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Determination of natural estrogens in the sediment of coastal area in Japan

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Abstract—The concentration of natural estrogens such as 17 β-estradiol (E2) and estrone (E1) in the sediment of coastal areas in Japan was determined using an enzyme-linked immunosorbent assay (ELISA). The samples of sediment were collected in Osaka Bay and Otsuchi Bay, Yamada Bay and Ofunato Bay, in Iwate Prefecture, Japan. In addition, core samples were taken in Otsuchi Bay and Yamada Bay. Estrogens in sediments were extracted by sonication in acetone followed by liquid-liquid extraction with dichloromethane. The levels of E1 were generally higher than those of E2 in sediments, whereas the concentrations of E2 were higher than E1 at some sampling sites. The levels of estrogens in the sites located near estuaries tended to be higher, and levels decreased at the sampling stations nearer to the mouth of the bay. However, the estrogen concentration in the sediment collected from near the mouth of bay was highest of all samples in Osaka Bay, both in summer and winter. Core samples were sliced every 2 cm from the surface to 20 cm deep for the measurement of estrogen. Although the concentrations of estrogens decreased with the depth of core samples, fairly high levels of estrogens were again noticed at the layer deeper than 16 cm.

Key words: 17 β-estradiol, estrone, ELISA, sediment and core sample

Introduction

Endocrine disrupting chemicals (EDCs) have received increasing attention in recent years, and concentrations of EDCs in the environment and their influence on wildlife have been investigated in many countries (Helle et al. 1976, Matthiesen 2003a, Oehlmann et al. 2003). Especially, many effects of estrogenic substances to wildlife in aquatic environment have been reported. (Matthiesen 2003b)

Estrogenic substances have been detected frequently in high levels in rivers and coastal areas, by using *in vitro* assays such as E-Screen (Soto et al. 1995, Zava et al. 1997), YES (Routledge et al. 1996) and ELISA (Goda et al. 2001), and chemical analyses such as GC/MS and HPLC (Matsuoka et al. 2004). It was also reported that the natural and synthetic estrogens comprised a majority of estrogenic substances in the aquatic environment (Desbrow et al. 1998, Matsuoka et al. 2004). Estrogenic substances flow into rivers through sewage treatment plants or directly, and are adsorbed to suspended solids. After partial degradation by aquatic bacteria and sunlight (Jürgens et al. 2002), the estrogenic substances finally settle down to bottom of the sea. The sediment is relatively stabilized in the aquatic environment compared with the river water and seawater whose qualities change daily, and many kinds of benthic organisms live in the sediment of the river and coastal area. Therefore, estrogenic substances may affect the lives of them, and it is necessary to investigate the levels of estrogenic substances in sediment as well as in the water. However, there are few reports about estrogenic substances in sediment (Tashiro et al. 2004, Legler et al. 2003, Kannan et al. 2000).

In this study, an enzyme-linked immunosorbent assay (ELISA) was used to determine the concentrations of natural estrogens, 17 β-estradiol (E2) and estrone (E1) in the sediment. It is possible to measure estrogens easily using an available kit, and to analyze estrogens of many samples in a short time. Sediment samples were collected from Osaka Bay and the coastal area of Iwate Prefecture.

Materials and Methods

Chemicals

ELISA kits for 17 β-estradiol (E2) and estrone (E1) assays were purchased from Japan Enviro Chemicals, Ltd. Acetone, dichloromethane, sodium sulfate anhydrous, ethanol and methanol were purchased from Wako Pure Chemical Industries, Ltd. These organic solvents used are pure grade ones for the analysis of residual pesticides.

Sampling of sediments and extraction of estrogens

Sediment samples were collected in Osaka Bay, which is surrounded by heavily populated areas, from which large

quantity of river water flow into the bay every day. Samples were also collected in Otsuchi Bay, Yamada Bay and Ofunato Bay, Iwate Prefecture, which are surrounded by areas that are less populated than the Osaka metropolis. Core samples of sediment were also taken in Otsuchi Bay and Yamada Bay to investigate the transition of the estrogen levels in each time period.

