial concentration of 1 μmol dm^{-3} as a function of dose by LC-MS analyses. Inset : Selected ion chromatogram monitored at the *m/z* of 235 amu after irradiation to 12.5 Gy.

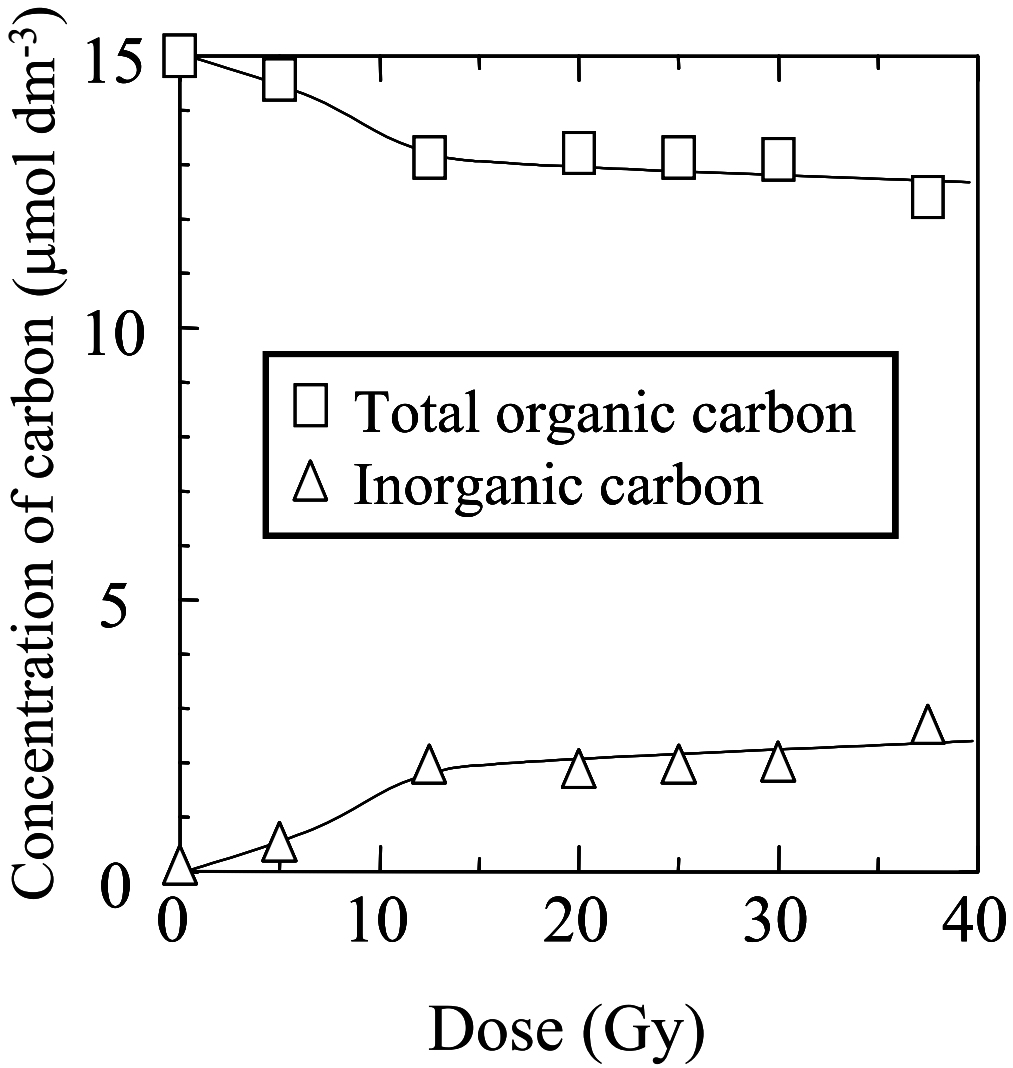


Fig. III-8 Amount of total organic carbon (□) and inorganic carbon (△) in air saturated *p*-nonylphenols solution at the initial concentration of 1 μmol dm^{-3} as a function of dose.

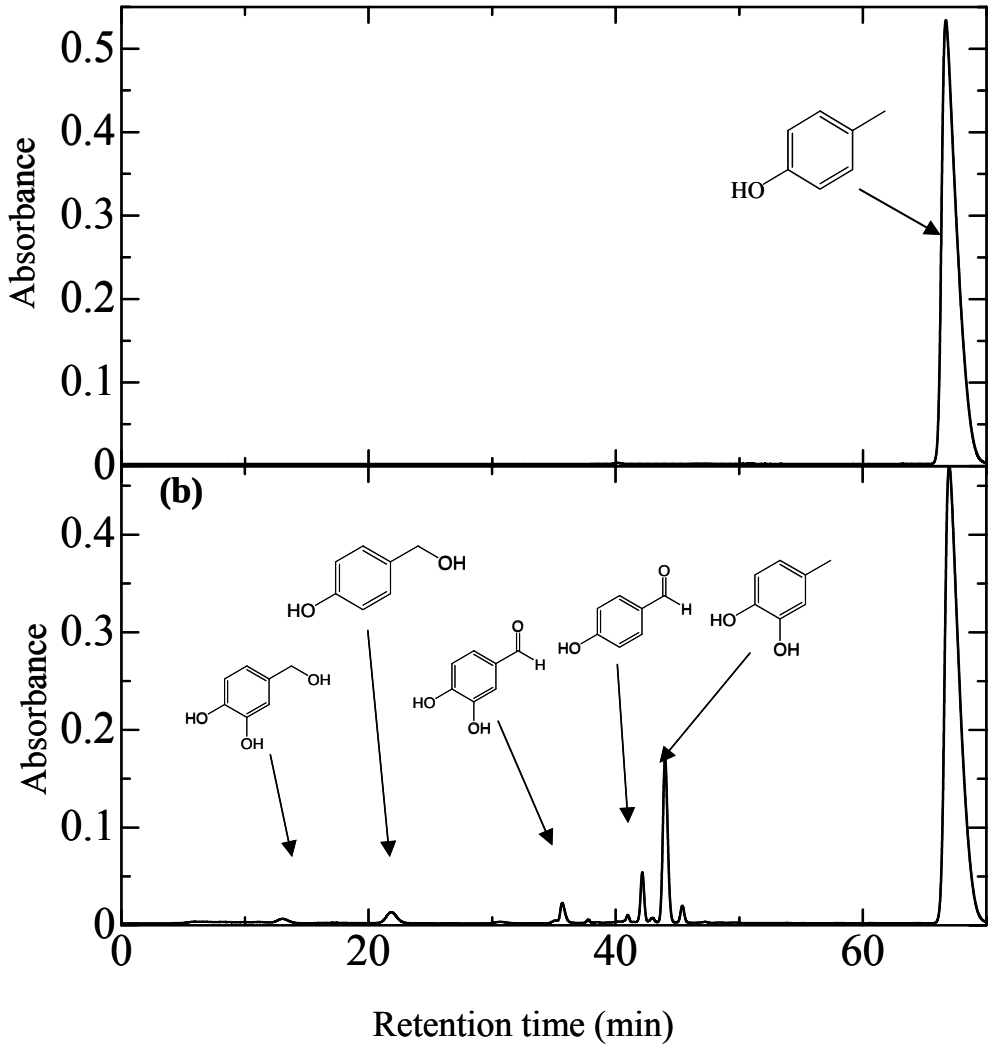


Fig. III-9 Chromatograms of aqueous *p*-cresol solution at the 1-mmol dm^{-3} initial concentration under the air saturated condition before (a) and after (b) the 1500-Gy irradiation.

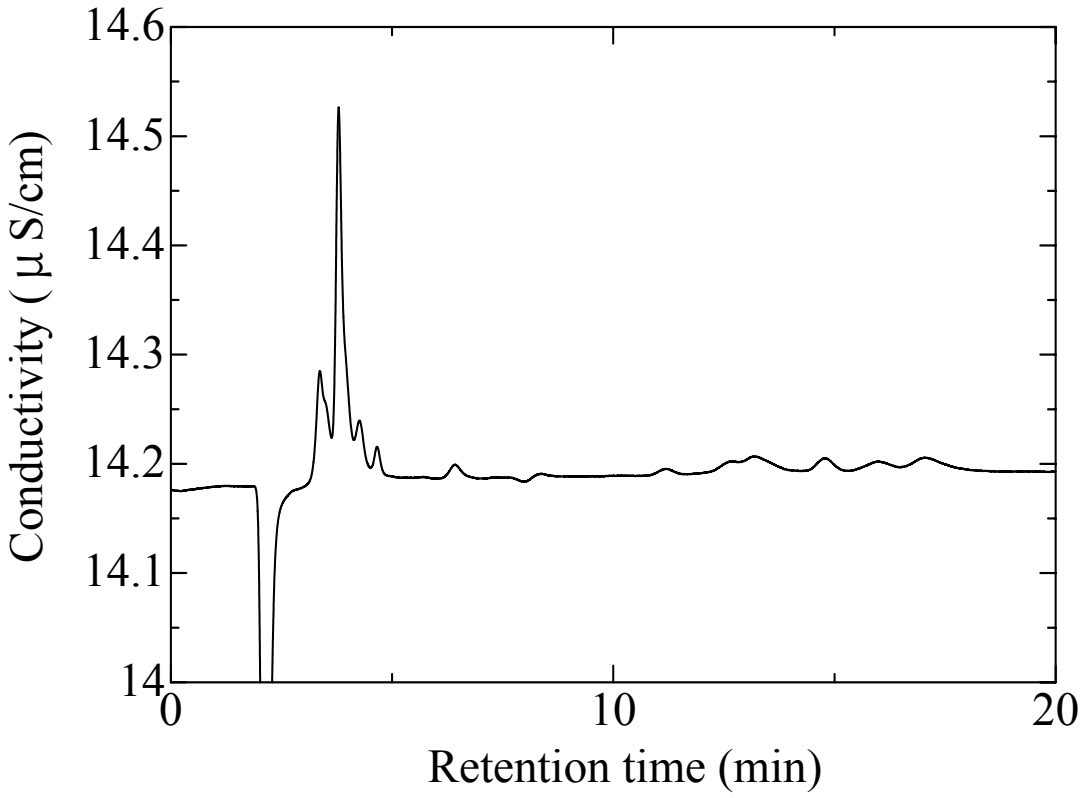


Fig. III-10 Ion chromatogram of aqueous *p*-cresol solution at 1 mmol dm^{-3} after the 1500-Gy irradiation.

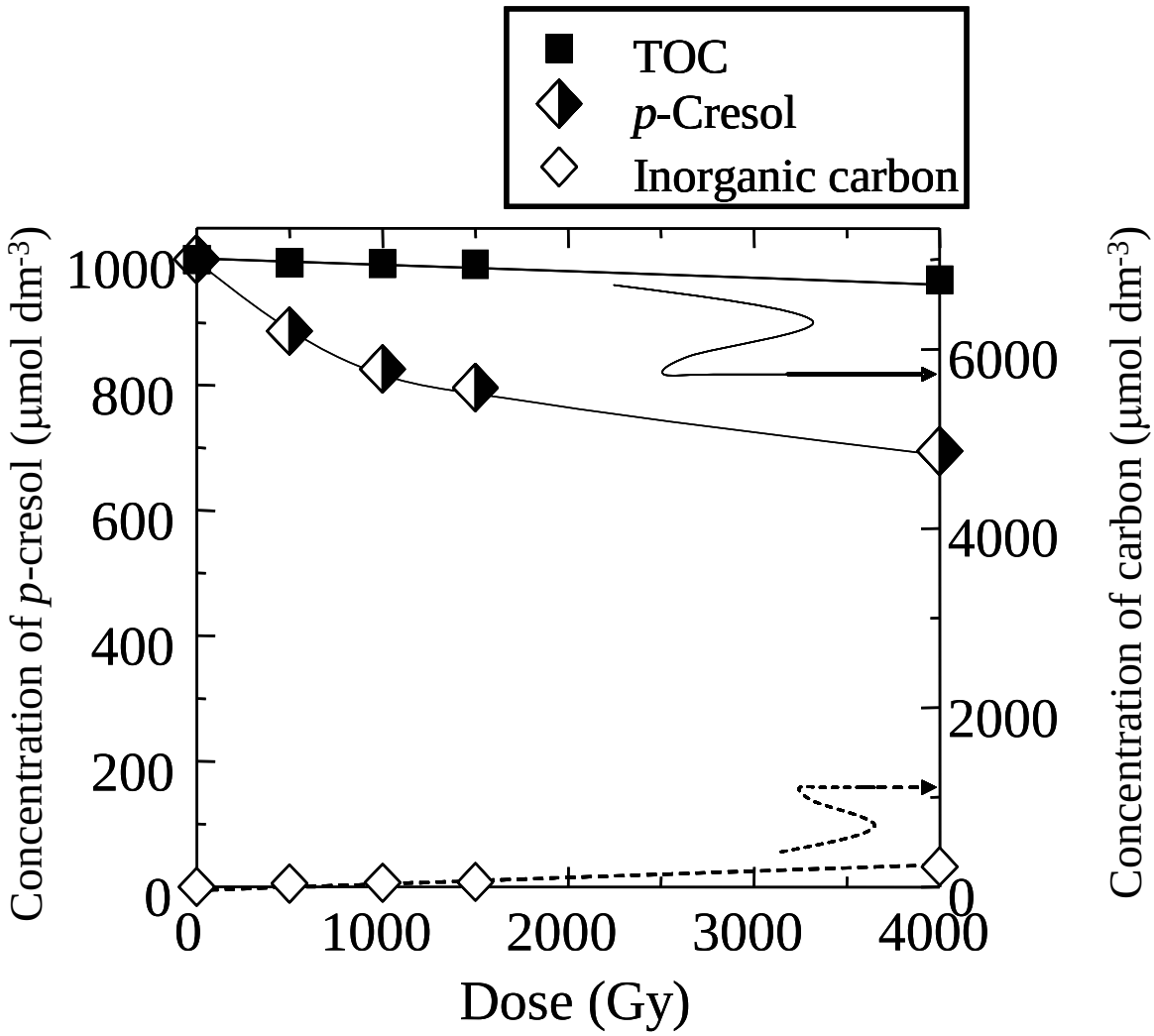


Fig. III-11 Concentration of *p*-cresol and amount of total organic carbon (TOC) and inorganic carbon (IC) under the air saturated condition as a function of dose.

