

EVALUATION OF ESTROGENIC ACTIVITY IN ROCKY REEFS SURROUND THE SHIMA PENINSULA IN JAPAN

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ABSTRACT

A survey of concentrations of estrogenic substances and other pollutant indexes of water and sediment was undertaken in the Shijima territorial sea around the Shima Peninsula in Japan.

The Shijima territorial sea is at the entrance of Ise Bay, which is a typical enclosed sea area, and the seabed is rocky and overgrown. We surveyed the sea area twice (survey no1 and survey no2). At the time of survey no1, the waves were high and the current was fast, therefore, the sediment on the seabed was churned up, and consequently, the suspended solid (SS) value of the bottom water was higher than the surface water. During survey no2, the sea condition was calm, and the water pollutant index values were low. Total organic carbon, total nitrogen and total phosphorus values in the sediment samples were relatively higher than those in the enclosed sea area. Estrogenic substances were detected in the water and sediment samples at survey no1 and survey no2. The water sample value at survey no1 was high, particularly solid estrogenic substances. There were small differences between the sediment sample values at survey no1 and survey no2. Water and sediment samples fractionated by HPLC were assayed, and estrogenic substances were detected retention times coinciding with estrone (E1) and 17 β -estradiol (E2). The origin of the estrogenic substances detected in this survey was probably domestic waste water and sewage treatment water.

RESUME

Une étude sur la concentration de substances œstrogéniques et d'autres indices d'agents de pollution de l'eau et des sédiments a été entreprise dans les eaux territoriales de Shijima, autour de la péninsule de Shima au Japon. Les eaux territoriales de Shijima se trouvent à l'entrée de la baie d'Ise, qui est une zone marine close, et le fond de la mer est rocheux et recouvert. Nous avons étudié deux fois cette zone marine (études N° 1 et N° 2). Au moment de l'étude N° 1, les vagues étaient hautes et le courant rapide. De ce fait, les sédiments au fond de la mer bouillaient et, par conséquent, l'indice des matières solides en suspension (SS) au fond de l'eau était plus élevé qu'à la surface de l'eau. Pendant l'étude N° 2, la condition de la mer était calme et les teneurs d'indices d'agents de pollution de l'eau étaient faibles. Les teneurs totales en carbone organique, en azote total et en phosphore total dans les échantillons des sédiments étaient relativement plus élevées que dans celles de la zone marine close. Des substances œstrogéniques furent découvertes dans l'eau et l'on a recueilli des échantillons de sédiments lors des études N° 1 et N° 2. La teneur de l'échantillon de l'eau pendant l'étude N° 1 fut élevée, avec particulièrement des substances œstrogéniques solides. Il n'y avait que de faibles différences entre les teneurs des échantillons de sédiments aux études N° 1 et N° 2. Des échantillons d'eau et de sédiments fractionnés par HPLC (chromatographie liquide au rendement élevé) furent analysés, et des substances œstrogéniques furent détectées au moment de la rétention, coïncidant avec l'œstrone (E1) et le β -œstradiol (E2). L'origine des substances œstrogéniques détectées dans cette étude provenait probablement d'eaux de décharge domestiques et d'un traitement des eaux d'égout.

1. INTRODUCTION

The Shima Peninsula, located roughly in the center of the Japan Archipelago, is hill shaped and has a winding coastline. It faces the Pacific Ocean and is in the Kuroshio (Japan) Current zone. This peninsula area has a good natural landscape and is known as an Ise Shima National Park. The north east coast of this peninsula is located an entrance to Ise Bay, which is a typical enclosed sea. The seabed in the inshore area of the Shima Peninsula consists mainly of rock reef, and is heavily overgrown with Ecklonia Kelp and another seaweeds. There is an abundance of fish and shellfish in the area forming a complex biodiversity. There are many abalone and spiny lobsters among these shellfish, and they command a high price at auction in Japan. In this region, there is a unique and traditional fisher-

woman (sometimes a man) called an ama (or an ama diver). Ama divers dive to depth of over ten meters while holding their breath to catch these shellfishes. They restrict their fishing area, season, and equipment to prevent the area from becoming overfished, although, catches of abalone have been decreasing since the 1990s. The cause of this decrease is still unknown, but there has been growing concern recently about the wide usage of chemicals because of their toxicity. This area is a popular tourist resort, and a major fishing ground in Japan. Therefore, it is of importance to research the chemical pollutants in this sea area from the point of view of economic activity and environmental protection.

