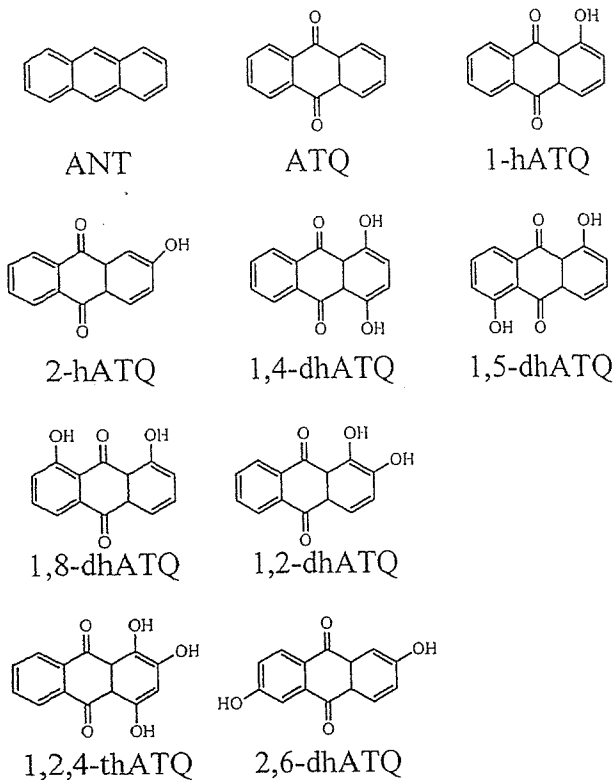

Fig. 1. Structures of anthracene (ANT) and nine of its derivatives used: ATQ = 9,10-anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid.

attracted attention because it allows detection of the presence of endocrine disruptors in a relatively short period of time [17]. Subsequently, a modified method has been developed as a more rapid and simpler detection system [22].

Our primary objective was to develop a method of determining the concentrations of ANT and its photochemical reaction products, including hATQs, in seawater and to use it to test seawater samples off the coast of Japan. Our secondary objective was to evaluate the estrogenic activity of the photochemical reaction products of ANT and their contribution to environmental estrogenic contamination.

MATERIALS AND METHODS

Chemicals

The structures and abbreviations of our target compounds are shown in Figure 1. Anthracene (99% pure), ATQ (97% pure), quinizarin (1,4-dihydroxyATQ; 96% pure), anthrarufin (1,5-dhATQ; 85% pure), danthron (1,8-dhATQ; 96% pure), alizarin (1,2-dhATQ; 97% pure), purpurin (1,2,4-trihATQ; 90% pure), and anthraflavic acid (2,6-dhATQ; 90% pure) were purchased from Sigma-Aldrich (Tokyo, Japan). The 1-hATQ (>95% pure) and 2-hATQ (90% pure) were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). These reagents were used without further purification. Pesticide residue grade solvents and chemicals were obtained from Wako Chemical (Tokyo, Japan).

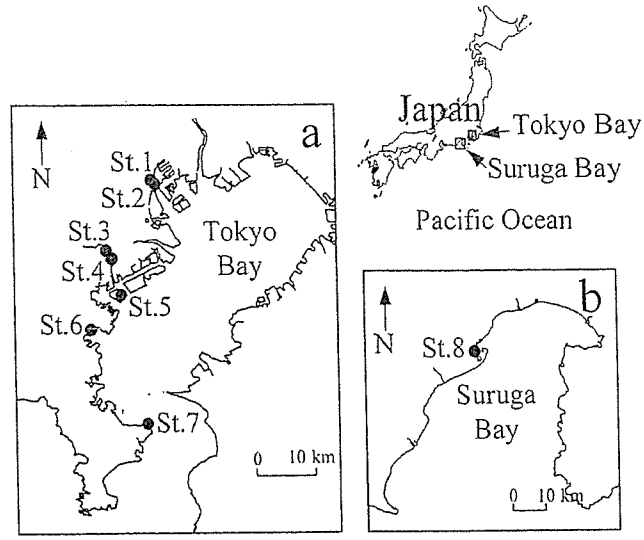

Fig. 2. Sampling locations: (a) Tokyo Bay and (b) Suruga Bay, Japan. Seawater was collected at stations 1 to 8.

Study sites and water collection

Tokyo Bay, with a surrounding population of nearly 26 million, is one of the most polluted areas in the world and has been the subject of many studies of environmental contaminants. For example, studies of fluorescent whitening agents [23] and haloacetic acid [24] have been reported in the past decade. Additionally, histological abnormalities in the gonads of konoshiro gizzard shad (*Konosirus punctatus*) [25] and flounder (*Pleuronectes yokohamae*) [26] were found in samples from inner areas of Tokyo Bay. For our current study, surface seawater samples from Tokyo Bay and Suruga Bay were collected in a stainless-steel bucket. The sampling sites are shown in Figure 2, and their latitude, longitude, collection date, depth, water temperature, and pH are shown in Table 1. Precleaned brown glass bottles (5,000 ml) were rinsed with seawater three times, then filled with sample water and capped.

Table 1. Sampling locations and characteristics of seawater samples (Japan)

Sampling station	Location	Collection date	Depth (m)	WT[a] (°C)	pH
1	35°37.894′N, 139°45.044′E	July 16, 2003	5.0	24.2	6.8
2	35°37.690′N, 139°45.445′E	July 16, 2003	2.1	23.2	7.0
3	35°31.008′N, 139°40.766′E	July 16, 2003	2.9	22.8	7.0
4	35°30.678′N, 139°41.031′E	July 16, 2003	2.7	23.0	6.9
5	35°27.362′N, 139°41.450′E	July 16, 2003	4.1	25.6	8.2
6	35°24.133′N, 139°38.282′E	July 16, 2003	3.5	24.8	8.3
7	35°15.309′N, 139°44.678′E	July 16, 2003	1.3	22.1	8.3
8	35°00.311′N, 138°30.034′E	June 27, 2003	12.2	24.2	8.1

[a] Water temperature.

Samples were brought to the laboratory within 1 d and stored at 4°C.

Chemical analysis procedure

Chemical analysis was performed with a gas chromatograph (GC; HP 5890 series II, Agilent Technologies, Wilmington, DE, USA) mass spectrometer (MS; HP5971 MSD) using selected ion monitoring. Standard and sample extract solutions were analyzed by injecting 1 μl of solution using a 7673-series autoinjector (Agilent Technologies). Separation was done in a 30-m DB-5MS capillary column (0.25-mm i.d., 0.25-μm film, J&W Scientific, Folsom, CA, USA). The GC oven temperature was programmed with a starting temperature of 90°C for 2 min, followed by a 10°C/min temperature increase to a final temperature of 290°C, which was held for 5 min. Identification was made by retention times and ion ratios. The hATQs were detected after trimethylsilylation with *N,O*-bis(trimethylsilyl)trifluoroacetamide with 10% trimethylchlorosilane ([BSTFA] + 10% TMCS, Pierce, Rockford, IL, USA). Standardization was done with anthracene-d_{10} for all the compounds. Silylation reaction time was optimized and found to be 60 min.