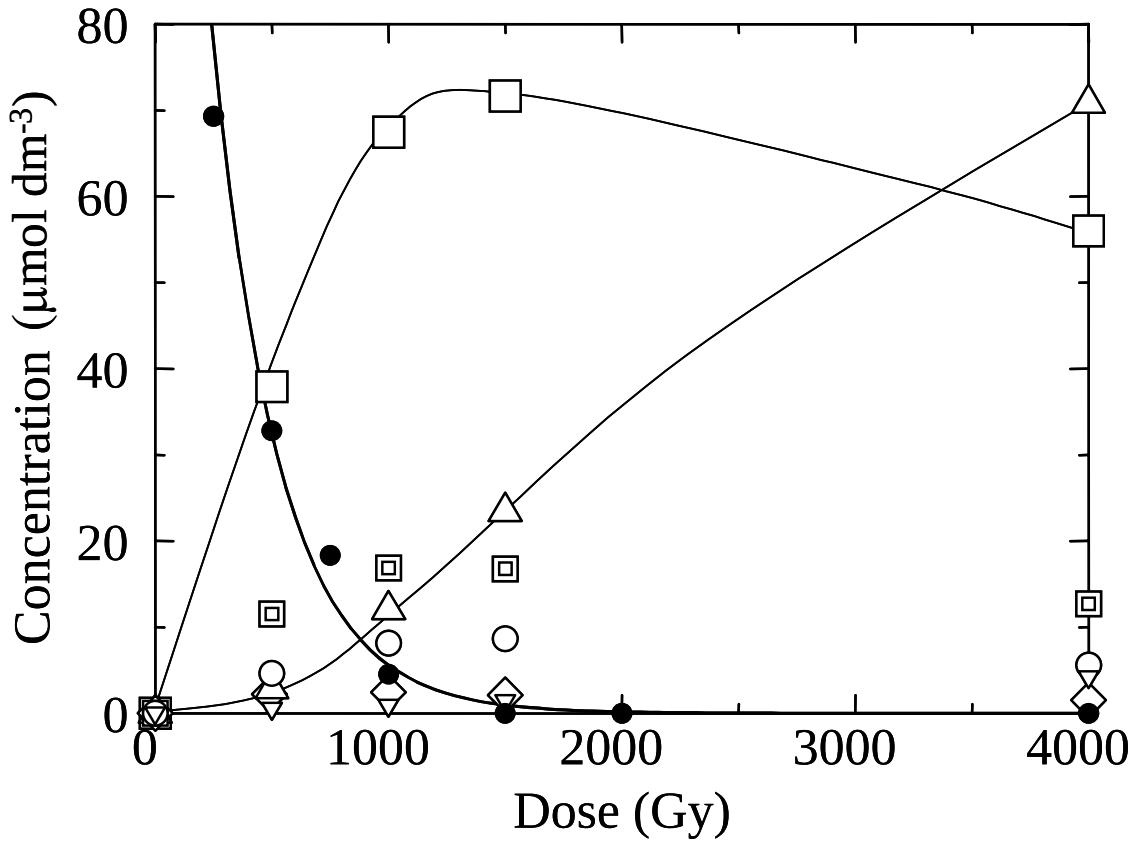


Fig. III-12 Concentrations of 4-methylcatechol (4MC, □), 4-hydroxybenzyl alcohol (4HBA, △), 4-hydroxybenzaldehyde (○), 3,4-dihydroxybenzyl alcohol (▽), 3,4-dihydroxybenzaldehyde (◇), formic acid (⊡), and dissolved oxygen (●) in the solution for decomposition of *p*-cresol at the 1-mmol dm^{-3} initial concentration under the air saturated condition as a function of dose.

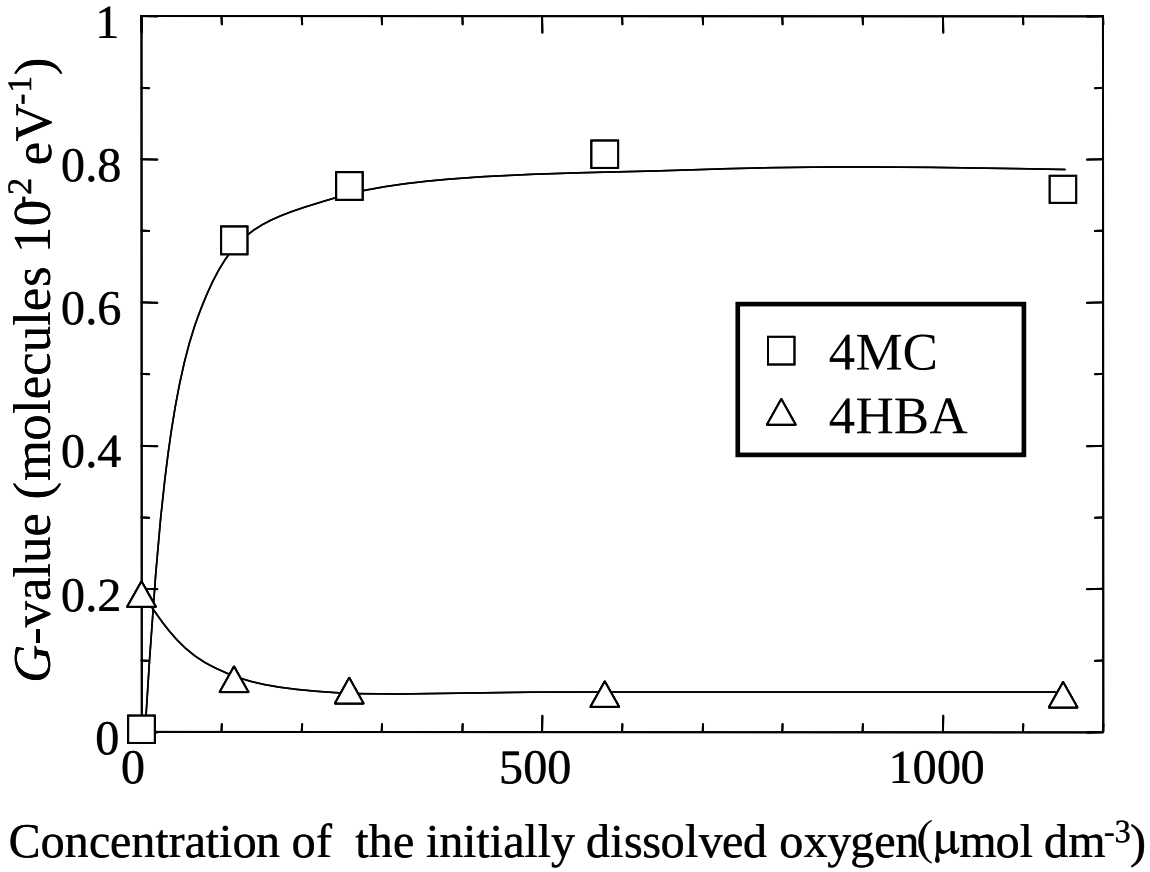


Fig. III-13 Dissolved oxygen dependence of *G*-values of 4-methylcatechol (4MC, □) and 4-hydroxylbenzyl alcohol (4HBA, △) formation from *p*-cresol by γ-ray irradiation.

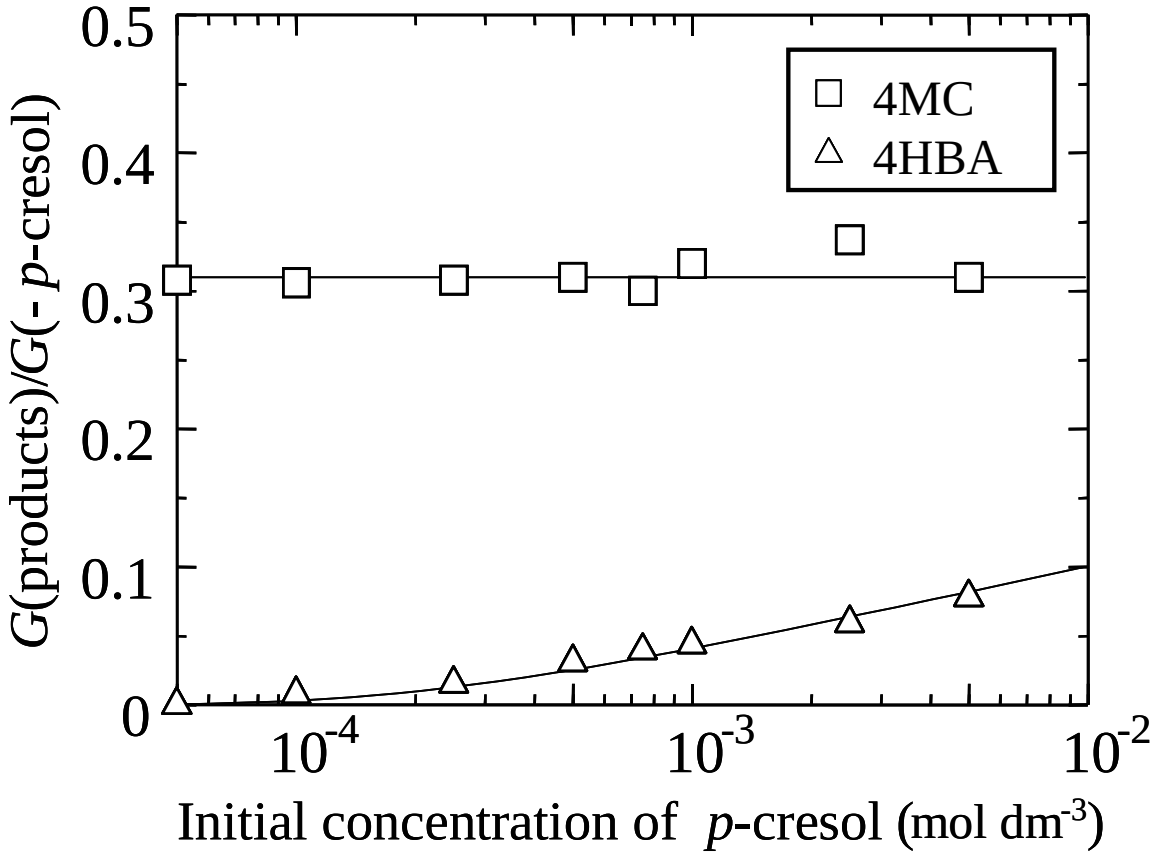


Fig. III-14 Dependence of the value of *G*(products) / *G*(- *p*-cresol) on the initial concentration of *p*-cresol.

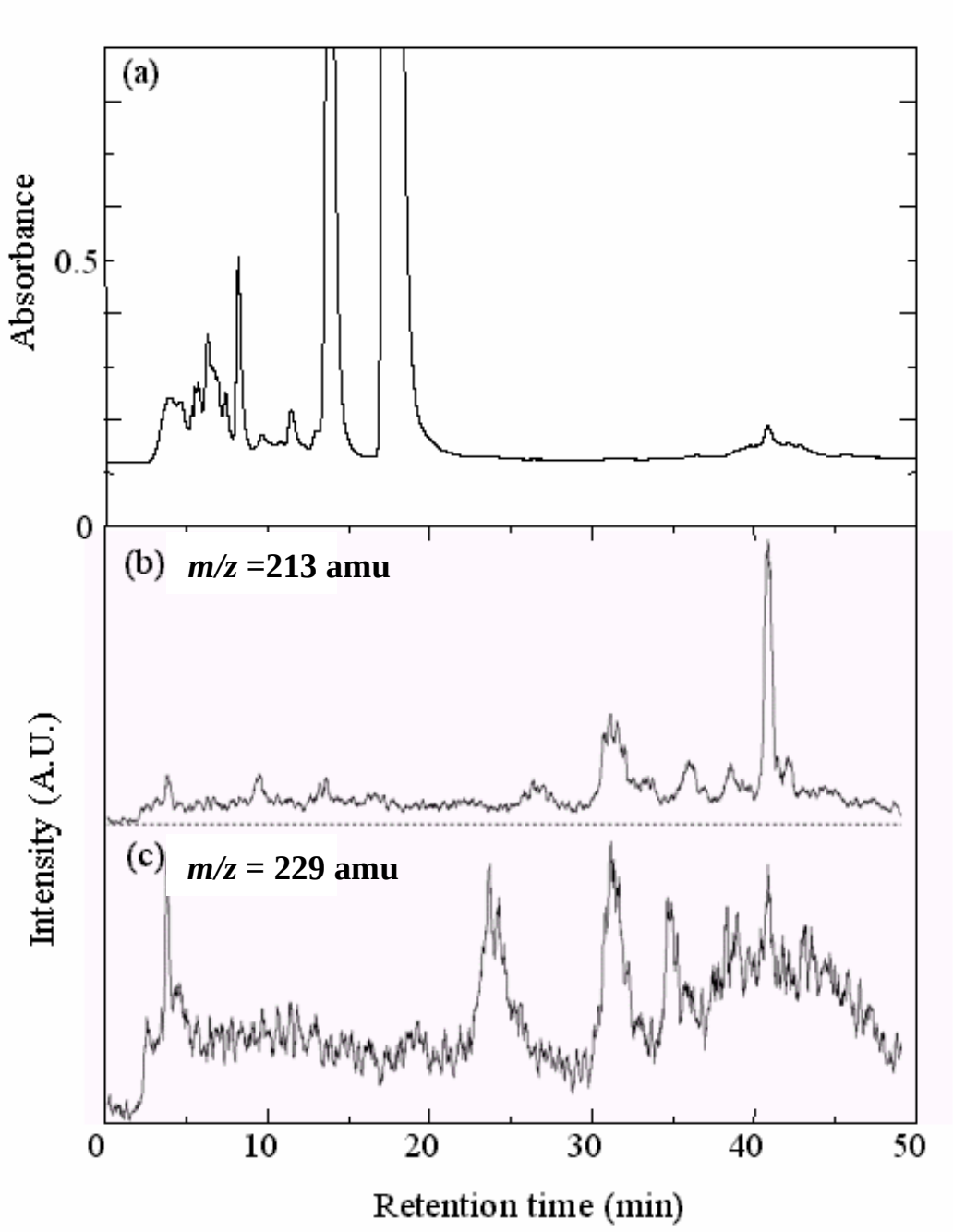


Fig. III-15 Chromatogram of *p*-cresol at 1mmol dm^{-3} after irradiation at a dose of 1500 Gy recorded on HPLC at 280 nm (a) and LC-MS of *m/z* = 213 amu (b) and *m/z* = 229amu (c)