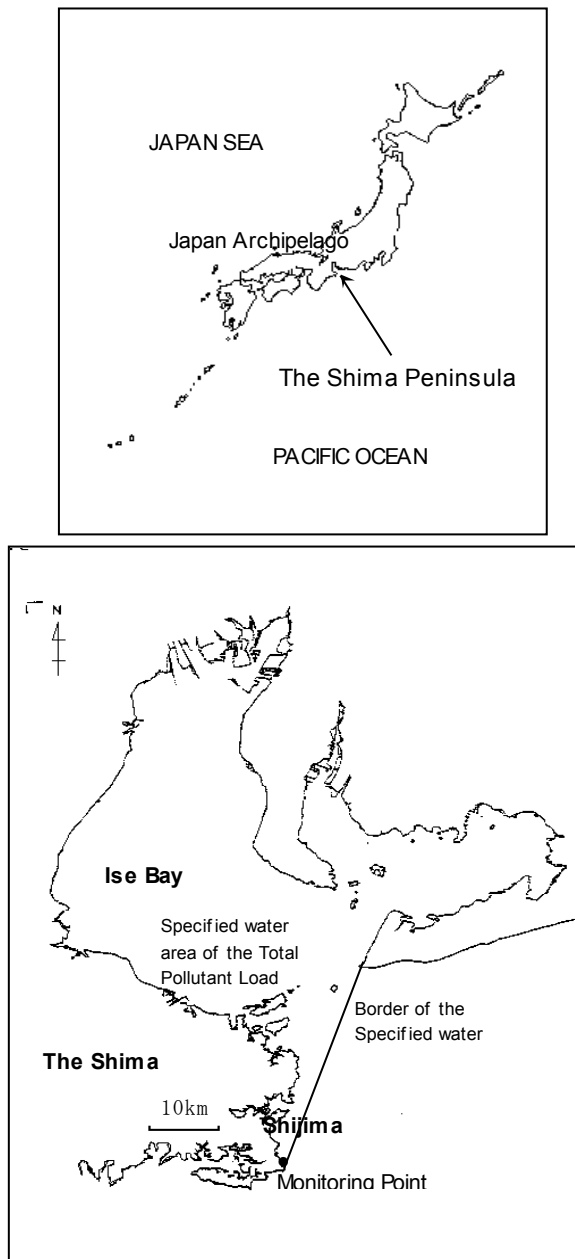


Figure 1. Location of water and sediment quality monitoring point (●).

Latitude 34°18'20"N and Longitude 136°54'00"E.

Sea area inside the dotted line is Specified water area of the Total Pollutant Load.

It is already well known that many kinds of chemicals have been detected in the aquatic environment (Iwasaki S. et al. 1999, 2000). These hazardous synthetic chemicals released in to the environment have hormone-like actions. Most of them have estrogenic activity, and chemicals such as

nonylphenols and ester phthalates have already been detected in the aquatic environment. Recent studies have revealed that natural estrogens excreted by humans are present in domestic sewage effluent and that the concentrations found are high enough to induce vitellogenin production (Desbrow C. et al. 1998, Routledge E. J. et al. 1998). They exert a great influence on aquatic organisms, although, analytical studies have not kept up with monitoring demand, and the full extent of the effects on the aquatic environment is unknown. Therefore, we used a bioassay system to examine estrogenic substances. The method developed by Prof J. P. Sumpter et. al. In which the DNA sequence of the human estrogen receptor is integrated into the yeast genome can be used to determine whether chemicals possess estrogenic activity with high sensitivity (Routledge E. J. et al. 1996).

In this study, we surveyed the levels of estrogenic substances by the yeast screen assay in water and sediments in this sea area, investigated the behavior of these pollutants, and comprehensively evaluated aquatic environment in the sea area around the Shima Peninsula. Moreover, we observed the underwater environment of this sea area by diving.