The rates of recovery of the target compounds were also optimized. Each test compound was dissolved in acetonitrile to 5 mg/L, and 100 μl of this solution were added to 500 ml of seawater from Suruga Bay (station 8) to give a final concentration of 1 μg/L of each test compound. The 500 ml of spiked seawater were filtered through a glass microfiber filter GF/C 47 mm (Whatman International, Maidstone, UK) and extracted by a 47-mm solid-phase Empore disk (3M, St. Paul, MN, USA), either after acidification with 1 ml concentrated HCl to about pH 2 or with no acidification. Two solid-phase disks, octadecyl silica (C_{18}) [27] and polystyrene–divinylbenzene copolymer (SDB-XC) [28], were used for the extraction. The solid-phase disks were cleaned with 5 ml dichloromethane, 5 ml methanol, and Milli-Q® water (Millipore, Bedford, MA, USA). After the seawater had been extracted by the solid phase, the target compounds were eluted with 5 ml acetone and 15 ml dichloromethane. The extract was filtered and dehydrated by passage through a column of anhydrous sodium sulfate. The organic solution was concentrated in a rotary evaporator, and the solvent was changed to acetonitrile under a nitrogen stream at less than 28°C. After derivatization of the sample with BSTFA (50 μl), the concentrations of ANT and its derivatives were measured by means of GC-MS. The extraction procedure was done in triplicate.

Application of the procedure for measurement of ANT and its derivatives in seawater

We used 1,000 ml of seawater to detect ANT and its derivatives. Ultimately, the organic layer was concentrated to 200 μl. In the case of the sample extraction by means of the C_{18} disk, half of each sample was used for the analysis of ANT and ATQ. The BSTFA was not added to these samples because BSTFA increased the background level of the chromatogram. The rest of the concentrated organic layer was used for the analysis of 1-hATQ, 1,5-dhATQ, and 1,8-dhATQs after derivatization with BSTFA (50 μl). Similarly, the samples extracted by SDB-XC were used for analysis of 2-hATQ, 1,4-dhATQ, 1,2-dhATQ, 1,2,4-thATQ, and 2,6-dhATQs. Each sample was measured in duplicate or triplicate.

Stability of ANT and its derivatives in seawater

The stability of ANT and its derivatives stored in seawater was examined by modifying the methods that are used for the study of estradiol and ethinylestradiol degradation [29]. Briefly, the ANT and its derivatives (each concentration: 1 μg/L) were incubated in the dark at 20.0 ± 0.15°C in precleaned glass bottles with seawater collected at station 8, and the concentrations of these compounds were measured in a 1,000-ml sample 0, 2, 4, 8, and 24 d after incubation began.

Estrogenicity assay

We used a rapid and simple operational estrogenicity assay system with the yeast two-hybrid system, based on the ligand-dependent interaction of two proteins (a hormone receptor and a coactivator), and detected hormonal activity on the basis of β-galactosidase activity [22,30,31]. Recombinant yeast with the estrogen receptor ERα and coactivator TIF2 was provided, and a method of screening for chemicals with hormonal activity was developed using the yeast Y190 (selected by growth on medium lacking tryptophan and leucine) in which two expression plasmids, the pGBT9-estrogen receptor ligand-binding domain (pGBT9-ER LBD) and pGAAD424-TIF2, were introduced [32]. Subsequently, we developed a modified procedure to simplify this method and improve its sensitivity by adapting the 96-well plate-culture method and the chemiluminescent reporter-gene assay method [22]. An Aurora GAL-XE kit containing chemiluminescent substrate, reaction buffer, and accelerator was purchased from ICN Pharmaceuticals (Costa Mesa, CA, USA). Zymolyase 20T was purchased from Seikagaku (Tokyo, Japan). Rat liver S9 was purchased from Kikkoman (Tokyo, Japan).

The estrogen agonist activities of ANT, ATQ, and the eight hATQ compounds in Figure 1 were examined. In addition, the activities of other low-molecular-weight photochemical reaction products of ANT, such as 2,5-dihydroxybenzoic acid, 2-hydroxy-1,4-naphthoquinone, and salicylaldehyde, were assessed [10]. Anthrarobin, 2-hydroxydibenzofuran, 2-hydroxyfluorene, 2-hydroxy-9-fluorenone, and 9-phenanthrol were also assessed to compare the estrogenic activities of target compounds with those of other hydroxyl-PAHs; 2-hydroxydibenzofuran, 2-hydroxyfluorene, and 2-hydroxy-9-fluorenone have been reported to have estrogenic activity [20]. Differences in the intensity of the chemiluminescent signal between samples (four or more times) and a blank control were tested by means of one-way analysis of variance (ANOVA). The chemiluminescent signals were significantly higher in the samples (four or more times) than that of the blank control ($p < 0.002$, ANOVA). Therefore, we defined a positive agonist as occurring when the target chemical increased the chemiluminescent signal to four or more times that with a blank control.

To evaluate the estrogenic activity of the metabolites of test chemicals, we also carried out an estrogenicity assay with S9 metabolic activation using rat liver S9. To analyze the estrogenic activity of the metabolic compounds, we also performed the +S9 test based on rat liver S9. The result of our bioassay of the S9 fraction indicated the estrogenic activity of the metabolic compounds.