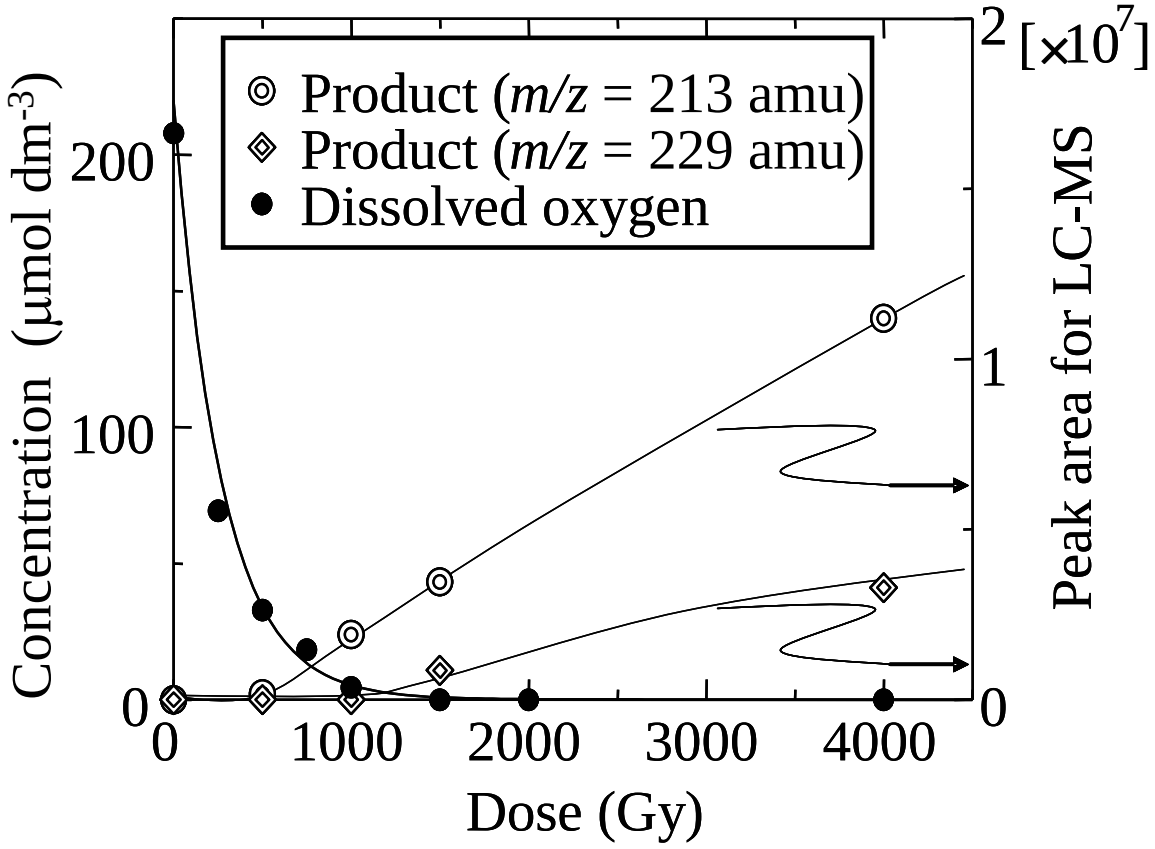


Fig. III-16 Peak area of products having molecular weight of 214 amu and 230 amu as a function of dose in air saturated *p*-cresol solution.

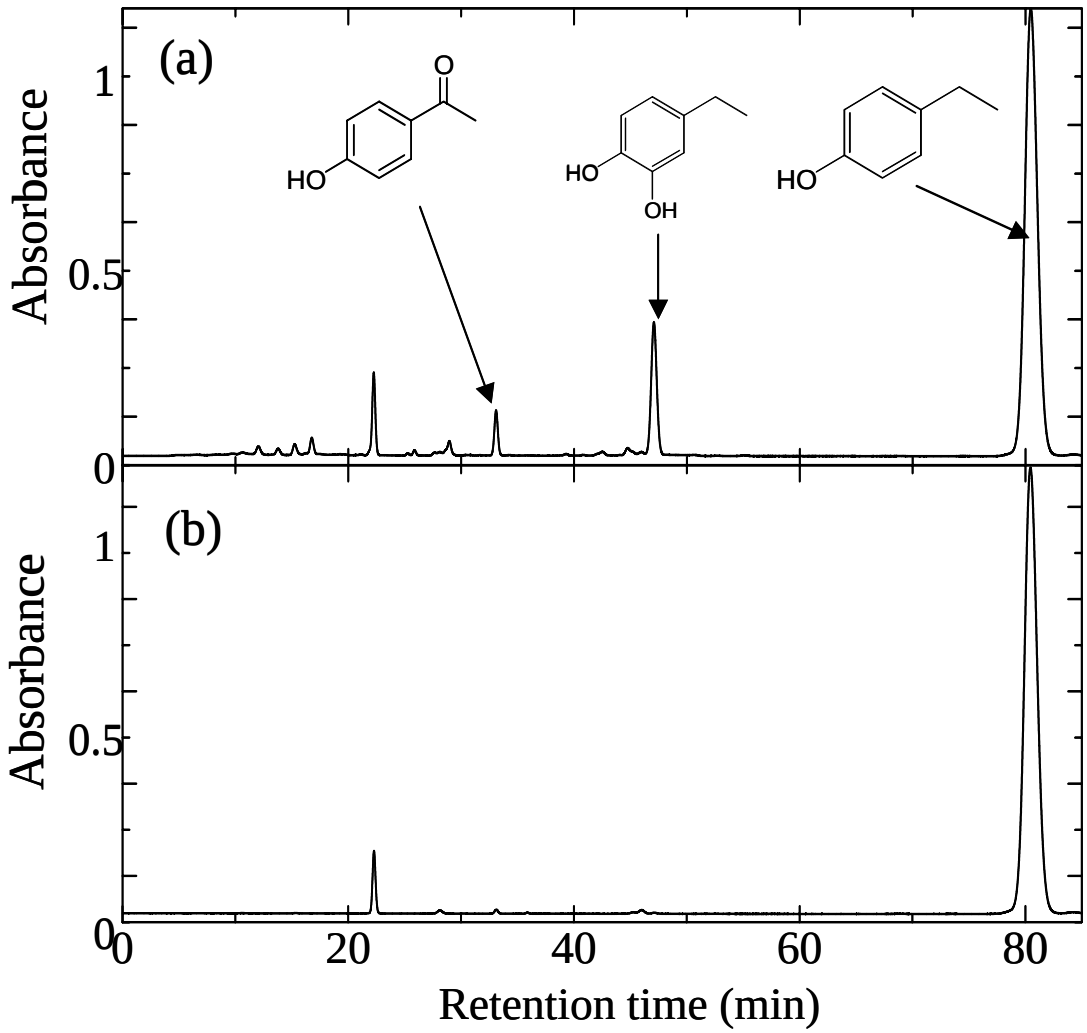


Fig. III-17 Chromatogram of aqueous 4-ethylphenol solution at 1 mmol dm^{-3} under the air (a) and helium (b) saturated conditions after the 1500-Gy irradiation.

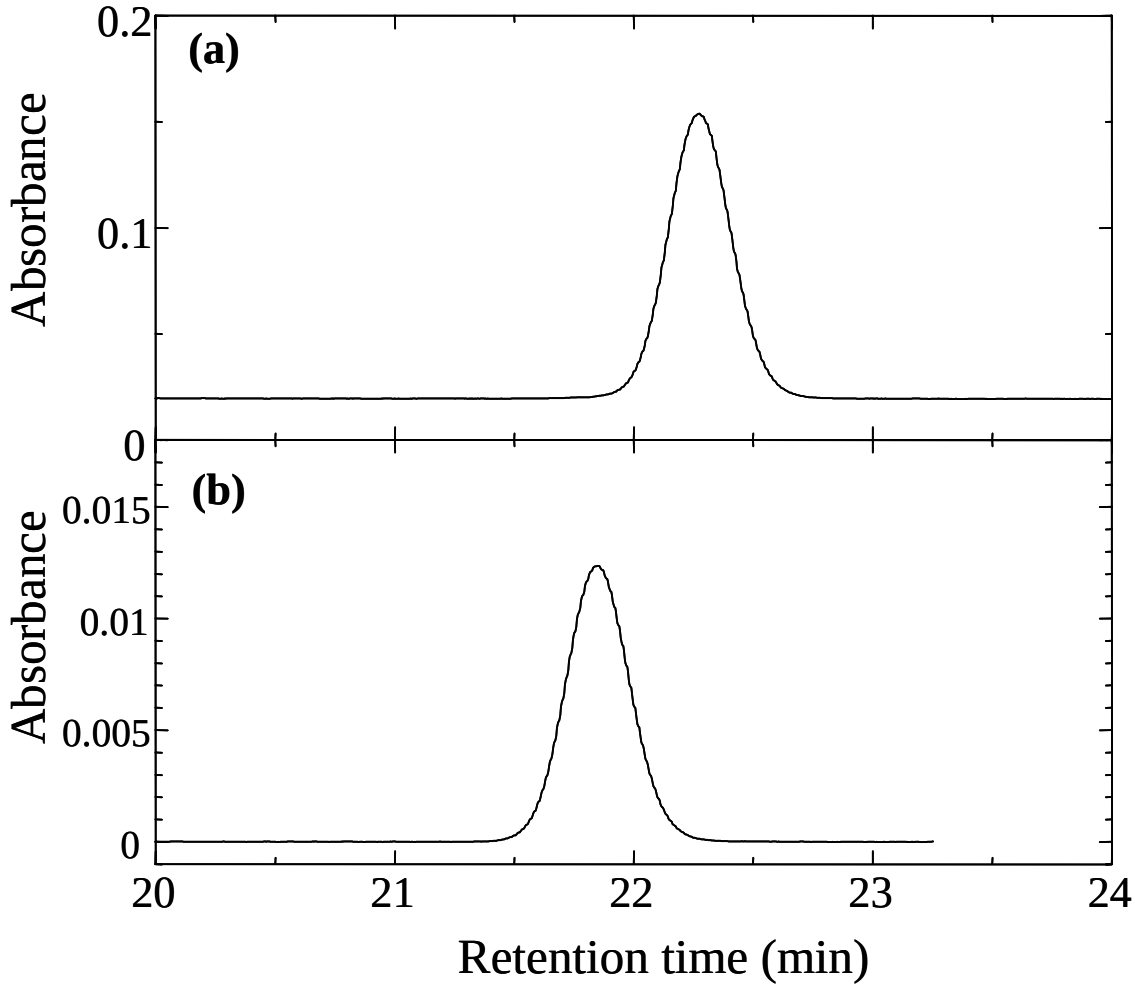


Fig. III-18 Chromatogram of 4-ethylphenol aqueous solution saturated with helium after the 1500-Gy irradiation (a) and aqueous 4-hydroxyphenylethanol solution (b).