Sediments were collected at each sampling site in Osaka Bay, in July and December, 2003, and Otuchi Bay, Yamada Bay and Ofunato Bay, Iwate Prefecture in July, 2000 and 2001 by using a Smith-McIntyre mud-sampler. Core samples of 50 cm in depth were collected in Otsuchi Bay and Yamada Bay in 2000 by using a piston core sampler (Figs. 1, 2). Ten grams of wet sediments were extracted by sonication in 40 ml of acetone for 30 min, and centrifuged at 2800 rpm for 10 min. This process was then repeated. The acetone extract was added to 500 ml of 5% NaCl and 100 ml of dichloromethane, and was shaken vigorously for 5 min. This process was then repeated. The dichloromethane extract was dehydrated and filtered by sodium sulfate anhydrous, and then concentrated by a rotary evaporator to until all moisture was removed. The extract was dissolved in 2 ml of ethanol. Percentage of water content of the sediments was also measured as follows: Ten grams of wet sediments were weighed and dehydrated at 100°C by water bath for an hour, followed by dry sterilization at 115°C for 2 hours, and then weighed again. The core samples were used from the surface to 20 cm layer for extraction of estrogens.

ELISA method for the measurement of estrogens

The 17 β-estradiol (E2) ELISA kit and estrone (E1) kit (Japan Enviro Chemicals, Ltd) use a monoclonal antibody which binds exclusively with an analyte and an enzyme-labeled antigen, and are able to detect each estrogen at a very low level in a few hours. The sediment extracts solved in ethanol were evaporated completely under a gentle stream of nitrogen gas and diluted tenfold in 10% methanol. The concentrations of E2 and E1 of the extracts were determined by the ELISA method following the assay procedure shown in the kit. One hundred μl of estrogen standard or the extract of sediment sample and 100 μl of conjugate solution were mixed in each well of a 96-well microplate. Hundred μl aliquots of the mixture were dispensed into each well of a microplate and incubated for 60 min at room temperature. After incubation, each well was rinsed with approximately 300 μl of wash solution and then this step was done twice more. One hundred μl of the coloring reagent was added into each well and incubated for 30 min at room temperature. After incubation, 100 μl of stop solution was added to terminate the reaction and the absorbance of yellow color was measured at 450 nm. The concentrations of E2 in samples were calculated from an absorbance reading and interpolation from the standard curve. The quantitative analysis of E2 ranged from 50 to 1000 ng/l, and that of E1 ranged from 50 to 5000 ng/l. Recovery rates of E2 and E1 were from 90 to 100% in these kits. The levels of estrogens were expressed as ng/g · dry.

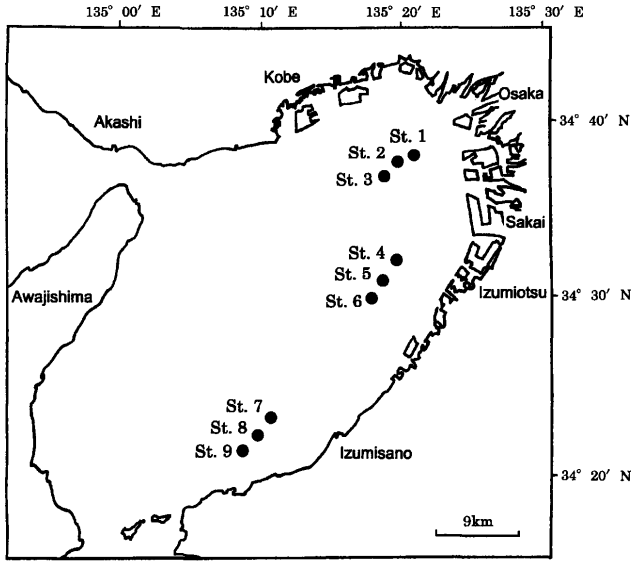

Fig. 1. Sampling sites in Osaka Bay.