Antagonistic assay

We used an antagonist assay system for estrogen receptors that employ a yeast two-hybrid system in which a yeast toxicity test (Ytox test) is incorporated. The principle of the yeast antagonist test for estrogen receptor is measurement of the inhibition of expression of β-galactosidase by competitive estrogen receptor–binding reaction between the test chemical substance and 17β-estradiol added to the medium at 300 pM

Table 2. Ions used for identification and quantification and retention times (RTs) for anthracene (ANT) and its derivatives in seawater

Analyte	Molecular ion/ characteristic fragment ion (*m/z*)[a]	Qualifier ion (*m/z*)	RT (min)
Anthracene	178.1	152.1	13.77
Anthraquinone (ATQ)	208.2	152.1/180.1	15.71
1-Hydroxyanthraquinone (1-hATQ)	281.2	282.2	18.40
2-Hydroxyanthraquinone (2-hATQ)	281.3	282.2/296.3	19.25
Quinizarin (1,4-dhATQ)	354.2	369.2	20.17
Anthrarufin (1,5-dhATQ)	354.2	369.2	20.52
Danthron (1,8-dhATQ)	369.2	370.3	20.67
Alizarin (1,2-dhATQ)	369.2	370.3	20.93
Purpurin (1,2,4-thATQ)	442.3	443.3/457.3	21.81
Anthraflavic acid (2,6-dhATQ)	369.2	384.3	22.20

[a] The *m/z* values correspond to the trimethylsilyl derivatization of the compounds if free hydroxy groups are present.

as a ligand for the receptor [31]. The yeast toxicity test, which measures the residual activity of β-galactosidase using a test chemical alone, is designed to indicate the toxicity of a test chemical to yeast. Thus, general toxicity and estrogen receptor antagonist activity can be distinguished by comparing the two results. The yeast used in the toxicity test has the activity of producing β-galactosidase without estrogen. Where a test chemical is not toxic to yeast, the β-galactosidase activity increases even in the absence of estrogen. Where a test chemical is toxic to yeast, the expression of β-galactosidase decreases. Therefore, a decrease in the expression of β-galactosidase in the Ytox test means that the test chemical was toxic to yeast. We defined the test chemical as an estrogen antagonist when the median effective concentration (EC50) value was less than 25% of the median inhibitory concentration (IC50) value in the Ytox test. We used the estrogen receptor yeast antagonist test to examine the estrogen receptor antagonist activities of ANT and its derivatives. A positive antagonist was defined as a chemical that decreased the chemiluminescent signal to 60% or less of that value for a blank control.

Vibrio *assay*

The toxicity test based on the response of *Vibrio fischeri* (the VF test) measures the inhibition of bacterial luminescence by test substances. We also performed the VF test to detect acute toxicity of the test chemicals. This test was performed as described previously [33].

Briefly, the *V. fischeri* cultures were seeded into a 96-well culture plate with T medium prepared with peptone, glycerol, NaCl, $MgSO_4$, KCl, and K_2HPO_4. Assays were established in triplicate with the test chemicals and blank (T medium containing 4% dimethyl sulfoxide). The intensity of chemiluminescence was measured with an AB2100 Luminescencer (ATTO, Tokyo, Japan). Chemicals were defined as nonactive if the luminescent signal remained between 90 and 100% of the level measured for the control. Differences in the intensity of the chemiluminescent signal between the samples (80–89%) and the blank control were tested by means of ANOVA. The chemiluminescent signals were lower in the samples (80–89%) than in the blank control ($p < 0.05$, ANOVA). Therefore, weak was defined as a statistically significant effect at a concentration of 10μM.

Data analysis

We applied a sigmoidal dose–response regression model to calculate EC50 using the GraphPad Prism 4® software (Ver 4.02; GraphPad Software, San Diego, CA, USA). The EC50 is the concentration at which the chemiluminescent signal of 17β-estradiol was inhibited by 50%. The 10× is the concentration at which the ratio of the chemiluminescent signal of the sample to that of a blank control equals 10. The IC50 values (the concentration at which the chemiluminescent signal was inhibited by 50%) were calculated for those chemicals that inhibited the luminescent signal to a value equal to 0 to 79% of the control value. Statistical analysis of the chemiluminescence results was tested by means of ANOVA. We used Bonferroni posttest to identify the concentrations that are significantly different from the control.

RESULTS

Chemical analysis of ANT and its derivatives

The ions we monitored and their retention times (RTs) are shown in Table 2. The recovery rates of each compound, together with the solid-phase disk used and the detection limit, are presented in Table 3. The recovery rates of some compounds were increased by acidification. For example, the recovery rates without acidification of seawater were 77.5% (1-hATQ), 50.9% (2-hATQ), 21.2% (1,4-dhATQ), 32.9% (1,5-dhATQ), 51.0% (1,8-dhATQ), 18.2% (1,2-dhATQ), 0.0% (1,2,4-thATQ), and 8.2% (2,6-dhATQ). Recovery rates with acidification from test samples from station 8 at Suruga Bay are shown in Table 3. Thus, acidification of seawater increased the recovery rate of hATQs.

The analytical procedure used to detect each compound is summarized in Figure 3. Target compounds were extracted from seawater by using two different solid-phase disks. The results indicated that extraction with both C_{18} and SDB-XC was needed for the measurement of these compounds in seawater. Therefore, we developed an analytical procedure that involved both the acidification of seawater before solid-phase extraction and the use of both C_{18} and SDB-XC disks. The recovery rates of some compounds varied with the disk used. For instance, when 1,8-dhATQ was extracted by SDB-XC, the recovery rate was 38.1% (100% with C_{18}; Table 3). On the other hand, when 1,2-dhATQ and 1,2,4-thATQ were extracted

Table 3. Mean recovery values and detection limits (DLs) of anthracene (ANT) and its derivatives from samples taken from station 8 in Suruga Bay (Japan)[a]

Compound[b]	Solid phase	Recovery rate[c] (%; n = 3)	DL (ng/L)[d]
ANT	C_{18}[e]	78.2 (±0.6)	0.2
ATQ	C_{18}	109 (±7.3)	2.9
1-hATQ	C_{18}	111 (±3.6)	0.9
2-hATQ	SDB-XC[f]	108 (±7.6)	1.2
1,4-dhATQ	SDB-XC	86.3 (±12.2)	0.4
1,5-dhATQ	C_{18}	101 (±0.8)	0.5
1,8-dhATQ	C_{18}	100 (±2.1)	0.8
1,2-dhATQ	SDB-XC	90.2 (±16.0)	1.8
1,2,4-thATQ	SDB-XC	149 (±27.3)	4.8
2,6-dhATQ	SDB-XC	137 (±17.1)	0.4

[a] After acidification with 1 ml concentrated HCl to about pH 2.
[b] ANT = anthracene; ATQ = anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid.
[c] Determined from spiked seawater (1 μg/L) using an Empore disk, followed by derivatization with *N,O*– bis(trimethylsilyl)trifluoroacetamide (BSTFA) and gas chromatographic/mass spectrometric analysis.
[d] Signal-to-noise ratio of 3 : 1 was define as being the detection limit.
[e] C_{18} = octadecyl silica.
[f] SDB-XC = polystyrene–divinylbenzene copolymer.

by C_{18}, the recovery rates were 34.5% (90.2% with SDB-XC; Table 3) and 5.9% (149% with SDB-XC; Table 3), respectively.

We used a 1,000-ml sample of Milli-Q water (n = 3) as a blank. No target compound peaks were observed in the chromatogram of this blank, confirming that no contamination occurred during the extraction procedure.