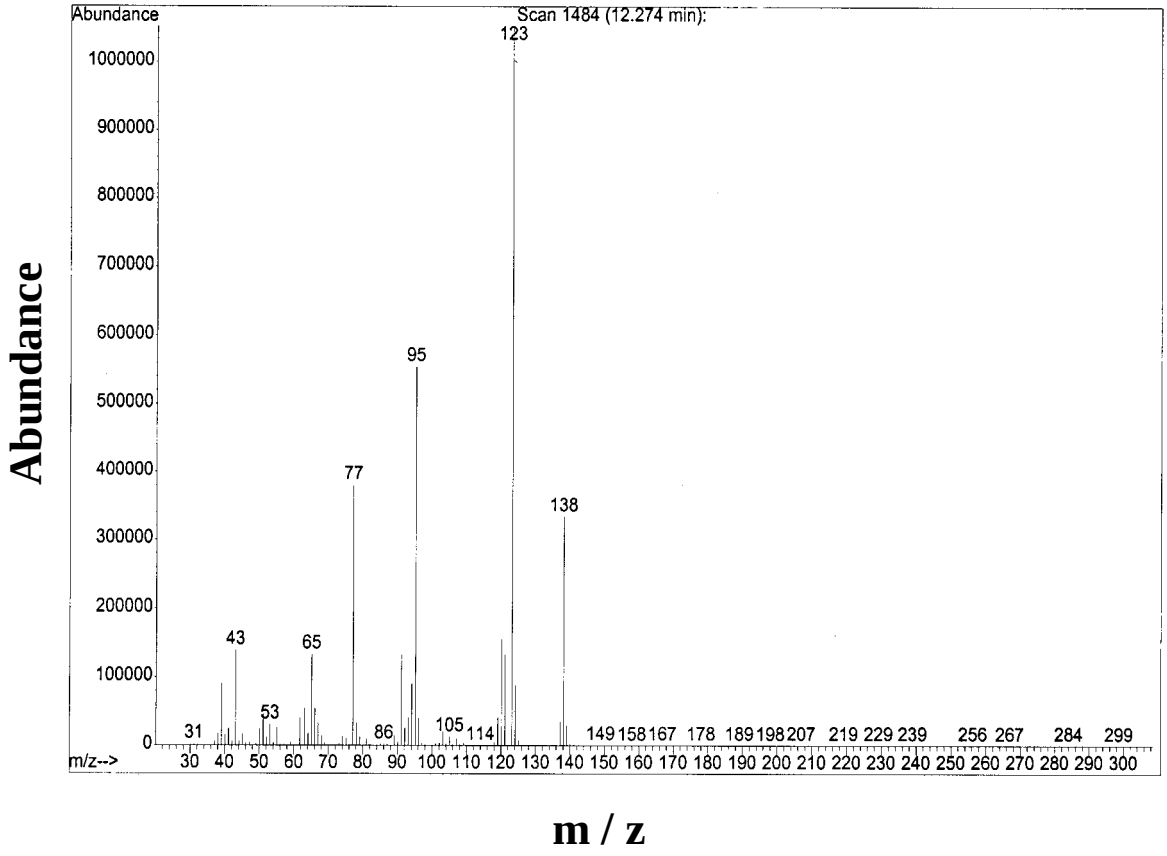


Fig. III-19 Mass spectrum of the product observed at 22.3 min on liquid chromatogram in Figure 3-18 (a).

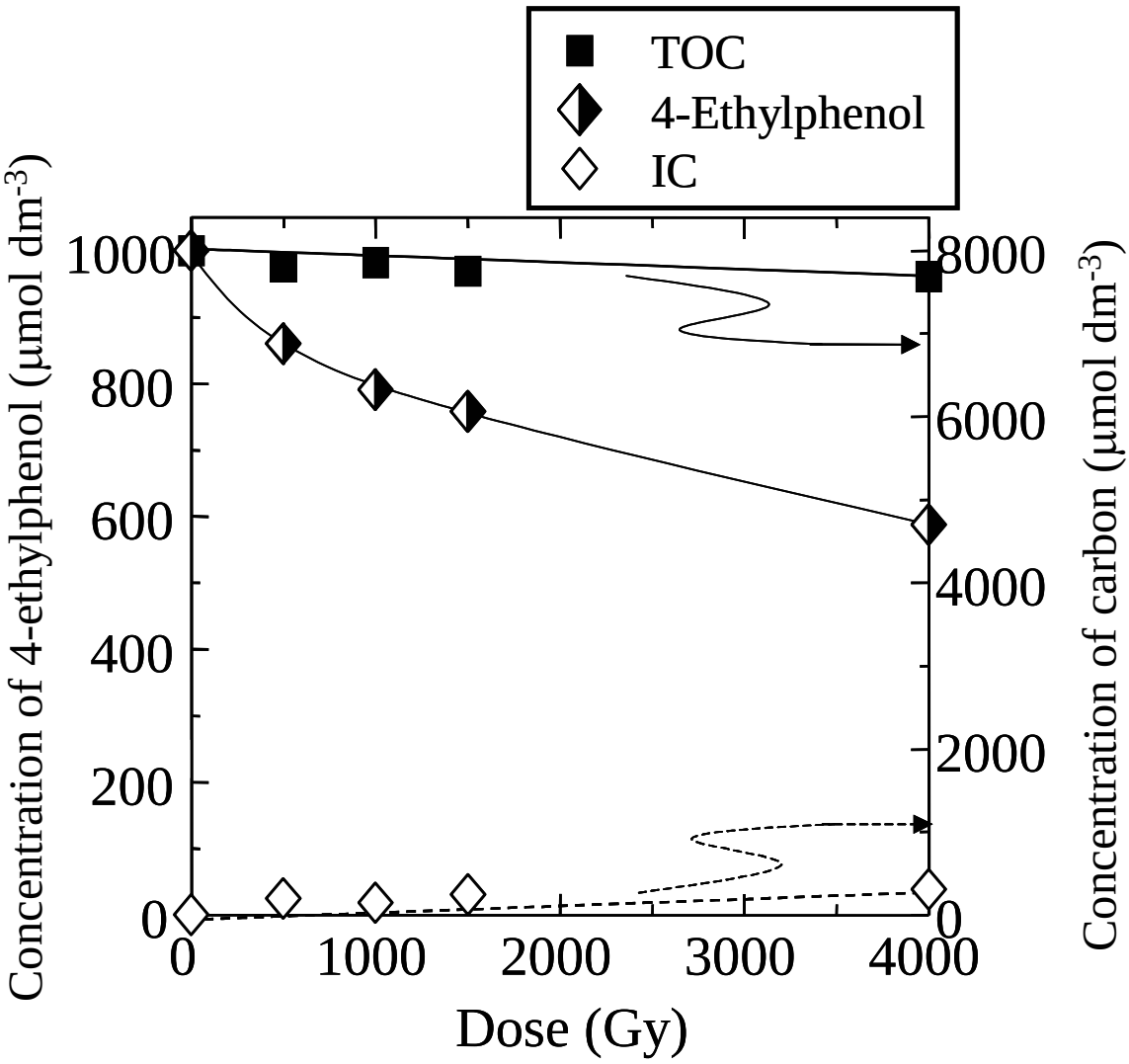


Fig. III-20 Concentration of 4-ethylphenol and amount of total organic carbon (TOC) and inorganic carbon (IC) as a function of dose.

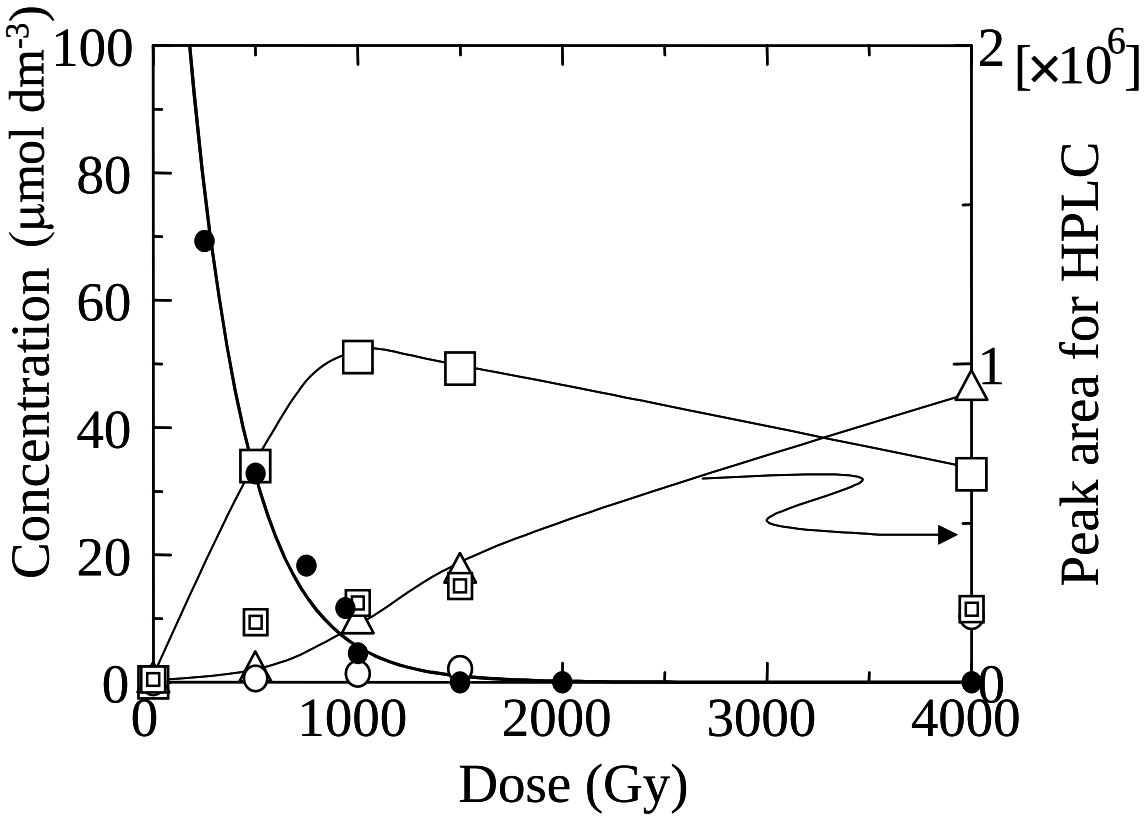


Fig. III-21 Dose dependence of concentration of 4-ethylcatechol (□), 4-hydroxyacetophenone (○), formic acid (⊡) and dissolved oxygen (●), and peak area of 1-(*p*-hydroxyphenyl)-1-ethanol (△), for decomposition of *p*-cresol in water at 1 mmol dm^{-3}.

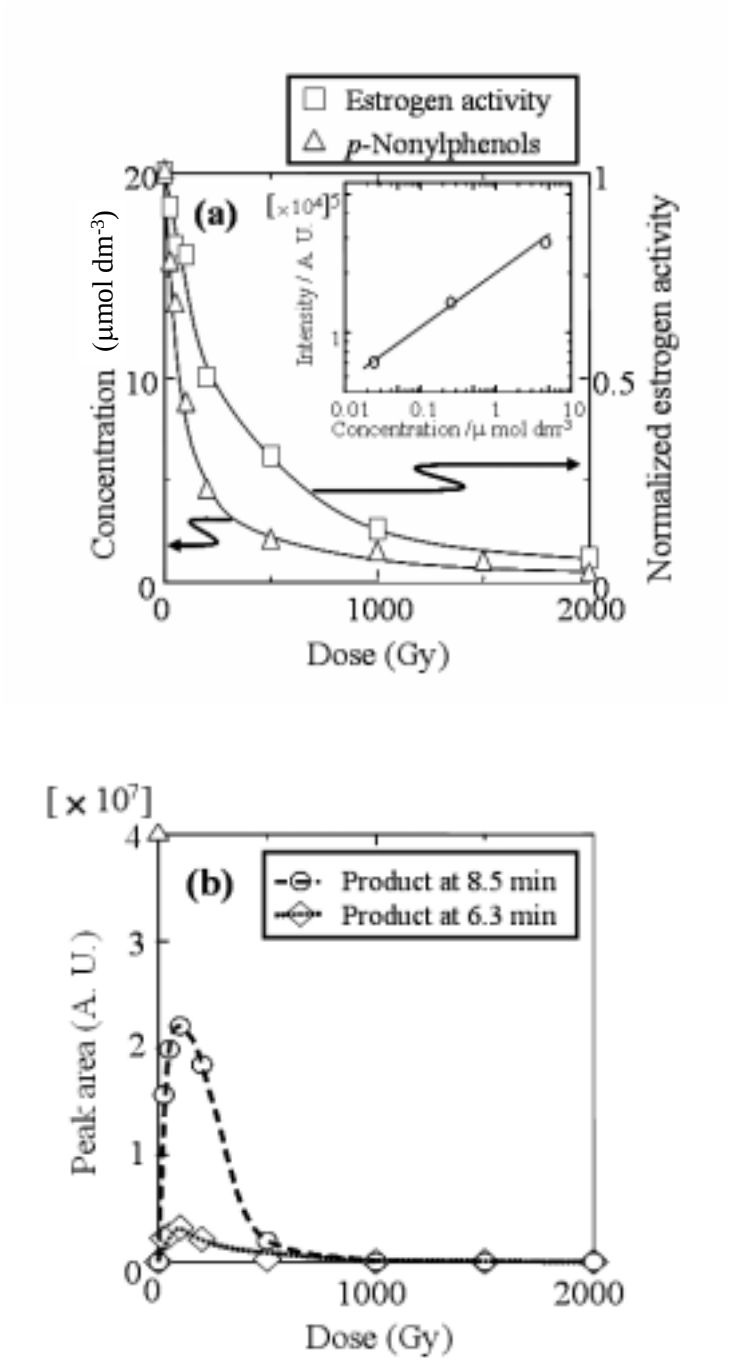


Fig. III-22 (a) Decreases in *p*-nonylphenols concentration (△) and estrogen activity of the *p*-nonylphenols solution (□) by γ-ray irradiation. Inset : plots of intensity of fluorescence for the yeast two-hybrid assay against *p*-nonylphenols concentration. (b) The peak area of the irradiation products having molecular weight of 236 given by SIC at the retention times of 6.3 (◇) and 8.5 (○) min are plotted against dose (see Fig. III-7).