Results and Discussion

Figure 3 shows the concentrations of E2 and E1 in the sediments collected at Osaka Bay in July and December of 2003. The concentrations of E1 and E2 in all samples ranged from 0.1 to 1.33 (mean 0.63) ng/g · dry, and from 0.17 to 2.33 (mean 0.63) ng/g · dry, respectively. The levels of both estrogens at St. 9, located at the mouth of Osaka bay were very low. This is because the sediment at St. 9 was gravel, showing a low water content, and estrogens were not adsorbed to sediment as much as at other stations. The levels of E1 tended to be higher than those of E2 generally; however, the level of E2 at St. 7, located at the southern part of the bay, was much higher than that of E1 in both months. Although the major cause of high levels of estrogen at St. 7 remains obscure, the possibility that some sort of input of estrogen might have occurred there was considered. Figure 4 shows the levels of estrogens in sediments collected in Iwate Prefecture at Otsuchi Bay in July, 2000 and 2001, at Yamada Bay in July, 2000, and at Ofunato Bay in July, 2001. Otsuchi Bay has a wide mouth, where there is good water exchange. The levels of E1 also showed the tendency to be higher than that of E2 at three bays in Iwate prefecture. The levels of E1 and E2 ranged from 0.21 to 0.87 (mean 0.51) ng/g · dry and from 0.15 to 0.49 (mean 0.33) ng/g · dry, respectively. The levels of E1 at St. 3, St. 5 and St. 6 of Otsuchi Bay were relatively higher than those of other sites, but remarkable differences in estrogen levels were not observed. The levels of E1 and E2 at

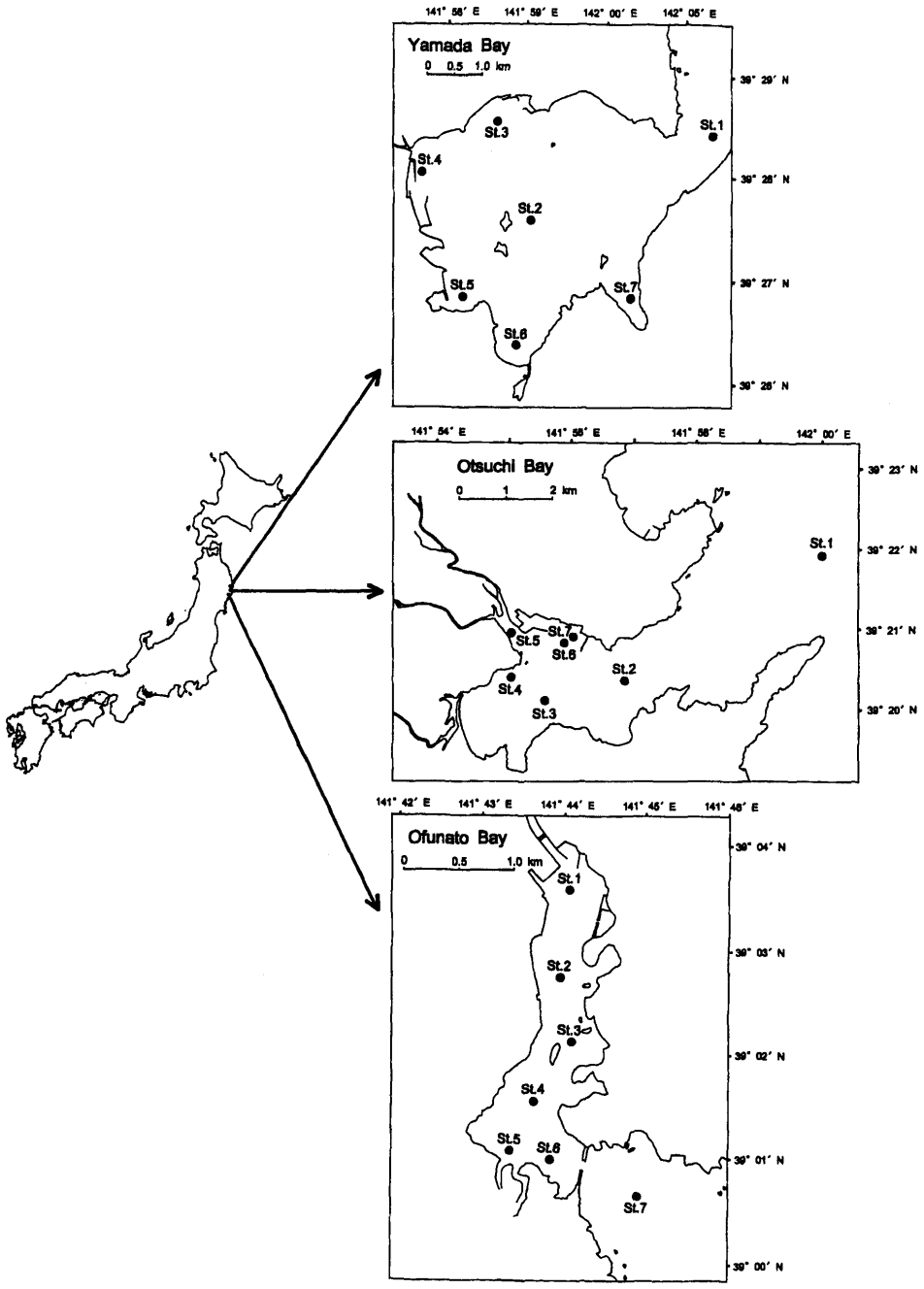

Fig. 2. Sampling sites in three bays, Iwate Prefecture.