Concentration of ANT and its derivatives in seawater

Table 4 shows the concentrations of ANT, ATQ, and the eight hATQs in seawater from Tokyo Bay and Suruga Bay. Anthracene, ATQ, and four hATQs were detected in the seawater. These compounds were present at concentrations in the order of nanograms per liter in the sampled areas. The ATQ was detected at high concentrations at stations 3 and 4, where low conductivity was measured (station 3: 5.8 ms/cm, station 4: 9.8 ms/cm). Concentrations of ATQ, 2-hATQ, and 2,6-dhATQ were higher than those of the parent compound ANT, except at stations 2 and 4. The relative molar amount of ATQ ranged from 5.6 to 120 times higher, 2-hATQ from 0.74 to 5.4

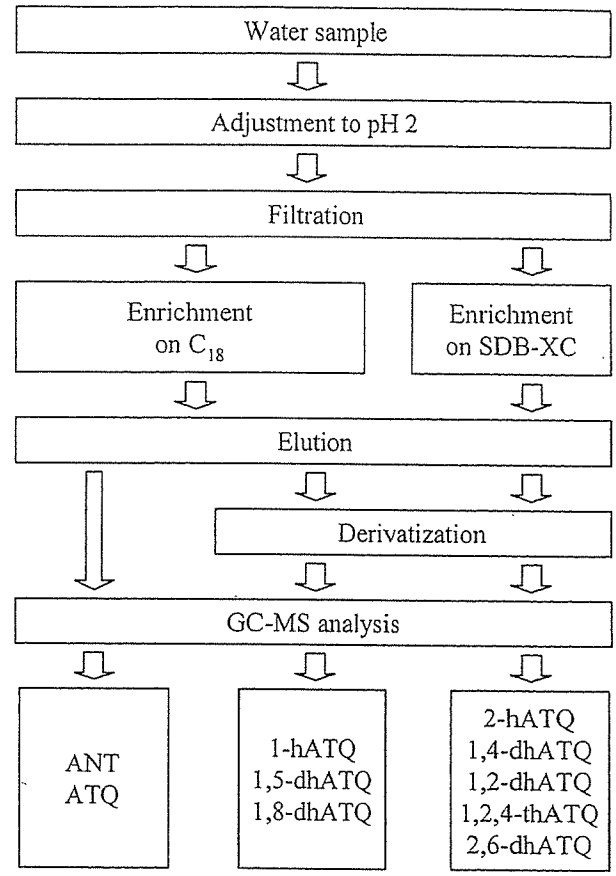

Fig. 3. Analytical procedure used to determine concentrations of anthracene (ANT) and its derivatives in seawater. C_{18} = octadecyl silica; SDC-XC = polystyrene–divinylbenzene copolymer; GC-MS = gas chromatography/mass spectrometry; ANT = anthracene; ATQ = 9,10-anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid.

Table 4. Concentration of anthracene (ANT) and its derivatives in seawater from Tokyo Bay and Suruga Bay (Japan) (ng/L)[a]

Compound analyzed[b]	Sampling location (station)[c] 1	2	3	4	5	6	7	8
ANT	2.2	4.7	1.4	1.7	0.4	0.4	BD[d]	0.4
ATQ	42	31	190	200	9.0	6.7	3.9	22
1-hATQ	1.9	BD	5.3	4.4	BD	BD	BD	BD
2-hATQ	3.6	4.4	4.4	5.5	2.7	2.6	1.7	1.6
1,4-dhATQ	BD	BD	BD	BD	BD	BD	BD	BD
1,5-dhATQ	BD	BD	BD	BD	BD	BD	BD	BD
1,8-dhATQ	BD	BD	0.9	BD	BD	BD	16	7.1
1,2-dhATQ	BD	BD	BD	BD	BD	BD	BD	BD
1,2,4-thATQ	BD	BD	BD	BD	BD	BD	BD	BD
2,6-dhATQ	3.4	7.0	2.0	1.7	1.1	1.4	0.6	1.4

[a] Values were averages of concentration found in double or triplicate analysis.
[b] ATQ = anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid.
[c] See Figure 2 for details.
[d] BD = below the limit of detection.

Table 5. Degradation rates for 1-μg/L target compounds in seawater from a sample at station 8 at 20°C

Compound[a]	Half-life (d)[b]	Adjusted r^2
ANT	8.9	0.953
ATQ	240	0.851
1-hATQ	5.0	0.961
2-hATQ	5.1	0.925
1,4-dhATQ	2.5	0.979
1,5-dhATQ	20	0.972
1,8-dhATQ	3.1	0.965
1,2-dhATQ	4.7	0.909
1,2,4-thATQ	0.3	0.894
2,6-dhATQ	3.9	0.915

[a] ANT = anthracene; ATQ = anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid.

[b] Calculated for target compounds as a simple first-order model.

times higher, and 2,6-dhATQ from 0.74 to 2.6 times higher than ANT values observed at stations 1 to 8.

Stability of ANT and its derivatives in seawater

The stability of ANT and its derivatives were analyzed in seawater collected at station 8. Table 5 shows the half-lives of ANT and its derivatives in seawater. The results indicated that ATQ was the most stable compound in the seawater. Concentrations of eight of the compounds (excluding ATQ and 1,5-dhATQ) decreased to less than 20% of their initial values after 24 d. Concentrations of ANT, 2-hATQ, and 2,6-dhATQ decreased only slightly during the first 8 d, then decreased rapidly. The slight decrease during the first 8 d may reflect the length of time taken for the microbial system to adapt sufficiently to be able to efficiently decompose these compounds. Similar results were obtained in a decomposition experiment that studied anthracene in soil [34].