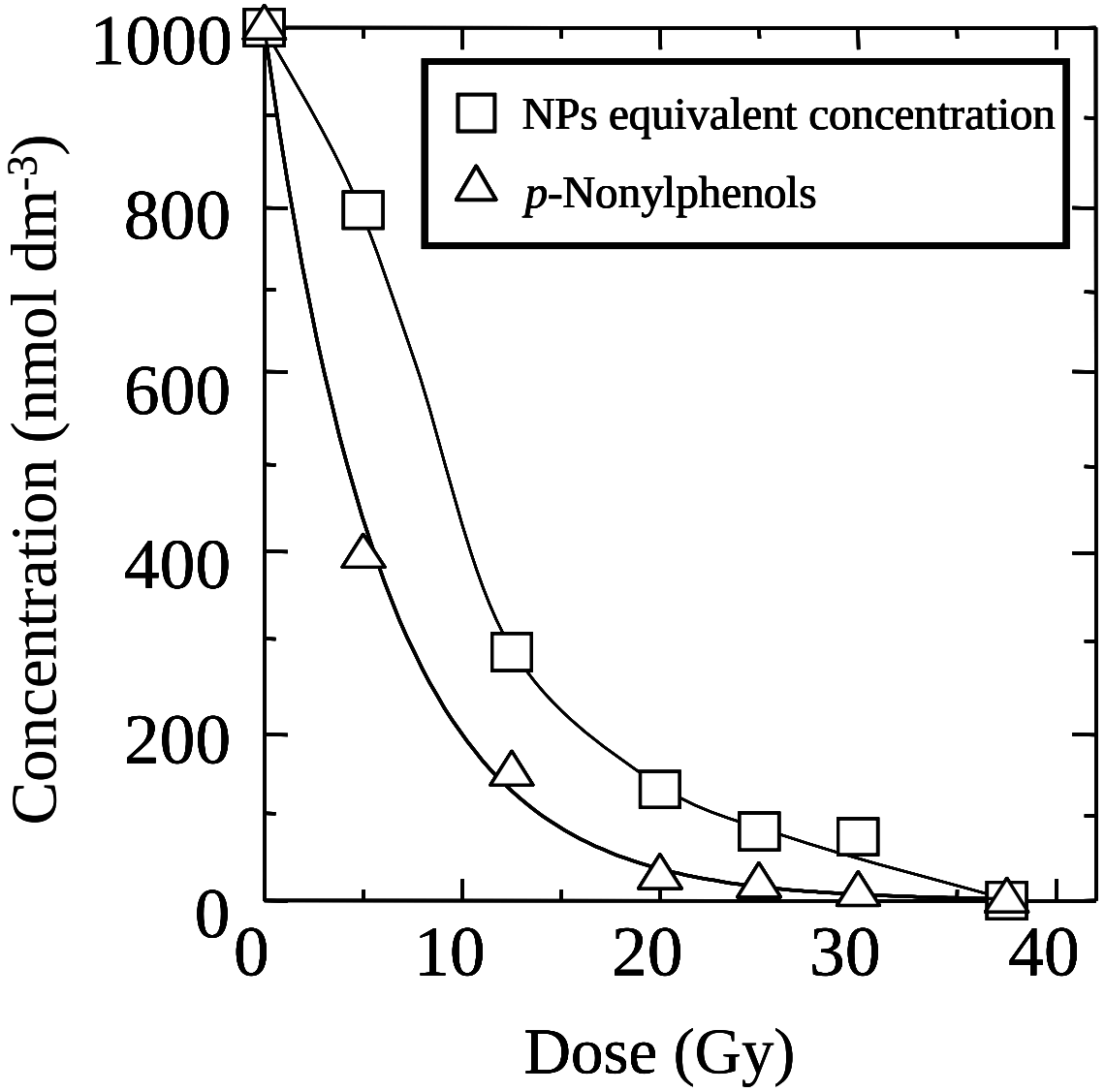
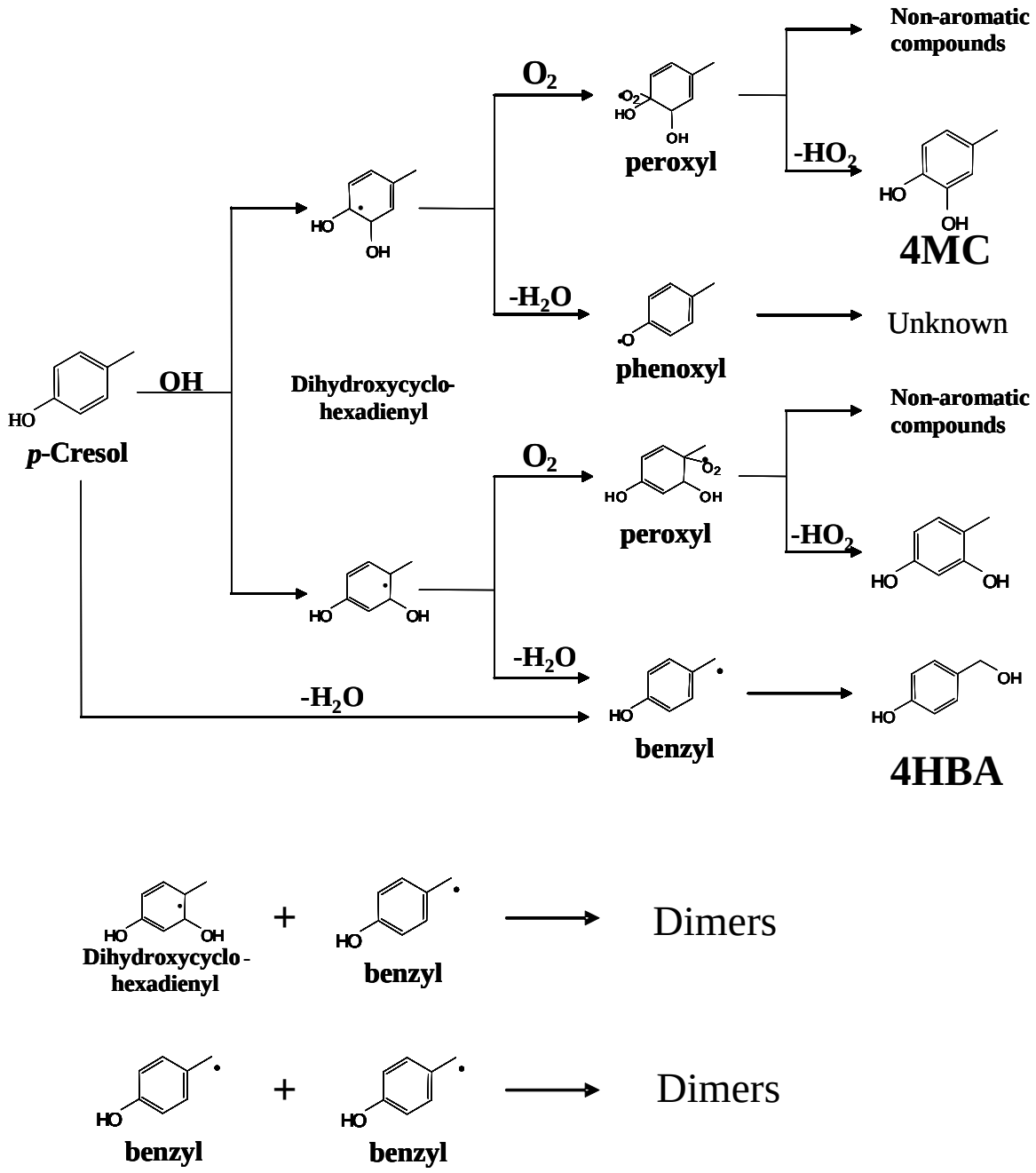
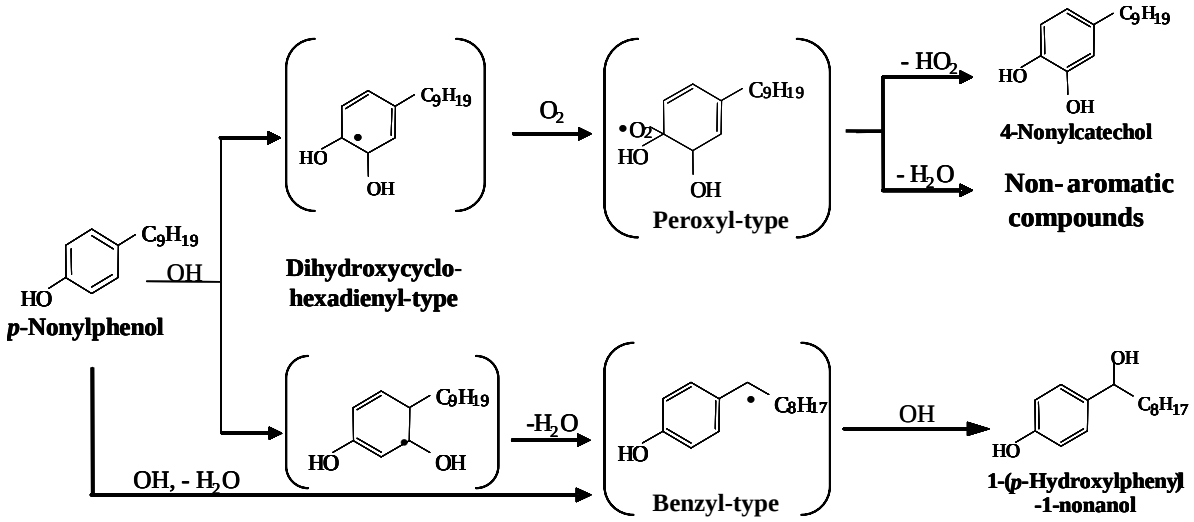


Fig. III-23 *p*-Nonylphenols (△) and *p*-nonylphenols equivalent concentrations (□) as a function of dose under the air saturated condition.



Scheme III-1 The oxidation mechanism of *p*-cresol by hydroxyl radicals in water.



Scheme III-2 The oxidation mechanism of *p*-nonylphenols by hydroxyl radicals in water.

Chapter IV

Treatment of wastewater having estrogen activity

IV-1 Introduction

Water treatment by ionizing radiation was already tried, and a pilot plant with electron beam combined with biodegradation process has been operated at the papermill factory and the dye industrial complex [IV-1]. The electron beam irradiation promotes the decrease of TOC, BOD and COD in wastewater [IV-2].

In this Chapter, treatment of real wastewater having the estrogen activity was studied based on the results mentioned in Chapter II and Chapter III. The cost for the water treatment plant with electron beam was estimated based on the results of experiment and simulation.

Estrogen activity of the wastewater was analyzed by the yeast two-hybrid assay which can estimate estrogen activities using human estrogen receptor (hERα) and medaka estrogen receptor (mERα). hERα has a high reactivity with estrogenic compounds such as estrone, E2 and estriol released by animals. mERα reacts not only with the estrogenic compounds but also artificial EDCs such as NPs. By making use of this analysis, the rate of elimination of the estrogen activity of the wastewater by radiation-induced hydroxyl radicals was studied. E2 and NPs were dissolved into the wastewaters having no estrogen activity, i.e., the model wastewater, to clarify the effects of total organic carbon in real wastewater. The decomposition efficiencies of E2 or NPs were obtained from their decomposition curves by γ-ray irradiation, and were found to depend on the amount of total organic carbon (TOC) in model wastewater. These results enable to estimate doses for the practical treatment of EDCs in wastewater.

IV-2 Experimental

Amounts of the TOC were measured by a TOC analyzer (Shimadzu, TOC-Vwp). The pH value of the wastewater was determined using a pH / DO meter (HORIBA, D-25). The γ-ray irradiations were carried out at 298 K using ^{60}Co γ-ray sources at JAEA, Takasaki, to doses in the range from 133 to 1000 Gy at dose rates ranging from 5 to 2000 Gy h^{-1}.

Real wastewater samples were obtained from the three different secondary effluents of water treatment plants with the activated sludge system in the agricultural areas. The amounts of TOC of these samples were 7.25, 8.66 and 25.4 mg dm^{-3}, and the pH values were 8.24, 8.28 and 7.49, respectively. Each sample at 0.1 dm^3 was used after filtration with a paper (Whatman, Qualitative Circles 150 mm ϕ) to remove dusts. The real wastewaters before and after γ-ray irradiation prepared at pH value of about 3.5 by adding hydrochloric acid were extracted with solid phase column (Waters, Sep-Pak plus PS-2). The samples were eluted with acetone (for RP, PCB Anal., Kanto Chemical Co., Inc.). The effluents were evaporated to dryness with a gentle stream of nitrogen, and reconstituted by addition of 3×10^{-3} dm^3 of borate buffer solution prepared by mixing of boric acid (GR, Wako) with sodium hydroxide (GR, Kanto Chemical Co., Inc.) of the pH value of 9.0. The solutions were extracted twice with 2×10^{-3} dm^3 of ethylacetate (for RP, PCB Anal., Kanto Chemical Co., Inc.). The extracted solutions were purged with nitrogen gas, and were added to 6×10^{-6} dm^3 of dimethylsulfoxide (for Biochem., Wako) and 1.44×10^{-4} dm^3 of modified Sabouraud's dextrose medium. The estrogen activities of the solutions were measured by the yeast two-hybrid assay with hERα or mERα. Detailed procedure of the assay was described in III-2-2, Chapter III [IV-3]. The real wastewaters after γ-ray irradiation were also extracted by dichloromethane (for

Dioxin Anal. Wako), and dried overnight at room temperature. Each sample was dissolved into 10% aqueous methanol solution and analyzed by ELISA (E2 ELISA Kit, Japan EnviroChemicals) to estimate E2 equivalent concentration [IV-4]. In this Chapter, the E2 equivalent concentration by ELISA with E2 antibody which selectively reacts with E2 is estimated to confirm the value of estrogen activity estimated by the yeast two-hybrid assay with hERα. The cross reactivity of hERα is suppressed by the hormone blocking chemicals, antagonists, in the real wastewater.