2. EXPERIMENTAL

2.1 Description of Investigated Field Site and Sample Collection

2.1.1 Field Site

Water and sediments were sampled from the Shijima territorial sea area around the Shima Peninsula (Latitude 34°18'20"N, Longitude 136°54'00"E, Figure 1). The depth of the sampling site was twelve meters.

2.1.2 Sample Collection

The surface layer water was sampled from a boat, and the bottom layer water and the sediment was sampled by SCUBA (Self-Contained Underwater Breathing Apparatus) diving. These samples were collected on Oct the 30th (Survey no1) and Nov the 23rd (Survey no2), in 2001.

The sediment in this sea area floated easily and was churned up, and there was only a very small quantity. Therefore, the sediment was carefully gathered using a spoon and put into a 1-L wide-mouth polyethylene bottle. The sediment was collected by centrifugation at 3,000 g for 15 min.

2.2 Analytical Method

2.2.1 Water Sample

Hydrogen ion concentration (pH), salinity, transparency and water temperature were examined according to the Manuals for Oceanographic Observation. Chemical oxygen demand (COD), solid suspended (SS), total nitrogen (T-N) and total

phosphorous (T-P) were examined by the Japanese Industrial Standard.

2.2.2 Sediment Sample

Total organic carbon (TOC), T-N and T-P were examined according to the Manuals for Sediment Monitoring Methods.

2.2.3 Yeast screen assay

Estrogenic substances were examined by the yeast screen assay developed by Routledge et al. (Routledge E. J. et al. 1996). The recombinant yeast was obtained from Prof J. P. Sumpter (Brunel University, Uxbridge, UK). Assay of water samples was performed according to the method of Takigami et al. (Takigami H et al. 1998). Two liters of water sample was filtered through a Whatman GF/C filter. The filtrate was concentrated onto a Sep-Pak C18+ cartridge (Waters Co. Ltd.) and the analytes were eluted with 10 ml of methanol. The eluate was reduced in volume to approximately 2 ml using a vacuum evaporator at 40 °C. The concentrate was dried by nitrogen gas flow and dissolved in 200µl of dimethyl sulfoxide. Finally, the assay sample was concentrated 10,000-fold. The filter residue was weighed after being dried for 2 hours at 40 °C, and the analytes were extracted with 10 ml of methanol by ultrasonic agitation. The extract was concentrated to 200µl in dimethyl sulfoxide by the same method used for the filtrate. The estrogenic substrate detected from the filtrate was defined as "soluble estrogenic substrate", that detected from the filter residue was defined as "solid estrogenic substance", and the sum of the soluble and solid estrogenic substances was defined as "total estrogenic substance". Assay of sediment sample was performed by the same method used for the filter residue of water samples.

2.2.4 Calibration Standard of Estrogenic Substances

Calibration standards were prepared in ethanol at a concentration of 100µg/L of 17 β-estradiol (E2) and serially diluted (x3) in ethanol. Samples were diluted serially by the same method as the standards. Ten-micro liter aliquots of each diluted standard and sample were transferred to a 96-microwell plate. The solvents were dried by allowing them to stand and assay medium (containing recombinant yeast and substrate for β-galactosidase CPRG(Chlorophenol red-β-D-galactopyranoside)) was added to each well and incubated for 3-4 days at 32 °C. After that, the absorbance of the medium developed red was measured.

Concentration of an estrogenic substance was determined from the concentration of E2 and assay sample that produced 50% induction of β-galactosidase activity (EC50) and was calculated by dividing the E2 EC50 by the concentration factor of the assay sample EC50. For example, if we assumed that E2 EC50 is 1µg/L and the concentration factor of the assay sample EC50 is 10,000, then the value of the estrogenic substance is 1µg/L /10,000 = 0.1 ng/L.

2.2.5 Fractionation of the Assay Sample by HPLC

The sample was fractionated by injecting 50µl through an HPLC column Lichrosorb RP-18 10µm 4.6x250mm (GL Sciences) at a flow rate of 1 ml/min using acetonitrile-acetic acid-water (70:5:25) (Lønning P. E. et al. 1989). The separate fractions were collected at 0.1 ml intervals, and dried by nitrogen gas flow. They were dissolved in 20µl of dimethyl sulfoxide and assayed for estrogenic substances. Ten milligrams per liter of estron(E1) and E2 standard solution as applied to the same HPLC column, and these compounds were monitored through an ultraviolet detector at 280 nm to determine their retention times.