Estrogen agonist activities of ANT and its derivatives

Figure 4a and b and Table 6 show the estrogen agonist activities of ANT and its photochemical reaction products. The 10× is the concentration at which the ratio of the chemiluminescent signal of the sample to that of a blank control was 10. The positive controls were 17β-estradiol for the −S9 test and *trans*-stilbene for the +S9 test. No activity of *trans*-stilbene occurs in this assay without S9 metabolic activation [17]. Of the ANT derivatives, 2-hATQ and 1,2-dhATQ showed estrogen agonist activity, with 2-hATQ having the strongest activity. We detected 2-hATQ at the test areas (Table 4). The estrogenic activity of 2-hATQ was similar to that of *p*-nonylphenol (10× = 1.2 ×10^5 ng/L) [22], a well-known environmental estrogen. The environmental concentrations of 2-hATQ were lower than that of *p*-nonylphenol (e.g., in Tamagawa River water entering Tokyo Bay, 50–170 ng/L [35]). However, PAHs and their derivatives become significant toxic chemicals that are already present as contaminants in petroleum and that form as by-product of the incomplete combus-

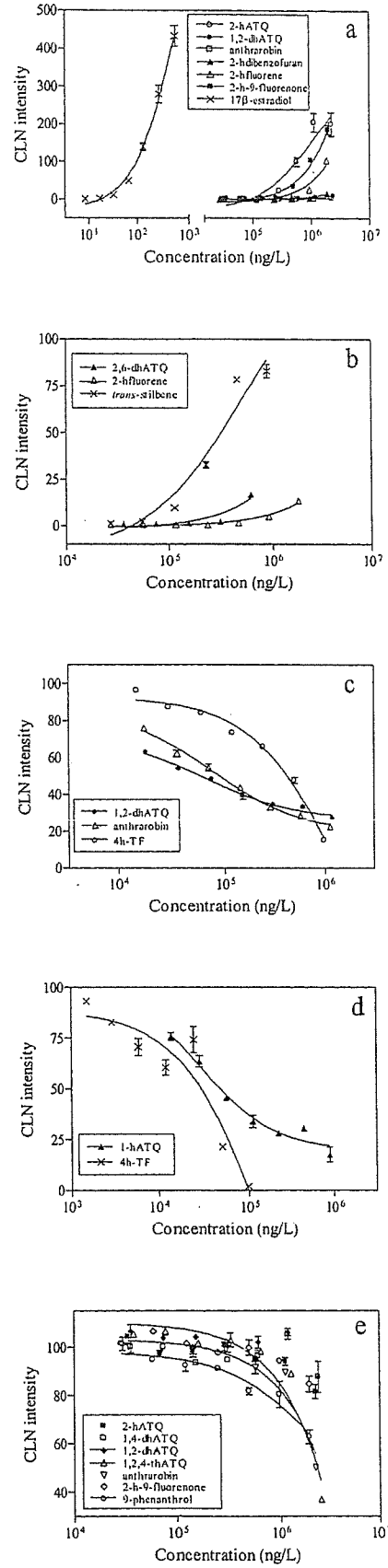

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Fig. 4. Dose–response curves of our target compounds and positive controls. (**a**) Estrogen agonist −S9 test. (**b**) Estrogen agonist +S9 test. (**c**) Estrogen antagonist −S9 test. (**d**) Estrogen antagonist +S9 test. (**e**) *Vibrio fischeri* (VF) test. CLN intensity = chemiluminescence intensity, defined as the ratio of the CLN value for a sample to the CLN value of the control. The CLN value of the control equals 1.0 in Figures 4a and b and 100 in Figures c to e. 2-hATQ = 2-hydroxyanthraquinone; 1,2-dhATQ = alizarin; 2,6-dhATQ = anthraflavic acid; 1-hATQ = 1-hydroxyanthraquinone; 1, 4-dhATQ = quinizarin; 1, 2, 4-thATQ = purpurin; 4-h-TF = 4-hydroxy-tamoxifen.

Table 6. Estrogen agonist activities, estrogen antagonist activities, and toxicities to yeast and *Vibrio fischeri* of anthracene (ANT), photochemical reaction products of ANT, and hydroxyl-polycyclic aromatic hydrocarbons

Compound[a]	Agonist 10× (ng/L)[b]				Antagonist EC50 (ng/L)[c]				Ytox test IC50 (ng/L)[d]				VF test IC50 (ng/L)[e]	
	−S9		+S9		−S9		+S9		−S9		+S9			
ANT	—[f]		—		—		—		—		Weak[g]		—	
ATQ	—		—		—		—		—		—		—	
1-hATQ	—		—		—		4.70×10^4	(0.959)	—		3.42×10^5	(0.942)	—	
2-hATQ	1.19×10^5	(0.902)[h]	—		—		—		Weak		3.15×10^5	(0.873)	Weak	
1,4-dhATQ	—		—		—		—		Weak		1.17×10^6	(0.717)	Weak	
1,5-dhATQ	—		—		—		—		—		Weak		—	
1,8-dhATQ	—		—		—		—		6.81×10^6	(0.943)	3.53×10^5	(0.964)	—	
1,2-dhATQ	2.62×10^6	(0.868)	—		5.90×10^4	(0.983)	—		Weak		2.12×10^5	(0.960)	Weak	
1,2,4-thATQ	—		—		—		—		2.05×10^6	(0.957)	1.72×10^5	(0.968)	2.34×10^6	(0.928)
2,6-dhATQ	—		4.27×10^5	(0.858)	—		—		—		Weak		—	
2,5-dhbenzoic acid	—		—		—		—		—		—		—	
2-h-1,4-Naphthoquinone	—		—		—		—		Weak		9.08×10^5	(0.553)	—	
Salicyladehyde	—		—		—		—		—		Weak		—	
Anthrarobin	5.36×10^6	(0.818)	—		8.93×10^4	(0.987)	—		1.46×10^6	(0.931)	2.25×10^5	(0.969)	2.49×10^6	(0.905)
2-h-Dibenzofuran[i]	1.28×10^6	(0.865)	—		—		—		—		1.90×10^5	(0.917)	—	
2-h-Fluorene[i]	3.41×10^5	(0.926)	1.46×10^6	(0.958)	—		—		—		1.60×10^5	(0.870)	—	
2-h-9-Fluorenone[i]	1.86×10^5	(0.982)	—		—		—		Weak		1.99×10^5	(0.938)	Weak	
9-Phenanthrol	—		—		—		—		Weak		1.40×10^6	(0.504)	4.23×10^6	(0.898)
17β-Estradiol	2.51×10	(0.987)												
trans-Stilbene			8.10×10^4	(0.944)										
4-h-TF					4.16×10^5	(0.985)	2.89×10^4	(0.891)	—		—			

[a] ANT = anthracene; ATQ = anthraquinone; 1-hATQ = 1-hydroxyanthraquinone; 2-hATQ = 2-hydroxyanthraquinone; 1,4-dhATQ = quinizarin; 1,5-dhATQ = anthrarufin; 1,8-dhATQ = danthron; 1,2-dhATQ = alizarin; 1,2,4-thATQ = purpurin; 2,6-dhATQ = anthraflavic acid; 4-h-TF = 4-hydroxy-tamoxifen.
[b] Values listed are the concentration at which the chemiluminescence is 10× the controls.
[c] EC50 (effective concentration 50%); the concentration at which the chemiluminescent signal of 17β-estradiol was inhibited by 50%.
[d] IC50 (inhibitory concentration 50%): the concentration at which the luminescent signal of the yeast was inhibited by 50%.
[e] IC50 (inhibitory concentration 50%): the concentration at which the luminescent signal of the bacterium was inhibited by 50%.
[f] Nonactive.
[g] Weak was defined as a statistically significant effect at a concentration of 10 μM.
[h] Goodness of fit, r^2.
[i] Compounds reported as estrogen agonists (Schultz et al. [20]).

of organic compounds, and these compounds are present ıghout the world.