Yamada Bay ranged from 0 to 2.16 (mean 0.66) ng/g · dry and from 0.16 to 0.82 (mean 0.36) ng/g · dry, respectively. Higher levels of E1 and E2 were noticed at St. 3 and St. 5 of Yamada Bay, because the sites were located at the inner part of the bay, where estrogens might tend to accumulate. Rather high concentration of natural estrogens in St. 5 might be responsible for the high water content. Ofunato Bay has a long shape with a tide embankment situated on the east of St. 6 for protection from tsunami as shown in Fig. 2. Therefore, it is possible that water pollution may occur because of reduced water exchange. The sampling sites from St. 1 to St. 6 are located inside Ofunato Bay, and St. 7 is located outside of the tide embankment (Fig. 2). The levels of estrogens decreased towards the mouth of the bay (mean of E1 and E2 were 0.59 and 0.35 ng/g · dry, respectively), and the level of estrogens at St. 7 was very low.

Judging from these results, the mean levels of estrogens in sediment collected at Otsuchi Bay were relatively lower than those in other two bays. Compared with the results of Osaka Bay, the levels of estrogens in sediment of three bays in Iwate Prefecture were rather lower than those of Osaka Bay except for a few sites. In other reports, the concentration of estrone in sediment collected at Manko Tidal Flat in Okinawa Prefecture, measured by LC/MS/MS, was 0.014 ng/g (Tashiro et al. 2004), and this concentration was lower than those of our results (mean 0.60 ng/g). In other case, the estra-

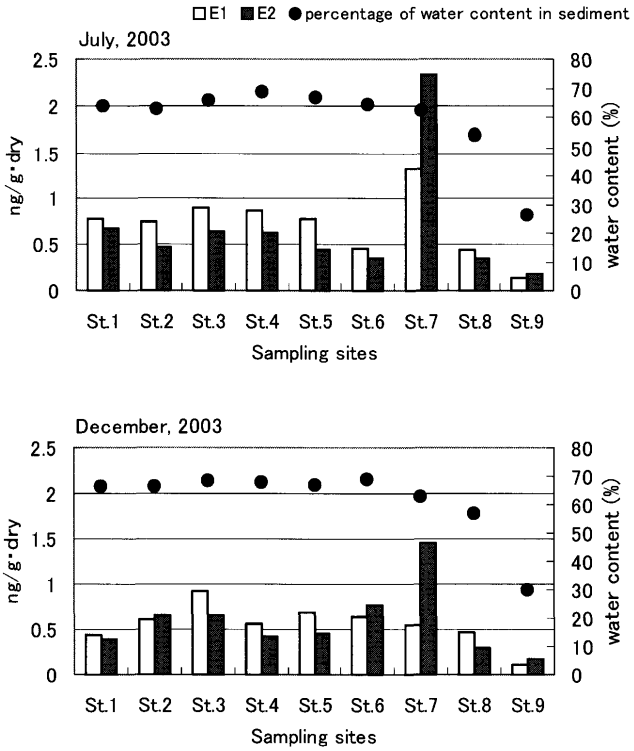

Fig. 3. The levels of estrogens in sediment in Osaka Bay.