3. RESULTS AND DISCUSSION

3.1 Current Status of the Shijima Territorial Sea around the Shima Peninsula

The annual climate of the coastline around the Shima Peninsula is warm, and the area is heavily populated with subtropical plants. The sea area we surveyed is located at the entrance to Ise Bay. The sea bed is mainly formed from rock, and there are numerous sea forest, fish and shellfish. The area faces the Pacific Ocean, and has a high frequency of stormy weather. On the day of survey no1, the wave scale was three, and the current was very fast (Figure 2). Therefore, the sediment was churned up, water was turbid, and the transparency was only 2.5 meters. On the other hand, on the day of survey no2, the wave scale was one, and the transparency was 10 meters. The conditions on survey no1 were not so unusual since 24% of days in 2001 had the same wave scale of three or higher.

3.2 Current status of water and sediments

The results of the seawater examination are shown in table 1. As noted above, the Shijima territorial sea is at the entrance of Ise Bay. It is an area regulated by the Total Pollutant Load Control program, which was implemented to reduce the total chemical oxygen demand (COD) loads in order to improve the water quality in a large enclosed water area. The concentration of saline in survey no1 was relatively lower than that in survey no2. This value is presumably the result of heavily rainfall several days before in this region. The seawater was diluted by the increasing run-off of river water. The values of COD and SS of survey no1 were higher than survey no2. The concentration of the seabed SS was higher than the surface SS. This was considered to be due to the waves being high at the day of survey no1, and the particles were churned up from the bottom. Another reason is that the inflow of pollutants had increased due to the heavy rainfall. The values of T-N and T-P were also higher on survey no1 than survey no2 because of the effect of a high concentration of SS. Although bad sea conditions such as survey no1 are not unusual as noted above. We should also consider the water pollution index values in bad conditions, to evaluate the annual sea water quality.

Table 1 Concentration of the seawater pollution indexes:
 at Shijima territorial sea.

	Survey no.	no1		no2	
		Date	Oct. 30th 2001	Nov. 23rd 2001	Date
	Weather	Fine		Fine	
	Scale of Wave	3		1	
		Upper	Bottom	Upper	Bottom
pH		8.11	8.17	8.34	8.34
Salinity	%	2.72	2.72	3.08	3.10
COD	mg/L	2.6	3.0	1.0	0.8
SS	mg/L	1.2	4.0	0.2	0.4
T-N	mg/L	0.21	0.26	0.09	0.15
T-P	mg/L	0.031	0.042	0.022	0.023
Transparency	m	2.5		10	
Water temperature	°C	20.0	20.0	17.5	18.0

The results of the sediment examination of are shown in Table 2. The values of the sediment contamination indicators of survey no1 and survey no2 were approximately equal, and they were relatively higher than the values in Ise Bay. The geography of this sea area is different from that of the enclosed sea area. The sediment in the enclosed sea area is like clay, although the sediment in the sea area is formed of floc. The sediment in the Shijima territorial sea area consists mainly of organisms, such as plankton and microbes and their remains. On the other hand, the sediment in Ise Bay contains many inorganic substances, for example clay or silt.

Table 2 Concentration of the sediment pollution indexes
 at Shijima territorial sea.

	Survey no.	no1		no2	
		Date	Oct. 30th 2001	Nov. 23rd 2001	Date
TOC	mg/g	190		156	
T-N	mg/g	31.5		29.6	
T-P	mg/g	2.84		2.56	

3.3 Estrogenic Substances

A standard curve of E2 is shown in Table 3. As shown, EC50 of E2 was approximately 1.0µg/L, and the concentration factor of the original water assay sample was

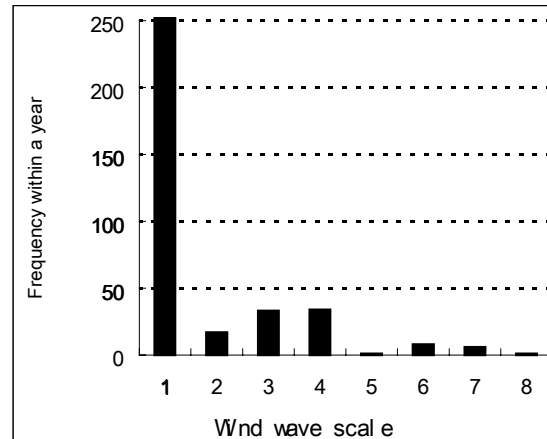


Figure 2. Annual wind wave scale in the Shijima sea area in 2001. Twenty-four % of all was above the scale of 3 in 2001.