'e compared the estogenicities of ANT derivatives with : of hydroxyl-PAHs, such as 2-hydroxydibenzofuran, 2- ɔxyfluorene, and 2-hydroxy-9-fluorenone, the estrogen- of which has been reported previously [20]. These three ɔounds also showed estrogenic activity by our methods e 6). The estrogen agonist activity of 2-hATQ ($1.19 \times$ ıg/L) was slightly stronger than that of 2-hydroxy-9-flu- ɔne (1.86×10^5 ng/L), which has been reported to be the gest estrogenic compound among the estrogenic PAHs

the estrogen agonist +S9 test, 2,6-dhATQ showed es- nic activity (Table 6). Its estrogenic activity was about ifth of that of *trans*-stilbene, the positive control in the test. This result suggested that 2,6-dhATQ is transformed trogenic compounds when it is metabolized in the liver. 2-hydroxyfluorene showed estrogenic activity in both the and the −S9 test, suggesting that it might be hard to bolize or may be transformed to other estrogenic com- ds by +S9 tests.

'gen antagonist activities of ANT and its derivatives

ıe estrogen antagonist activities of the test substances are n in Figure 4c and d and Table 6. The yeast antagonist neasured the inhibition of expression of β-galactosidase :d by competition between the test substance and 17β- diol. The positive control was 4-hydroxy-tamoxifen (4- . Toxicity to yeast was evaluated by the IC50 of the Ytox The compounds 2-hATQ, 1,4-dhATQ, 1,8-dhATQ, 1,2- 'Q, 1,2,4-thATQ, 2-h-1,4-naphthoquinone, anthrarobin, 2- uorenone, and 9-phenanthrol showed toxicity to yeast, ıese compounds were more toxic to yeast after they were :d with rat liver S9 than with no S9 treatment (Table 6). esults of the yeast estrogen antagonist test were evaluated ht of the results of the Ytox test. When the EC50 value : estrogen antagonist test was four times smaller than the value of Ytox test, the chemical was considered to be .ve in the antagonist test. Statistical significance was test- · means of two-way ANOVA. The result showed that the ical was positive in the antagonist test ($p < 0.0001$). :fore, we chose to define the chemical as an antagonist. ıen needed to decide whether the decrease in the chemi- ıescent signal was caused by antagonist activity or tox- to yeast. Some compounds had estrogen antagonist ac- with or without S9. In the +S9 test, 1-hATQ had estrogen onist activity. This compound was also detected in sea- · from some of the test sites (Table 4). This result suggests 1-hATQ might have estrogen antagonist activity when up by organisms.

·ity to V. fischeri

gure 4e and Table 6 show the toxicities of the test com- ds to *V. fischeri*. The VF test evaluated the acute toxicity : concentration at which the luminescent signal of these ria was inhibited to 50%; it was assessed for those chem- that, in the original samples, inhibited the luminescent l in the range 0 to 79%.

hen the tested chemical decreased the signal to a level to 80 to 89% of the control value, we regarded the icals as exhibiting weak toxicity. The 2-hATQ, 1,4- 'Q, and 1,2-dhATQ showed weak toxicity to *V. fischeri*. e target compounds, 1,2,4-thATQ was the most toxic to *V. fischeri*. These results showed that photochemical reaction of ANT produces a variety of toxic compounds, whereas the parent compound does not have these toxicities.

DISCUSSION

Little information exists on the action of hydroxyl-PAH-quinones, in particular hATQs, in the environment, though many reports have described the concentrations of intact PAHs in the atmosphere [3], marine core sediments [4], and living organisms [5]. Photochemical reaction products, such as 1-hATQ and 2-hATQ, have only tentatively been identified in previous reports on sediment pore water [15] and contaminated sediment [16]. The silyl derivatization method has been used in the measurement of low-volatility polar compounds. These polar compounds show low sensitivity and tailing in GC analysis. Highly volatile derivatives can be obtained by means of the silylation reaction. We found that analyzing hATQs using silyl derivatization of samples was successful, with high sensitivity (at the ng/L level). The BSTFA is widely used to react with hydroxyl compounds [36,37]. Silylation using BSTFA made it possible to perform highly sensitive analysis of hATQs by GC-MS. It was reported previously that the matrix-induced chromatographic enhanced detection of the chemicals was observed in the analysis by GC [38]. The matrix protects the compounds from adsorption and decomposition in the hot vaporizing injectors and thereby ensures a more complete transfer from the injector to the column compared with the results observed when standards dissolved in matrix-free solvent are used. This could be one reason why our recovery rates exceeded 100% in some cases.

Ours is the first report to identify and quantify eight kinds of hATQs in seawater. Our results showed high concentrations of ATQ, 2-hATQ, and 2,6-dhATQ relative to the parent compound ANT in seawater off a heavily populated area of Japan (Table 4). Of the target compounds, ATQ was detected at the highest concentration in seawater. McKinney et al. have reported the ratio of the concentration of ATQ to that of ANT in coastal marine sediments to be between 0.317 and 2.81 [14]. However, the ratios of the concentration of ATQ to that of ANT in our coastal Japanese samples were between 6.6 and 140, with the exception of the samples from station 7, where ANT was not detected. This disagreement may be related to differences in the octanol–water partition coefficients (log K_{ow}) of ANT and ATQ. The log K_{ow} of ANT is 4.45, and that of ATQ is 3.34 [39]. As ANT is more hydrophobic than ATQ, a disagreement might exist between the ratios of the concentrations of ANT and ATQ in sediment and seawater.

Stability against photochemical reactions and biodegradation would also contribute to the distribution of ANT and its derivatives in the marine environment. Our data showed that ATQ was the most stable compound in seawater (Table 5). Previous studies have examined the stability of ANT and its photochemical reaction products to SSR [8,10]. The ultraviolet radiation present in the solar spectrum will photomodify parent compounds as well as contribute to chemical degradation. These data showed that the half-lives of ANT and ATQ were 2.0 and 1.8 h, respectively [8,10]. The 2-hATQ and 2,6-dhATQ are stable to SSR and are end products of the photochemical reaction of ANT [8,10]. Hydroxyl-PAH-quinones detected in the environment may be formed by photochemical reactions or by biodegradation from PAHs, or they may be discharged in waste from industrial processes.