A model wastewater of pH value at 7.45 and the amount of TOC at 20.3 mg dm^{-3}, which has no estrogen activity, was collected at an effluent of a water treatment facility. A part of the model wastewater was diluted into one-second or one-twenties with pure water supplied from a Milli-pore Milli-Q system in order to prepare the samples containing different amounts of TOC. 17 *β*-estradiol (E2, GR, Tokyo Chemical Industry) at 1.8 nmol dm^{-3} or *p*-nonylphenols (NPs, mixture of isomers with branched side chains, Tokyo Chemical Industry) at 1 μmol dm^{-3} were added to each wastewater to prepare the model wastewater samples. An HPLC (Agilent, 1100 series) with a reversed phase column (Shodex, RSpak DE-613) was used for analyses of E2 or NPs solutions at 313 K before and after γ-ray irradiations. Mixed solutions of pure water and methanol (for HPLC Analysis, Wako) were used as an eluent. Mass spectrometer (JEOL, LC-mate) connected with the HPLC was used to determine the concentration of E2 before and after γ-ray irradiation.

IV-3 Results and Discussion

IV-3-1 Decrease in estrogen activity of real wastewater

Evaluation of estrogen activity is significant for the treatment of EDCs in wastewater because primary products of the EDCs formed by irradiation show estrogen activity as mentioned in Chapter II and Chapter III. Three samples of the real wastewater before irradiation were analyzed by the yeast two-hybrid assay, ELISA and TOC analysis as shown in Table IV-1. Subtraction of the estrogen activities (hEA) estimated by using hERα from the estrogen activities (mEA) using mERα gives activities of the artificial EDCs in wastewater. The E2 equivalent concentration by ELISA is estimated to confirm the cross reactivity of the hERα, which is suppressed by antagonists in the real wastewater.

The mEA and the hEA of sample 1 were determined to be 8.3 and 0.5 ng dm^{-3}, respectively, indicating that artificial chemicals are included in the wastewater. While the E2 equivalent concentration of sample 1 was 0.8 ng dm^{-3}, which was closed to that of hEA. The mEA of 3.1 ng dm^{-3} was higher than the hEA of 0.4 ng dm^{-3}, and the estrogen activity of sample 2 is also considered to come from the artificial chemicals. In contrast to the case of sample 1, the E2 equivalent concentration was estimated to be 2.8 ng dm^{-3}, suggesting that the cross reaction of hERα would be suppressed by antagonists. The mEA and the hEA of sample 3 were estimated to be 11.3 and 5.1 ng dm^{-3}. The E2 equivalent concentration was 3.3 ng dm^{-3} before irradiation, and the little difference between hEA and E2 equivalent concentration may be derived from estrogen activities of estrogenic compounds except E2.

Treatment of the wastewater is required to decrease the estrogen activity to less than 1 ng dm^{-3}; the lower limit concentration of appearance of endocrine disrupting property. Estrogen activities of the real wastewaters as a function of

dose of γ-ray irradiation are shown in Fig. IV-1. Every mEA (□) initially increased and then decreased by the irradiation, indicating that decomposition products in the real wastewaters also have the estrogen activity. The doses required to decrease in mEA of samples 1 to 3 below 1 ng dm^{-3}, D_{1ng}, were estimated to be 100, 200 and 150 Gy, respectively. The D_{1ng} of sample 2 was estimated by an extrapolation of the decomposition curves of mEA in Fig. IV-1 (b). Since the D_{1ng} of E2 at 500 ng dm^{-3} (1.8 nmol dm^{-3}) in pure water was estimated to be 5 Gy as mentioned in Chapter II, the elimination of estrogen activity of real wastewater is considered to be interfered by organic impurities.

IV-3-2 Decomposition of 17 *β*-estradiol and *p*-nonylphenol in model wastewater

The model wastewaters containing E2 or NPs were investigated because these samples are the typical chemicals in estrogenic compounds and artificial EDCs, respectively. Decomposition efficiency of EDCs by hydroxyl radicals would be interfered by the organic compounds in wastewater. In this section, reaction of EDCs with hydroxyl radicals in the real wastewater containing organic compounds (OC) is discussed. The EDCs react with hydroxyl radicals as follows:

$$\text{EDCs} + \text{OH} \xrightarrow{k_1} \text{Product 1a} + \text{Product 1b} \qquad \text{(IV-1)}$$

Product 1a has estrogen activity and aromaticity, but Product 1b does not have. Product 1a react with hydroxyl radicals to give secondary product (Product 2) having no estrogen activity.

$$\text{Product 1a} + \text{OH} \xrightarrow{k_2} \text{Product 2 (Secondary product)} \qquad \text{(IV-2)}$$

OC having no estrogen activity in wastewater also react with hydroxyl radicals:

$$\text{OC} + \text{OH} \xrightarrow{k_3} \text{Unknown products} \qquad \text{(IV-3)}$$

These reactions competitively occur and the rate of elimination of EDCs is given as follows,

$$-\frac{\mathrm{d[EDCs]}}{\mathrm{d}D} = \frac{G_{\mathrm{OH}}}{100 \cdot 1.6 \times 10^{-19} \cdot N_{\mathrm{A}}} \frac{k_1[\mathrm{EDCs}]}{k_1[\mathrm{EDCs}] + k_2[\mathrm{Product\,1a}] + k_3[\mathrm{OC}]} \qquad \text{(IV-4)}$$

where D, G_{OH} and N_{A} are absorbed dose of the wastewater, G-value of hydroxyl radicals and Avogadro's number, respectively. The k_1, k_2 and k_3 are considered to be about 10^7~10^{10} $\mathrm{mol^{-1}\ dm^3\ s^{-1}}$. Since the concentration of OC is about 10^7 times higher than those of EDCs and Product 1a, Equation (IV-4) is approximated as follows.

$$-\frac{\mathrm{d[EDCs]}}{\mathrm{d}D} = \frac{G_{\mathrm{OH}}}{100 \cdot 1.6 \times 10^{-19} \cdot N_{\mathrm{A}}} \frac{k_1[\mathrm{EDCs}]}{k_3[\mathrm{OC}]} = \frac{\mathrm{A}}{[\mathrm{OC}]}[\mathrm{EDCs}] \qquad \text{(IV-5)}$$

$$\mathrm{A} = \frac{G_{OH}}{100 \cdot 1.6 \times 10^{-19} \cdot N_{\mathrm{A}}} \frac{k_1}{k_3} \qquad \text{(IV-6)}$$

$$\therefore [\mathrm{EDCs}] = [\mathrm{EDCs}]_0 \exp\left(-\frac{\mathrm{A}}{[\mathrm{OC}]}D\right) \qquad \text{(IV-7)}$$

Decomposition efficiency of EDCs was represented as A/[OC]. The amount of TOC is the sum of the concentration of EDCs and the other OC. Since the concentration of EDCs was about 10^7 times lower than that of the other OC, the amount of TOC was used in place of [OC] in this research.

Decomposition of E2 in the model wastewaters by γ-ray irradiation at each TOC concentration is shown in Fig. IV-2. Although E2 at the initial concentration of 1.8 nmol $\mathrm{dm^{-3}}$ in pure water (TOC: 4×10^{-6} g $\mathrm{dm^{-3}}$) was decomposed at a dose of 10 Gy, decomposition of the model wastewaters

required doses ranging from 50 to 200 Gy. Each plot was fitted with Equation (IV-7). The decomposition efficiencies, A/[OC], in the model wastewaters at the amount of TOC of 20.3, 10.2, 1.02 and 4×10^{-3} mg dm^{-3} were estimated to be 1.7×10^{-2}, 2.8×10^{-2}, 1.9×10^{-1} and 0.45 Gy^{-1}. A/[OC] decreases sharply with the concentration of TOC up to 10 mg dm^{-3} and then slowly decreased. The value of A (the fitting parameter) was estimated to be 2.5×10^{-4} g dm^{-3} Gy^{-1} as shown in Fig. IV-3.

The decomposition efficiency of NPs in the model wastewater decreased with increase in the amount of TOC as shown in Fig. IV-4, and could be fitted with Equation (IV-7). The value of A was estimated to be 1.0×10^{-4} g dm^{-3} Gy^{-1}. The decomposition of the EDCs by hydroxyl radicals in model wastewater would take place following after the equations (IV-1) to (IV-3).

IV-3-3 Simulation of decrease in estrogen activity of real wastewater

Reduction of estrogen activities of the real wastewaters was simulated with Equations (IV-1) to (IV-7). The estrogen activity of the real wastewater, EA, is defined as follows:

$$\mathrm{EA} = CR_1[\mathrm{EDC}_1] + CR_2[\mathrm{EDC}_2] + \Lambda + CR_n[\mathrm{EDC}_n] \qquad \text{(IV-8)}$$

where *CR* is the cross reactivity of the EDCs with the estrogen receptors. Assuming that the Product 1a has estrogen activity but not the Product 1b and the secondary ones, the estrogen activity after irradiation is given as follows:

$$\mathrm{EA} = CR_1[\mathrm{EDC}_1] + CR_2[\mathrm{Product\,1a}] \qquad \text{(IV-9)}$$

Formation yield of Product 1b in Equation (IV-1) is assumed to be equal to that of Product 1a. Then, formation and decomposition of Product 1a is represented as follows:

$$\frac{\mathrm{d[Product\ 1a]}}{\mathrm{d}D} = \frac{\mathrm{A}}{\mathrm{[OC]}}[\mathrm{EDC_1}] - \frac{\mathrm{A}}{\mathrm{[OC]}}\frac{k_2}{k_1}[\mathrm{Product\ 1a}] \qquad \text{(IV-10)}$$

Dose dependence of hEA and mEA of the real wastewater are calculated with Equations (IV-7), (IV-9) and (IV-10) by FACSIMILE for Windows (version 2.0104, AEA Technology plc) as shown in Fig. IV-5. The k_1 was set at 1.6×10^{10} $\mathrm{mol^{-1}\ dm^3\ s^{-1}}$, which is the rate constant of E2 with hydroxyl radicals evaluated by the competition reaction method with phenol as mentioned in Chapter II. This value is the diffusion controlled limit of the reaction rate of aromatic compounds with hydroxyl radicals [IV-5-6]. We assume that Product 1a has the same rate constant as EDCs, because Savel'eva et al. [IV-7] reported the rate constant of aromatic compounds with hydroxyl radicals slightly increases in OH substitution products. The CR_1 and CR_2 for mEA are determined to be 1.0×10^{-4} and 4.0×10^{-4}, being consistent with the fact that estrogen activity of the irradiation products from NPs would have about 2 times higher than the NPs. The CR_1 and CR_2 for hEA are set at 1.0 and 0.1 since estrogen activity of the irradiation products would be less than that of E2. The values of A, 1.0×10^{-4} g $\mathrm{dm^{-3}\ Gy^{-1}}$ and 2.5×10^{-4} g $\mathrm{dm^{-3}\ Gy^{-1}}$, were employed for the simulation.

Simulation curves of mEA for samples 1 to 3 have a maximum, showing the formation of estrogen active products. Simulation curves of hEA of samples 1 to 3 describe the exponential decay function and the irradiation products from EDCs in wastewarter little contribute to increment in hEA. The elimination of estrogen activity in the real wastewater by γ-ray irradiation was explained by the simulated result within error at the early stage. Discrepancy observed as a later stage, may be ascribed to the reaction of Product 1a with other reaction species. However, the required dose for the elimination of estrogen activity of

wastewater by ionizing radiation can be easily estimated by the simulation.

Effect of pH on the decomposition of EDCs in wastewater was also simulated by FACSIMILE as shown in Fig. IV-6. *G*-value of hydroxyl radicals was set to be 2.95 at the pH values of 1 and 14 in this simulation [IV-8]. The reduction rate of estrogen activity at the pH values of 1 or 14 is almost the same with that at the pH values of 7.

IV-3-4 Economical evaluation

Application of radiation to the treatment of EDCs in secondary effluent from sewage treatment plants is discussed on the basis of the results of the decomposition of EDCs in wastewater by γ-ray irradiation. The irradiation system is assumed to be the electron beam accelerator which is rather easy to be newly established and to be attached to the existent wastewater treatment facilities. Electron beam was used to decrease chemical oxygen demand (COD) in the secondary effluent from the sewage treatment plant [IV-2]. Electron beam accelerator (5 MeV, total beam power 300 kW) has been applied for the commercial plant of wastewater from a papermill [IV-1]. Economical cost for decomposition of EDCs in wastewater by electron beam is evaluated on the basis of these pilot plant experiments as shown in Fig. IV-7. Consistency of dose evaluation by γ-ray and electron beam has been confirmed by Kojima et al [IV-9], and the replacement of γ-ray with electron beam is suitable for the pilot plant experiment. Elimination of the estrogen activity of wastewater by electron beam is accomplished at a dose of about 200 Gy. The treatment plant of electron beam for the recirculation of 10,000 m^3 day^{-1} requires electron beam accelerator at a total power of 280 kW (5 MeV, 56 mA) [IV-2]. The irradiation is carried out under the continuous flow condition, and the wastewater is supplied with the jet

nozzle as a thin layer. The initial investment is assumed to be about 600 million yen (15 yen m^{-3}) for the sum of the cost of the electron beam accelerator having the endurance life of 15 years and the personnel costs. The consumption of the electric power for an operation of the electron beam accelerator is estimated to be 2 yen m^{-3}, and the total running cost for the plant is estimated to be 17 yen m^{-3} in Japan. For example, the charges of the treatment plants in Gunma prefecture were set ranging from 30 to 140 yen m^{-3}. It is concluded that the electron beam treatment system is considered to have the potentiality of the attachment to the treatment plants.

IV-4 Conclusion

Ionizing radiation was tested for the treatment of wastewater having estrogen activities, which were estimated by the yeast two-hybrid assay using human or medaka estrogen receptors. The estrogen activity derived from the estrogenic compounds in the real wastewater decreased with increase in absorbed dose. On the other hands, estrogen activity of the real wastewaters containing artificial EDCs increased initially and then decreased. The D_{1ng} of the wastewater was estimated to be about 200 Gy. The results of the decomposition of E2 and NPs in model wastewater suggests that D_{1ng} is determined by the amounts of TOC in the wastewater. The elimination of estrogen activity in the real wastewater by γ-ray irradiation was satisfactory explained by the simulated result. The economic cost of the treatment plant of EDCs using electron beam was estimated to be 17 yen m^{-3}.