Wind wave scale

Class	Wave Height (m)	Wave Length
1	<2	short
2	<2	long
3	2-4	short
4	2-4	middle
5	2-4	long
6	>4	short
7	>4	middle
8	>4	long

Twenty four percent was over "3" in 2001.

10,000. Therefore, we calculated the detection limit by dividing 1.0µg/L by the concentration factor to obtain a value of 0.1 ng/L. Estrogenic substances in the sediment were extracted from 1 g of sediment, the assay sample volume was 500µL, and the sample volume applied to analysis was 10µL. The detection limit of this assay was 1.0µg/L, which corresponded to 10 pg per microwell (per 10µL), and was 500pg per 500µL. Finally, the detection limit of the sediment sample was 500 pg / 1 g = 0.5 ng/g. Table 2 shows the concentration of estrogenic substances in the water and sediment samples. Under the influence of a high concentration of SS, concentrations of SS-estrogenic substances were relatively high at survey no1, especially on the bottom layer of water. The concentrations of estrogenic substances of survey no2 were lower than survey no1, and SS-estrogenic substances were below the limit of detection.

The concentrations of estrogenic substances in the sediments were 13.3 ng/g and 11.0 ng/g, for survey no1 and survey no2, respectively. The relative abundance of estrogenic substances in the sediment samples was significantly higher than in the water samples (Taniguchi N. 1998). The estrogenic substances will tend to become associated with suspended solids, and ultimately sediments (Snyder S. A. et al.). As a result, the benthic fishes could be exposed to estrogenic substances directly from the

Table 3 Concentration of estrogenic substances of water and sediment

Survey no.	no1		no2	
Date	Oct. 30th 2001		Nov. 23rd 2001	
	Upper	Layer	Upper	Layer
Water Sample				
Unit	ng/L		ng/L	
Soluble	0.5	0.4	0.3	0.3
Solid	0.5	1.1	N.D.	N.D.
Total	1	1.5	0.3	0.3
Sedimentation sample	no1		no2	
Unit	ng/g		ng/g	
	13.3		11.0	

N.D. : Not detected. Detection limit of estrogenic substances of water sample was 0.1 ng/L, and that of sediment sample was 0.5 ng/g.

sediments in which they live, and by feeding on the sediment-dwelling invertebrates.

The concentrated water and sediment assay samples were fractionated by HPLC and assayed to determine the chemical species of the estrogenic substances. Retention times of the detected estrogenic substances fractionated by HPLC coincided with E2 and E1, and were found in all of the samples. It is well known that natural estrogenic substances such as E2 and E1 excreted by humans are present in domestic sewage. We found these substances in many rivers and sea areas in Mie Prefecture. Further studies are needed in order to fully understand the effects of widespread contamination of sea areas by estrogenic substances on fish populations.

4. CONCLUSION

Concentrations of estrogenic substances and other general water and sediment pollutant indexes of water and sediment samples collected in Shima Peninsula territorial sea area were measured on two occasions (survey no1 and survey no2). Estrogenic substances were measured using a recombinant yeast screen assay. The value of COD of survey no1 was higher than survey no2, both in the surface and bottom water. The SS value of survey no1 was also higher than survey no2, both upper and bottom of the water, and particularly, the SS value recorded in the bottom sample of survey no1 was very high (4.0 mg/L). As a consequence of the high value, the value of the transparency of survey no1 was 2.5 m, compared with 10 m