We also determined the estrogenic activities of the pho-

tochemical reaction products of ANT. For example, 2-hATQ had estrogen agonist activity, and 1-hATQ treated with rat liver S9 had estrogen antagonist activity, although the parent compound ANT did not show these activities (Table 6). Mechanisms for the estrogenicity of various compounds have been proposed. For example, most estrogen agonists have a phenol ring with a moiety of appropriate hydrophobicity at the *para* position, and substitution of a bulky moiety at the *ortho* position reduces their activity [17].

Photomodification reactions of PAHs could result in such toxicities not shown by the parent PAHs. Because of this phenomenon, a need exists to investigate the distribution and behavior of hATQs and to estimate the fate of these compounds in the marine environment. Moreover, in this study, toxic compounds, such as hydroxyl-PAH-quinones, may exist at higher concentrations than intact PAHs in the marine environment (Table 4). The literature shows many instances of photomodified PAHs in atmospheric particulate matter [40]; further studies of the environmental existence of derivatives of other PAHs are needed because contamination by PAHs is frequently reported in the marine environment [4,5]. Seawater samples contained 2-hATQ at concentrations ranging from 1.6 to 5.5 ng/L, and this chemical showed estrogenic activity similar to that of *p*-nonylphenol. The results suggest the importance of studying the distributions of PAH derivatives as well as of PAH in the environment and of the possible environmental effects of the compounds that demonstrated estrogenicity, toxicity, and estrogen antagonism.

CONCLUSION

We observed hATQs in seawater near two populated areas of Japan. The concentrations of photochemical reaction products of ANT, such as ATQ and 2-hATQ, were generally larger than those of ANT. Both estrogen agonist activity and estrogen antagonist activity were found among the hATQs that were the products of photochemical reactions of ANT. The photochemical reaction products of ANT had estrogen agonist or antagonist activity with or without being metabolized by rat liver S9. Our data indicate the importance of monitoring not only ANT but also hATQs. Photochemical reaction products may also exist for other PAHs in the marine environment, so both intact PAHs and hydroxyl-PAH-quinones should be monitored.

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Comparison of implantation and cytotoxicity testing for initial toxic biomaterials

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Abstract:

* Abstract

In order to evaluate the predictive value of cytotoxicity testing, the present study compares the *in vivo* tissue responses to *in vitro* cytotoxicity before and after implantation. Zincdiethyldithiocarbamate (ZDEC) is used as a catalyst for vulcaniizing latex. ZDEC is toxic and has been used as a standard for in vitro cytotoxicity testing. Polyurethane discs with the addition of 0.5% or 1% (ZDEC) as well as non-toxic discs were inserted in the abdominal wall of rats for 1 day up to 6 weeks. After explantation the foreign body response was analyzed immunohistochemically by light microscopy. *In vitro* tests of the explanted reference materials (RMs) revealed remaining high concentrations of toxic compounds after 1-week implantation, whereas no toxicity was seen after 6 weeks implantation. This was reflected in the foreign body response where a significantly thicker capsule and more inflammatory cells were seen at 1-week for the toxic implants. Over time, with decreasing toxicity, these differences disappeared. This study also indicates that *in vivo* implantation is more effective to extract the toxic compound than *in vitro* extraction at body temperature. It is concluded that many clinically useful implant materials may be unnecessarily rejected due to the results of *in vitro* tests.

* Author Agreement

All authors have seen and approved that the manuscript as submitted. The work is original and has not been submitted elsewhere.

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Introduction

The implantation trauma in itself initiates a local, acute host response at the site of the implanted biomaterial. The inserted biomaterial is then a persistent stimulus for a chronic reaction, the foreign body response that depends on factors related to the implant and the host tissue. It has been suggested [1] and is now widely accepted [2, 3] that the primary interactions taking place within a second, between the material's surface atoms, and the molecules of the surrounding tissue will influence the future cellular behavior and implant integration [4, 5]. According to this hypothesis, it would be devastating to for example use a material like bone cement, which is initially toxic [6, 7], and that also generates excessive heat during the curing process. Despite this, bone cement has been proven to establish an excellent clinical long-term integration in bone tissue [8].

Today, cytotoxicity testing of extracts from prospective biomaterials plays a crucial role for the initial decision, whether a proposed material is biocompatible or not. Materials intended for implant applications are regarded as biologically safe after *in vitro* cytotoxicity tests [9, 10] although additional testing to warrant their biological safety with respect to other concerns need to be performed [11]. The cytotoxicity test methods allow analysis of different cellular functions such as replication, secretion, phagocytosis, chemo taxis, chemo kinesis, surface adhesion, etc. [10]. However, since these *in vitro* tests only expose single cells in a culture, these results cannot be directly related to the outcome in such a complex biological system as living tissue [12, 13]. Therefore the predictive value

of various *in vitro* tests, like cytotoxicity, should be considered cautiously for actual implant materials.

At present, many candidate implant materials are rejected due to the results of *in vitro* tests [14, 15]. Some previous studies have shown that candidate implant materials that display a moderate in vitro cytotoxicity have an excellent biocompatibility *in vivo* relative to the controls [16]. Bone cement might for example never have reached clinical evaluation due to its adverse *in vitro* test results [17, 18].

In the present study polyurethane discs, plain or with a defined addition of the cytotoxic zincdiethyldithiocarbamate (ZDEC), an accelerator for vulcanization of latex rubber, were implanted in the abdominal wall of rats. Polyurethane was chosen due to its widespread use as an implant material, and lack of toxicity [19]. After explantation, the host response was evaluated and the explanted discs were reanalyzed with regard to *in vitro* cytotoxicity. These results were further compared to a corresponding *in vitro* extraction test of unimplanted reference materials.

Materials & Methods

Preparations of test sample

Negative reference material (RM): PU (polyurethane sheet, lot no.: 98001NP) and Positive RM: 0.5% ZDEC (zincdiethyldithiocarbamate) and 1% ZDEC PU sheets (lot no.: 96003F and P005, respectively) were used as test samples. All materials were produced as 1 mm thick sheets. These materials have been supplied worldwide as RMs for the safety test of medical devices from the Hatano Research Institute (HRI), Food and Drug Safety Center (FDSC), Kanagawa, Japan.

The sheets of materials were cut into discs using a punch. For the *in vivo* experiments discs of 5 mm diameter were used after ethylene oxide sterilization. Some of these discs were evaluated after explantation for *in vitro* cytotoxicity. These samples were immediately frozen after explantation. They were thawed and ethylene oxide sterilized again just prior to testing. The materials used for *in vitro* testing only, were made to § (right?XX) mm diameter and sterilized using Ethylene oxide (EO).