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Table IV-1 Estrogen activity, 17 β-estradiol equivalent concentration and the amount of total organic carbon (TOC) in wastewaters.

Sample	Estrogen activity (ng dm^{-3}) hEA	mEA	E2 equivalent conc. (ng dm^{-3})	TOC (mg dm^{-3})
1	0.5	8.3	0.8	7.25
2	0.4	3.1	2.8	8.66
3	5.1	11.3	3.3	25.4

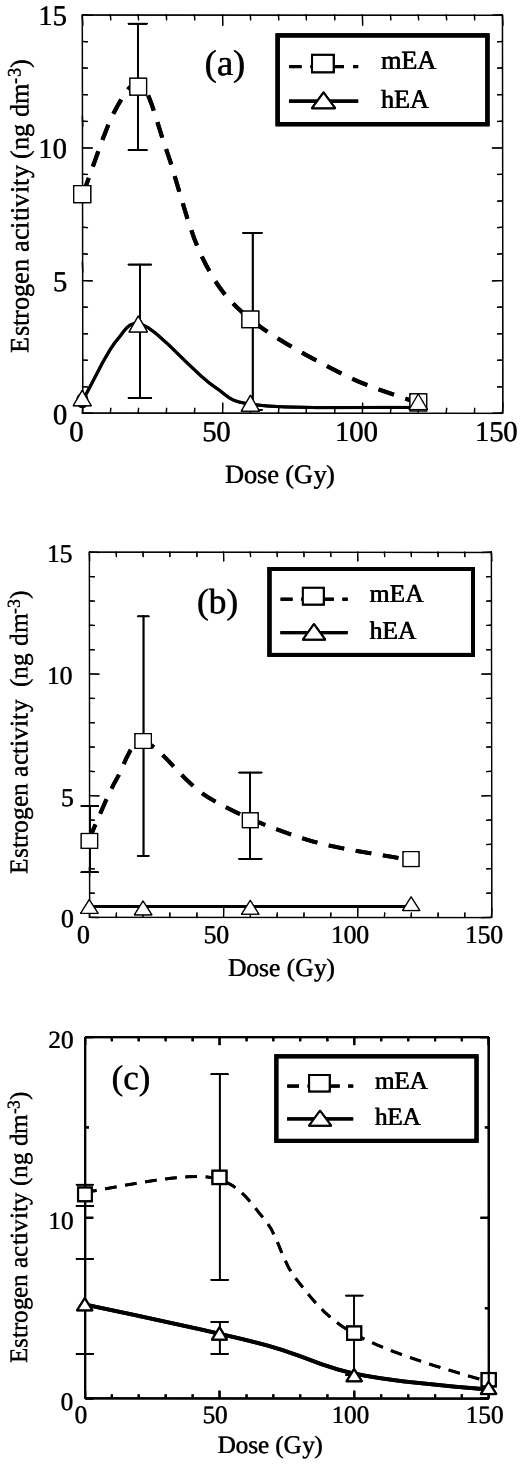

Fig. IV-1 Estrogen activities estimated by using medaka estrogen receptor (□) and human estrogen receptor (△) for real wastewaters by γ-ray irradiation.

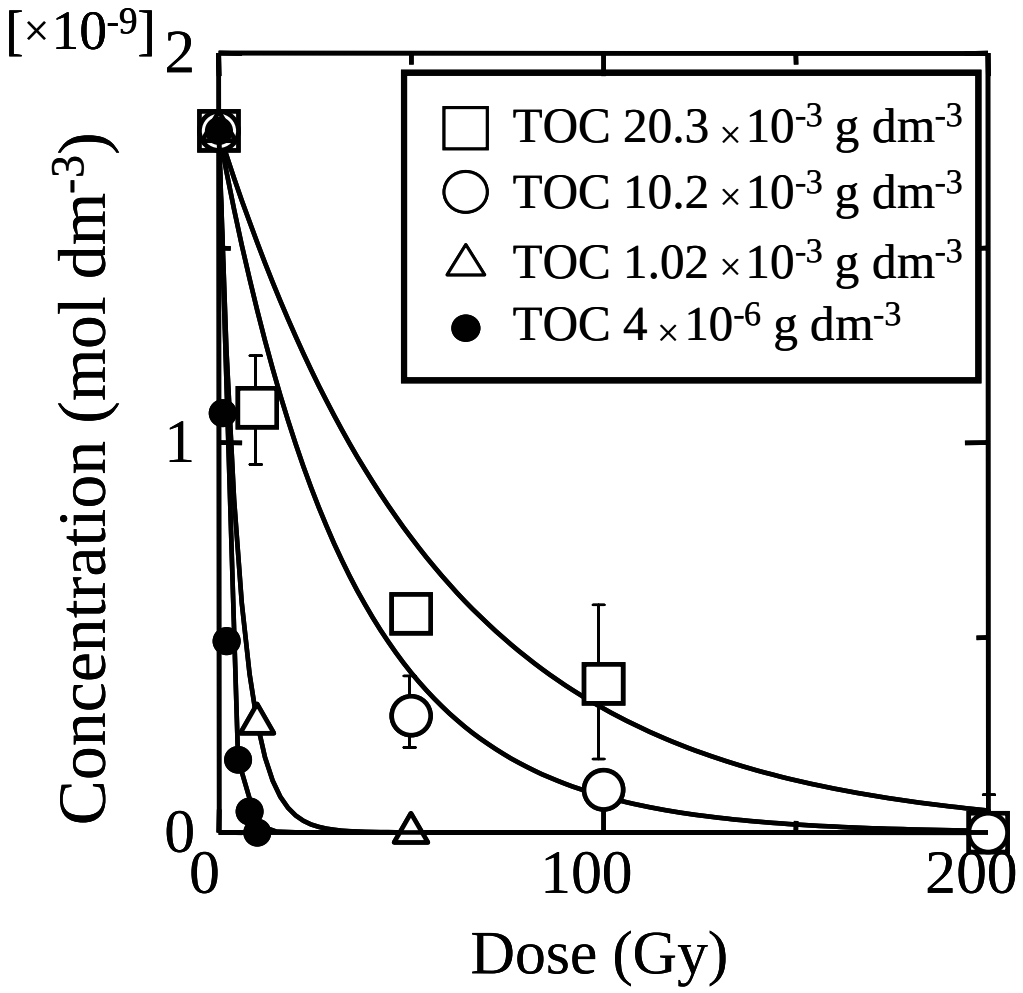

Fig. IV-2 Concentration of 17 β-estradiol in model wastewater after γ-ray irradiation: the amount of total organic carbon (TOC) in each sample was set at 20.3×10^{-3} g dm^{-3} (□), 10.2×10^{-3} g dm^{-3} (○), 1.02×10^{-3} g dm^{-3} (△) and 4×10^{-6} g dm^{-3} (●: Mill-Q water).

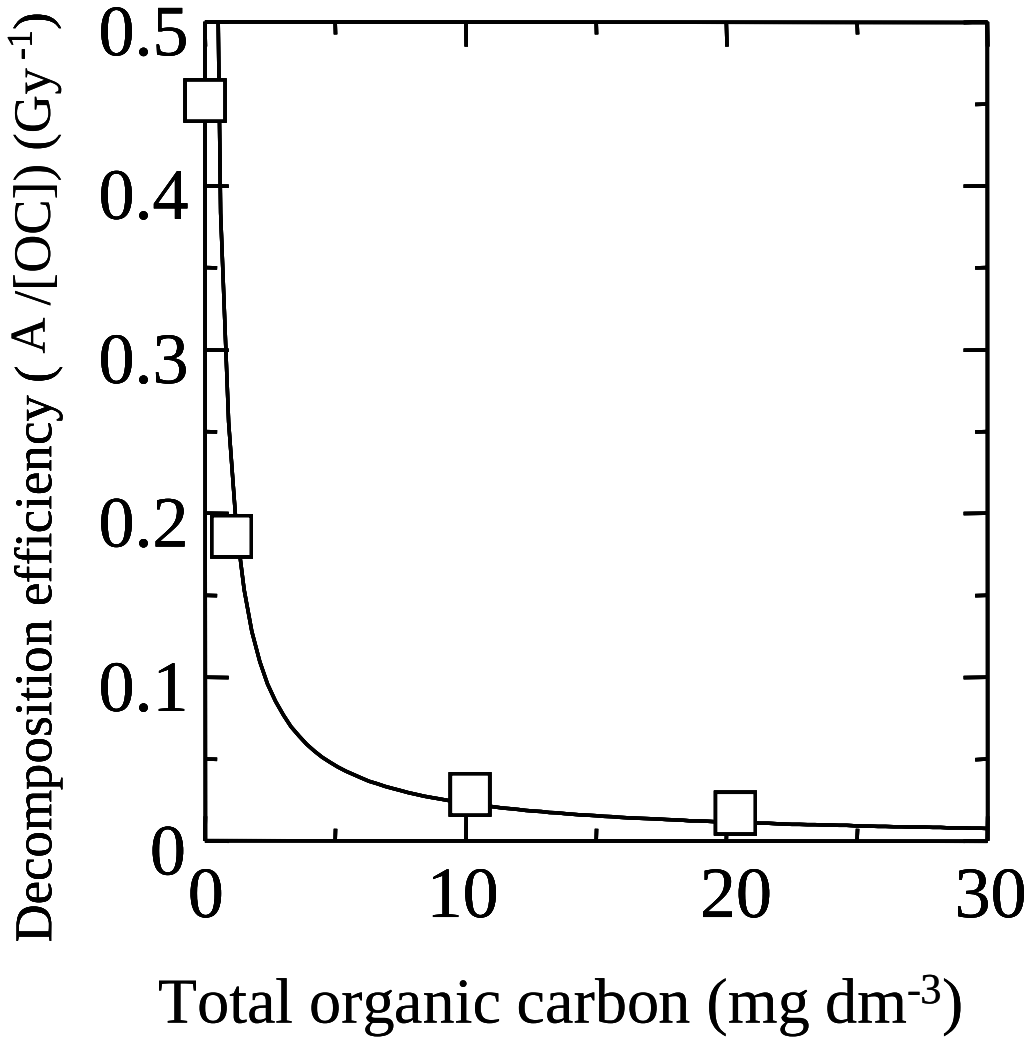

Fig. IV-3 Dependence of decomposition efficiency (A/[OC]) of 17 β-estradiol in model wastewater on the amount of total organic carbon.

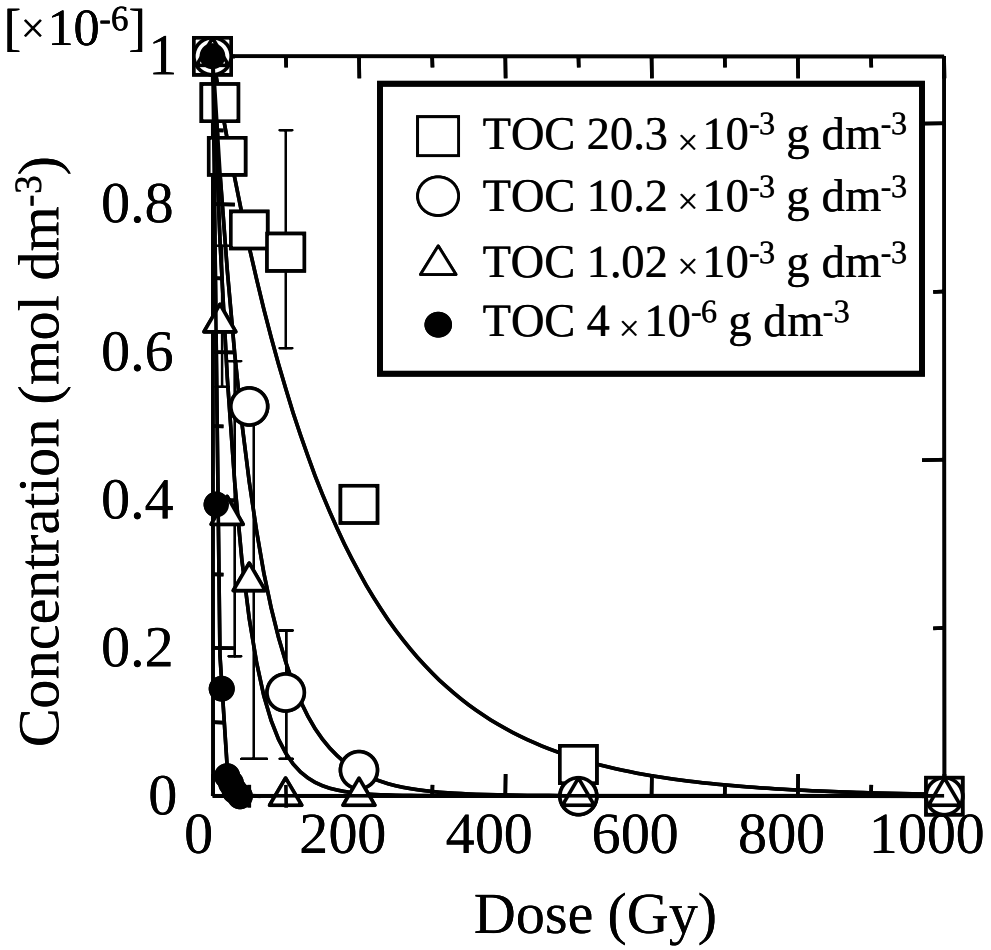

Fig. IV-4 Concentration of *p*-nonylphenol in model wastewater as a function of dose: the amount of total organic carbon in each sample was set at 20.3 × 10^{-3} g dm^{-3} (□), 10.2 × 10^{-3} g dm^{-3} (○), 1.02 × 10^{-3} g dm^{-3} (△) and 4 × 10^{-6} g dm^{-3} (●: Mill-Q water).