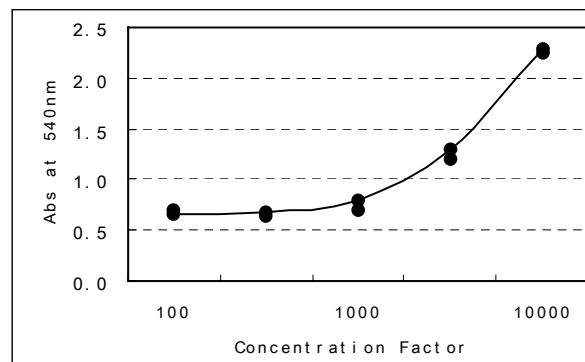
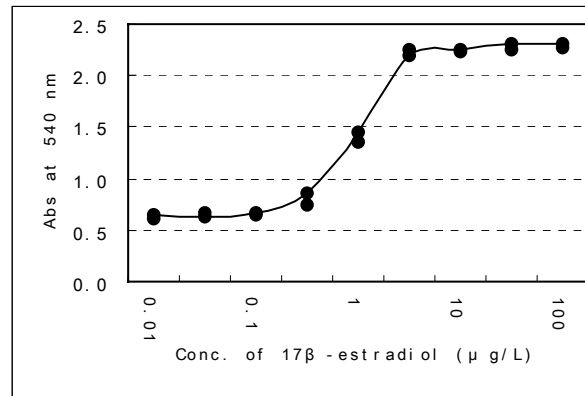


Figure 3. Calibration curve of 17- β estradiol (Top) and dose-response curve of concentrated water assay sample (bottom). The graph shows that EC₅₀ of 17- β estradiol is approximately 1.0 μ g/L. The sample shown in this figure is filtrated surface layer sea water collected in survey no1.

in survey no2. The difference in concentrations of T-N and T-P between survey no1 and survey no2 were generally smaller than those of SS and COD. Values of soluble estrogenic substances of survey no1 were generally higher than survey no2, although there was small difference between the values for the upper and bottom layer water, in both survey no1 and survey no2. Solid estrogenic substance, concentrations of survey no1 were high, particularly in the bottom layer sample. The value of the upper and bottom layer sample was under the limit of detection. The variation in the concentration of water pollutant indexes between survey no1 and survey no2 was presumably a result of the difference in the conditions of the sea area.

There was a small difference between the values of TOC, T-N and T-P of survey no1 and survey no2. These values were relatively high compared to the sediment in the enclosed sea area, although very little sediment was present in this sea area. There was also a small difference

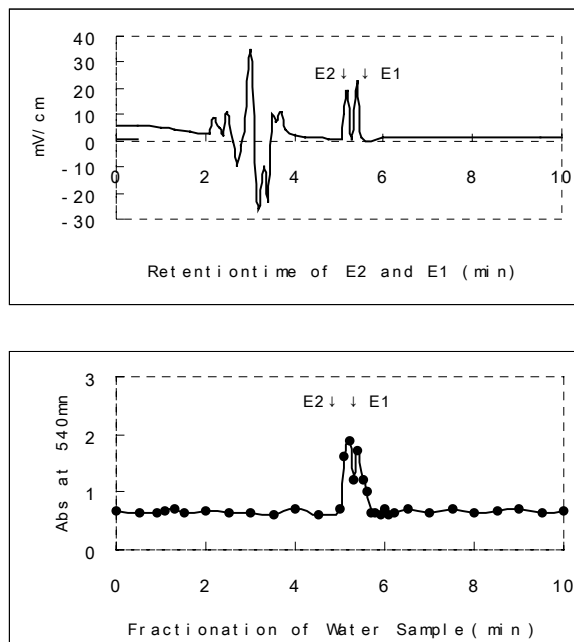


Figure 4. Estrogen standard (E2 and E1) chromatogram absorbance at 280 nm fractionated with HPLC (Top) and assay result of the fraction with HPLC (bottom). The sample was fractionated by injecting 50 μ L at a flow rate of 1 mL/min using acetonitrile-acetic acid-water (70:5:25). The separate fractions were collected at 0.1 mL intervals, and assayed by recombinant yeast screen assay.

between concentration values of estrogenic substances of survey no1 and survey no2.

The assay samples of water and sediment were fractionated by HPLC and the fractions were measured by a yeast screen assay, which revealed estrogenic substances in several fractions. The retention times of the substances coincided with E2 and E1. They are typical natural female hormones, and their origin is presumably domestic sewage and other wastewater containing feces and urine.

In conclusion, studies on estrogenic substances in aquatic environments are lacking, and additional surveys are necessary to provide information on the fate and behavior of these substances.

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