In vitro testing

All *in vitro* cytotoxicity evaluation was performed according to ISO 10993-5.

Cell and culture conditions

V79 cells (obtained from the Japanese Cancer Research Resources Bank in September 1988, used within ten passages after thawing, passage No.5 at obtaining and passage No.14 at present) were cultured in Eagle MEM medium supplemented with 10 vol% fetal calf serum (FCS, lot no: US192415, Life Technologies), in a humidified incubator at 37°C and 5 % CO_2. The cells were certified to be mycoplasma-free.

In vitro pre-extraction test

In vitro cytotoxicity tests were applied to compare decline in cytotoxicity of materials implanted in animals to *in vitro* extraction in water solutions. Implanted materials were retrieved 1, 2, 4, 8 days and 6 weeks after implantation for cytotoxicity testing. All materials were wiped clean from tissue and kept frozen at –70°C until shipped for toxicity test. *In vitro* analysis of decline in toxicity was done by incubation of the material

in a large volume of water for 0, 4, 8 hrs and 1, 2, 4, 8 days.. The materials were then subjected to two different cytotoxicity tests.

TC-insert method (Tissue Culture) (1)

RMs were extracted for various intervals as described above and used as test sample. Extract solutions were discarded. Fifty cells suspended with 0.5 ml of culture medium were inoculated in each well. Next day, TC-inserts were set in each well. Then a test RM was put in each TC-insert (added with 0.25 ml medium). After 6 days culture, the wells were fixed, stained with Giemsa's solution and the colonies counted.

Medium extract method (2)

In this method, each RM was re-extracted again with MEM medium (1 disc/ml) for 24 hr at 37°C, in a 5% CO_2 incubator. Several doses of extracted medium were prepared by serial dilutions with fresh medium to 0.049, 0.098, 0.2, 0.39, 0.78, 1.6, 3.1, 6.3, 12.5, 25, 50 and 100 % (original extract solution). The colony formation assay was performed by plating 50 cells in each well. The day after plating, cells were treated with the extracted medium and cultured for 6 days. After fixation with methanol and staining with 10% Giemsa solution, the colonies in each well were counted.

The number of colonies at each extraction concentration was counted and expressed as the relative percentage of the negative control. Thereafter the concentration giving 50% inhibition of colony formation (IC_{50}) was calculated.

In vivo implantation

Animals

Male Sprague-Dawley rats, weighing 200-250 g, fed on standard pellets and water ad libitum, were used. The rats were anaesthetized by i.p. injections of 1.0 ml/100 g body weight of a solution containing sodium pentobarbital (60 mg/ml) and NaCl (9 mg/ml) in 1:9 volume proportions. The experimental protocol was approved by the Animal Ethics Committee in Lund, Sweden .

Implantation procedure

The test samples were implanted in rat abdominal wall. Details of the implantation procedure have been described previously [20]. In brief, the rectus abdominis muscle sheath was opened and the muscle moved laterally. Two negative and two toxic discs were inserted on either side of the linea alba, outside the peritoneum without injuring the peritoneal membrane. Some animals solely received negative implants. The rectus abdominis muscle was slipped back to cover the implant and a suture was placed in the muscle sheath to secure the position of the implant. In a first set of experiments eight animals for each implantation time period (i.e. 1 or 6 weeks) were used. In the second set, similar test samples were inserted for 1, 2, 4 and 8 days in 3(XX)animals.

Implant retrieval and tissue fixation

The animals were euthanized after 1 or 6 weeks in the first experiment and after 1,2, 4 and 8 days in the second. Implants with surrounding tissue were removed en bloc. The

specimens were washed in ice-cold phosphate buffered saline (PBS pH 7.4),embedded in Tissue Tek® O.C.T. compound (Histolab Products AB, Sweden) and snap frozen for 30 s in 2-methylbutane at -70°C. The implants were then removed by dissection of the peritoneal membrane without letting the specimen thaw. The tissues were sectioned in a cryostat (6 µm in thickness) and collected on chromium-alum treated slides and allowed to air dry. The slides were kept at -70°C until analyzed.

The explanted implant materials were analyzed at the HRI, FDSC, Kanagawa, Japan for *in vitro* cytotoxicity (ISO 10993-5) and compared to unimplanted reference materials.

Immunohistochemistry

The staining procedures for the ED1 macrophage subclass were done according to Rosengren et al [21]. In brief, after removal of the endogenous peroxidase activity and blocking of unspecific binding the slides were incubated with mouse anti-rat ED1 (Serotec Ltd., Oxford, UK). Then, the specimens were incubated with a biotinylated horse anti-mouse IgG antibody followed by incubation with Vectastain ABC peroxidase standard PK-4000 kit (Vector Lab. Inc., Burlingame, CA, USA). The presence of peroxidase was detected using 3-Amino-9-Ethyl-Carbazole (Sigma Chemical Co, St. Louis, USA). The sections were counterstained in Mayers HTX. Mouse monoclonal primary antibodies directed to human cell surface antigens were used to control unspecific binding.

Image processing

All images were obtained using a RT color Spot digital camera (Diagnostic instruments, Rochester, NY) mounted on a Nikon FXA (Tokyo, Japan) microscope, using bright field

illumination. The images were retrieved and manually counted using Photoshop 5.5 software (Adobe Photoshop, Mountain View, CA, USA) on a Power Macintosh G4 computer (Apple Inc, Cupertino, CA).

Morphometry

Sections from the implants impregnated with 0.5% and 1% ZDEC-PU as well as the non-impregnated controls were evaluated for all implantation times (1 or 6 weeks; 1,2,4 or 8 days). The numbers of cells were determined by computer aided manual counting of all cell nuclei as well as cells stained for the ED1 antigen. The numbers of cell nuclei were used as a measure of the total numbers of cells, which thus includes macrophages, fibroblasts and other cells. A 10x10 square grid where each square covered a 40x40 μm large area was superimposed with one side aligned at the tissue border to the implant surface (magnification objective 20x). The thickness of the reactive capsule was determined using the grid and was defined as the distance between the tissue border to the implant and the muscle – foreign body capsule border. The numbers of cells in the measured capsule were manually counted in 5 orthogonal rows of squares from the implant surface to the border of the muscle.

Statistics

Nonparametric statistics was used (Statview 4.5 for the Macintosh, Abacus Concepts, Berkeley, CA.). Differences in tissue responses due to grade of toxicity were analyzed using the non-parametric Kruskal-Wallis test and a post hoc test was performed according to Siegel [22]. The Mann-Whitney U test was used for evaluating the effects