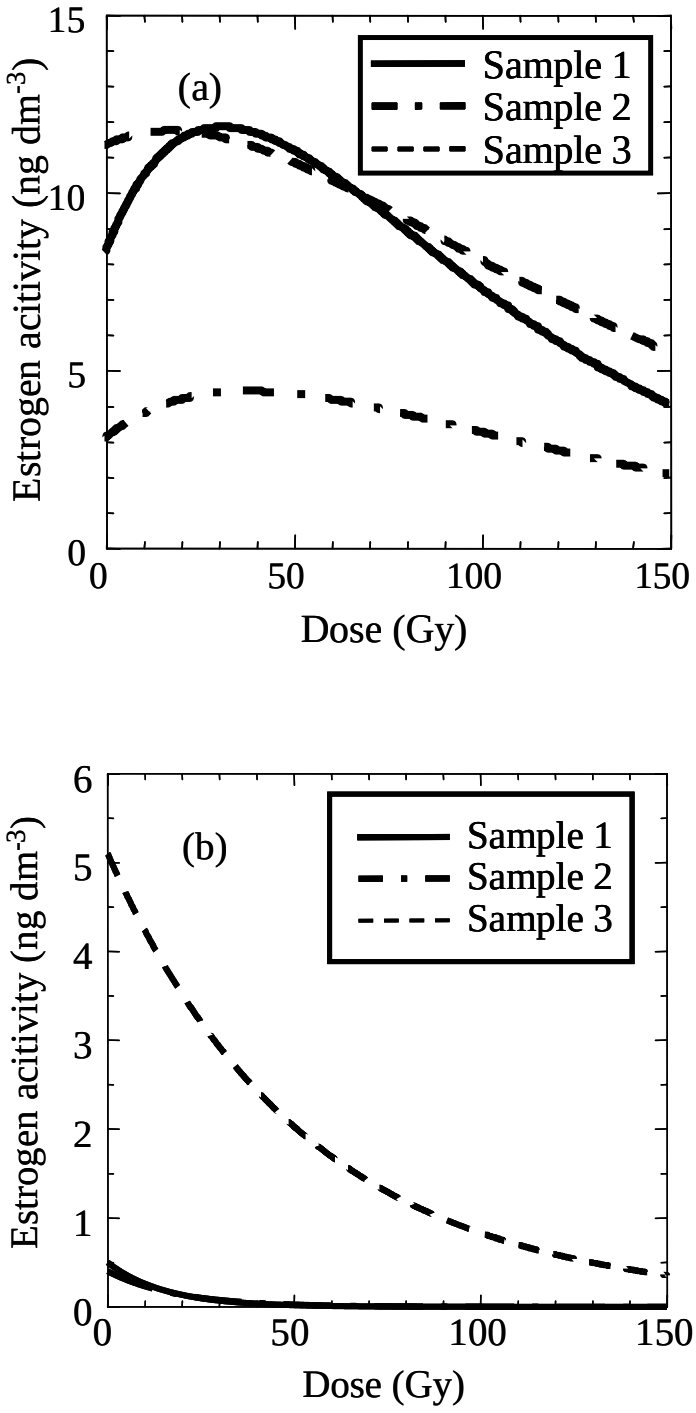

Fig. IV-5 Simulation of decrease in estrogen activity by making use of medaka estrogen receptor (a) and human estrogen receptor (b).

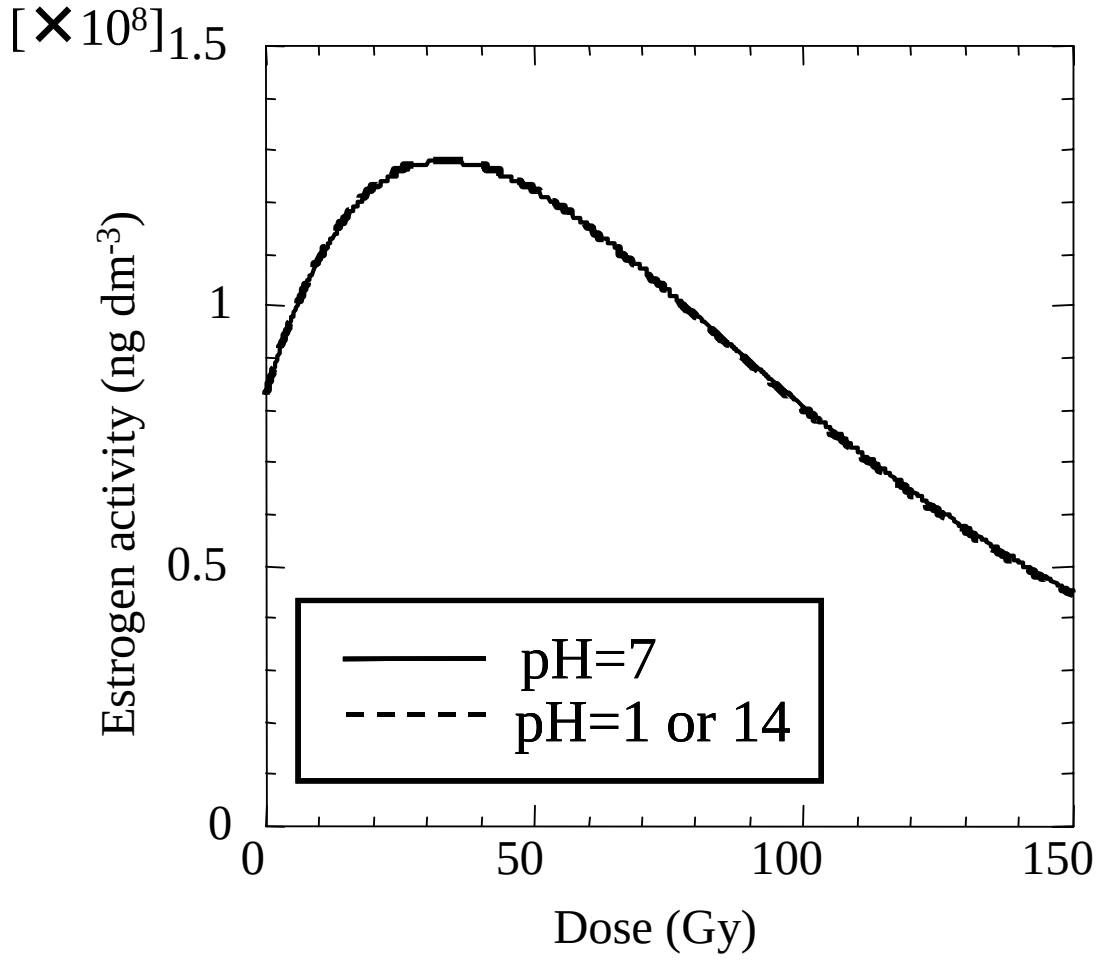

Fig. IV-6 Simulation for the decomposition of estrogen activity of Sample 1 by FACSIMILE under the neutral condition (solid line) and acidic or alkaline condition (broken line).

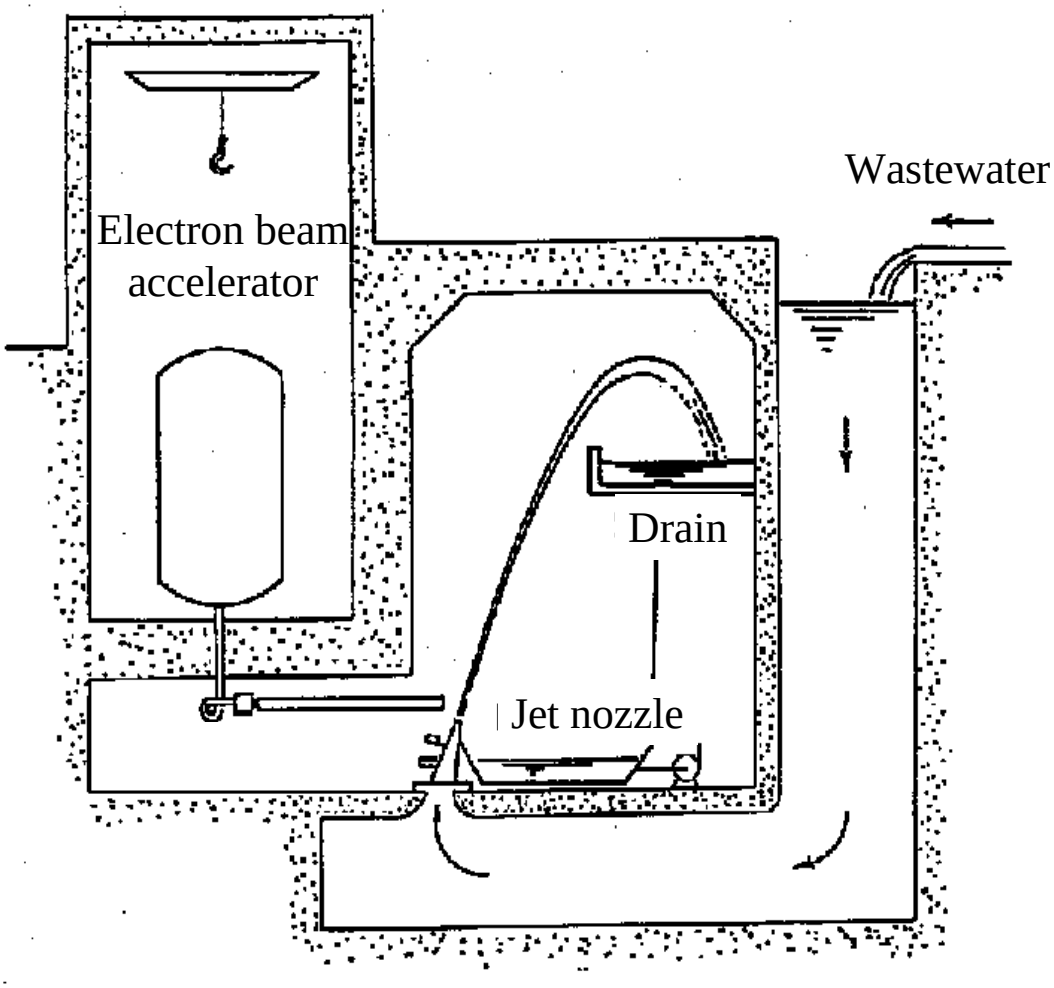

Fig. IV-7 The wastewater treatment plant using an electron beam accelerator to decrease chemical oxygen demand, referred from [IV-1].

Chapter V

Conclusion

V. Conclusion

Decomposition of E2 having one of the highest estrogen acitivity in all substances and NPs exhibiting one of the highest estrogen acitivity in artificial chemicals were studied by γ-ray irradiation. Treatment of real wastewater of the estrogen activity was investigated based on the results of decompositions of E2 and NPs.

Trace amounts of E2 in water was degraded almost completely by ^{60}Co γ-ray irradiations up to 10 Gy. The estrogen activity of the E2 solution, however, still remained after the 10-Gy irradiation. Further irradiations up to 30 Gy was able to decrease the estrogen activity to the level lower than the threshold level of contamination to induce some estrogenic effects on the environmental ecology.

Trace amount of NPs in water was mainly decomposed by radiation-induced hydroxyl radicals. Formation of formic acid, oxalic acid, inorganic carbon and two different products having molecular weight of 236 were identified with HPLC, LC-MS, ion chromatograph and TOC analyzer. Based on the oxidation mechanisms of *p*-cresol and 4-ethylphenol, the last two products are ascribable to *p*-nonylcatechol and 1-(*p*-hydroxyphenyl)-1-nonanol having high estrogen activity as evaluated by the yeast two-hybrid assay. NPs and two products were completely decomposed at 5000 Gy, and estrogen activities in NPs solution were also eliminated.

Ionizing radiation was tested for the treatment of real wastewater having estrogen activities which were estimated by the yeast two-hybrid assay using human or medaka estrogen receptors. The estrogen activity derived from the estrogenic compounds such as E2 in the real wastewater decreased with increase of the absorbed dose. On the other hands, estrogen activity of the real

wastewaters containing artificial EDCs such as NPs increased initially and then decreased. The doses required to decrease in estrogen activity below 1 ng dm^{-3}, D_{1ng}, of the wastewater was estimated to be about 200 Gy. The results of the decompositions of E2 and NPs in model wastewater suggest that the D_{1ng} depends on the amounts of TOC in the wastewater. The elimination of estrogen activity in the real wastewater by γ-ray irradiation was explained by the simulation within error. The cost of the treatment plant of EDCs using electron beam was estimated to be 17 yen m^{-3}.

The application of ionizing radiation to treat real wastewater containing the most hazardous EDCs, E2 and NPs, can be feasible. The required dose for the elimination of estrogen activity of wastewater by ionizing radiation can be easily estimated by the simulation, which is valuable to design economical wastewater treatment process practically. The practical use of water treatment by ionizing radiation is difficult to achieve at the present stage because the initial investment is too expensive to apply, and cost reduction of electron beam accelerator is needed. The ionizing radiation, however, has many advantages such as the low running cost, routine treatment of wastewater and high decomposition efficiency of EDCs. The wastewater from a papermill has been treated by electron beam irradiation on the commercial plant of the recirculation of 10,000 m^3 day^{-1} in Korea. The investigation of this study would support the practical use of electron beam accelerators for EDCs treatment in wastewater.

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