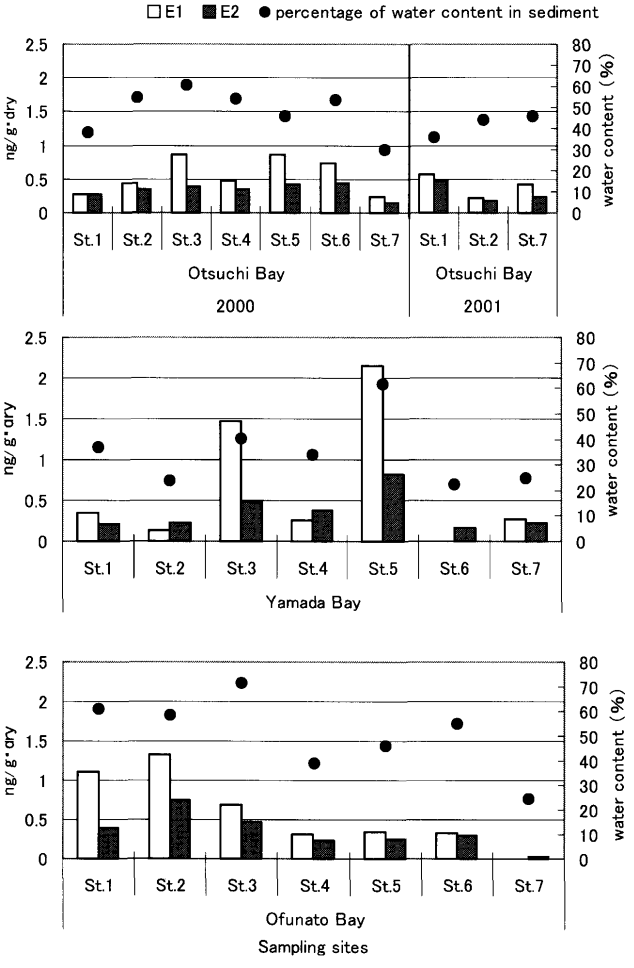

Fig. 4. The levels of estrogens in three bays, Iwate Prefecture.

diol equivalents of sediment collected in the Netherlands, measured by ER-CALUX, were about 0.23 ng/g (Legler et al. 2003), showing the similar order of estrogens as ours.

In our previous paper, the total estrogenic activities of seawater at three bays in Iwate collected on the same day were determined by *in vitro* assays including E-Screen, Ishikawa cell-ALP assay (Littlefield et al. 1990, Nishida et al. 1996) YES assay and enzyme immunosorbent assay (ELISA) (Kawai et al. 2002). The levels of estrogenic activities in seawater did not necessarily correspond to those of sediments, because the quality of seawater changes continuously, whereas sediments remain relatively stable.

Figure 5 shows the estrogen levels in core samples of Otsuchi Bay. The sample of 14–16 cm depth at St. 5 was lost. The levels of E1 were higher than those of E2. The levels of E1 of core samples collected from St. 5 decreased with depth, but the levels of E2 didn't change so much at any layer. And the levels of E1 increased again from the sample

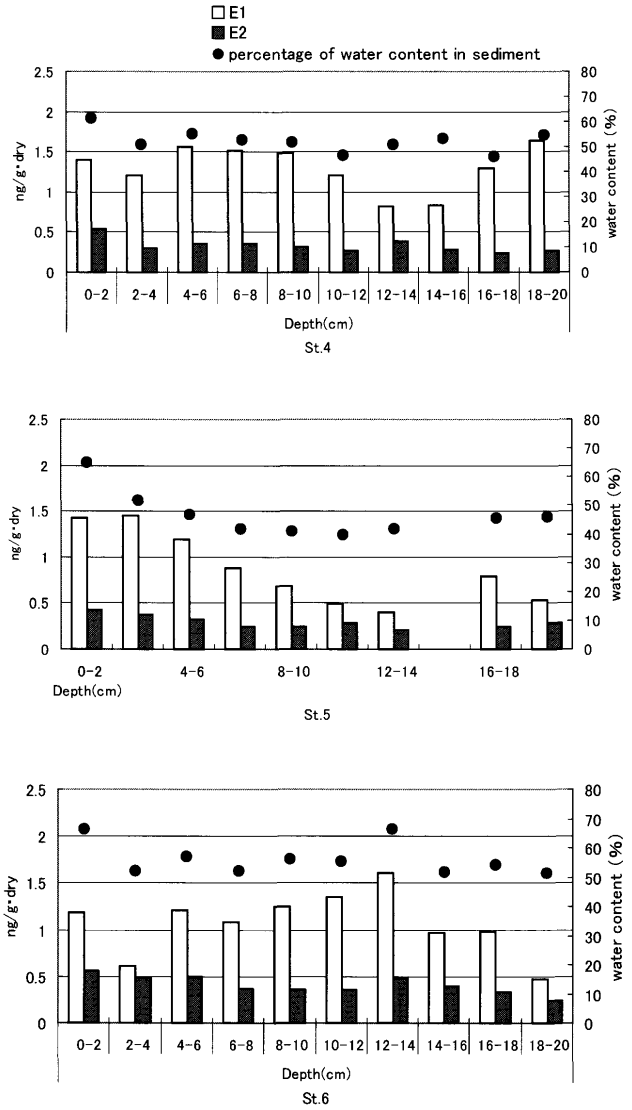

Fig. 5. Vertical profiles of estrogen concentrations in Otsuchi Bay.

of about 16–18 cm at St. 4 and St. 5. The core depth around 16–18cm corresponds approximately to the 1960s, judging from the sedimentation rate (0.34 cm/year) in Otsuchi Bay (Takahashi et al. 1999). In other reports, the levels of estrogens of core samples collected at Tokyo Bay measured by the increase in luciferase expression in MCF-7 cells were significantly high at the depth corresponding to the period from 1955 to 1960 (Kannan et al. 2000). This evidence means that the estrogens in sediment remain for forty years or more. E1 of core samples collected at Yamada Bay were also detected at all depths and were high depths of 16–20 cm at St. 5. (Fig. 6) The levels of estrogens at St. 7 were relatively lower than

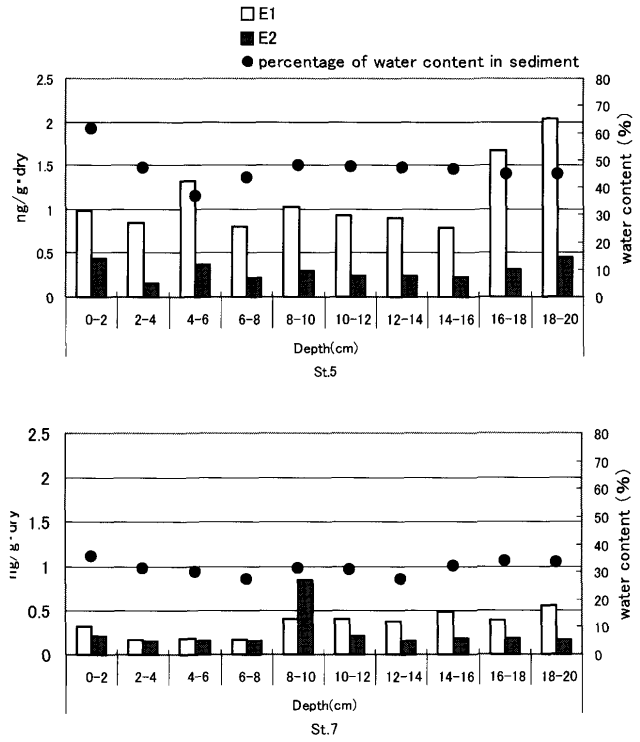

Fig. 6. Vertical profiles of estrogen concentrations in Yamada Bay.

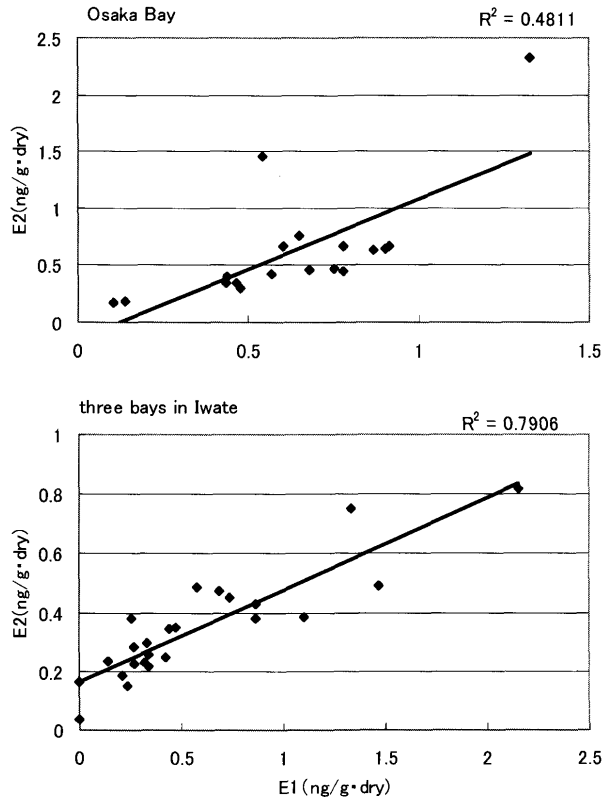

Fig. 7. Correlations between E1 and E2 of sediment in Osaka Bay and in three bays, Iwate Prefecture.

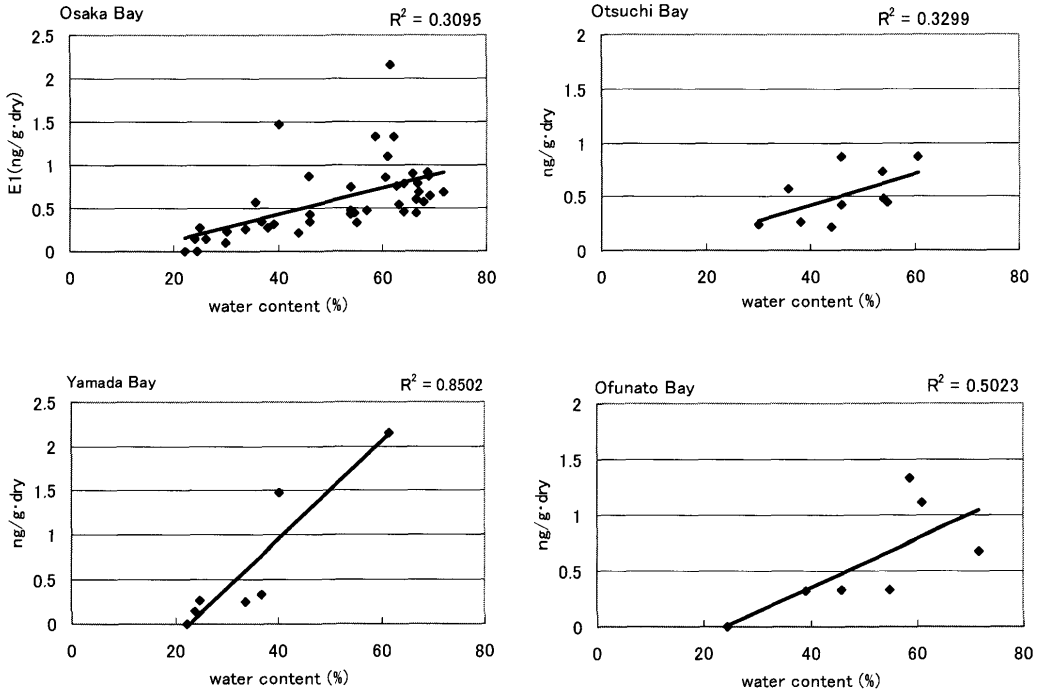

Fig. 8. Correlations between E1 and water content of sediment in Osaka Bay and in three bays, Iwate Prefecture.

those at St. 5.

The reason the E1 content in core samples of Otsuchi Bay and Yamada Bay increased at depths more than 16cm remains obscure.

Figure 7 shows the correlation between the levels of E2 and E1 of sediment collected at Osaka Bay and the three bays in Iwate. High positive correlation ($r^2=0.79$) was noticed between the levels of E2 and E1 in the three bays in Iwate. The correlation in Osaka Bay was not so high. ($r^2=0.48$) Furthermore, the estrogen levels of sediment showed a tendency to increase as the increase of percentage in water content of sediments (Fig. 8). High water content in sediment are observed in polluted area, generally. Therefore, rather high levels of estrogens might be contained in such sediment.

Sediment extracts were subjected to the measurement of total estrogenic substances using E-Screen and YES assay, however, the cells and yeast were killed by some chemicals in the sediment extracts by our extraction method, thus, estrogenic activity could not to be evaluated at all by *in vitro* assays in this study. Sediments include not only estrogenic substances but also other chemicals; for example, dioxin, polycyclic aromatic hydrocarbons (Vondracek et al. 2001), organochlorines, heavy metals and alkylphenols. Therefore, it is necessary to remove these toxic chemicals using HPLC or some treatments of fractionations for the determination of total and potential estrogenic substances using *in vitro* assay.

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