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Estrogenic substances in the surface seawater collected from Otsuchi Bay, Yamada Bay and Ofunato Bay, Iwate Prefecture

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Recently, there are many reports on the endocrine disrupting chemicals that cause adverse effects on endocrine systems of animals, especially on reproductive ones in man and other organisms. Though some synthetic chemicals are known to cause reproductive failure in wild animals, cause and effect relationships in endocrine disruption of various organisms are not clear. In order to evaluate the potential estrogenic substances in surface seawater collected from Yamada, Otsuchi and Ofunato Bays, Iwate Prefecture, three kinds of *in vitro* assays including E-screen (Human breast cancer cells), Ishikawa cell (Human endometrial adenocarcinoma cells) and YES (Recombinant yeast cells) were used. Estrogenic substances in seawater were extracted with Sep-pak C18 cartridge, and the extracts were applied to *in vitro* assays to calculate 17β-estradiol (E_2) equivalents. Commercial enzyme immunoassay (EIA) kit was also used for the measurement of E_2 in seawater. Though seawater extracts of Otsuchi Bay in July, 2000 showed rather high level of E_2 equivalent in the station located near mouth of the Bay, the values were very low in the samples of July, 2001. The high levels of E_2 equivalent value assayed by E-screen and Ishikawa cell-ALP was noticed in the station located in the estuary area of a small river in Yamada Bay. Estrogenic substances were detected in all stations of Ofunato Bay, and the values of E_2 equivalent were rather high in the stations near the mouth of Bay. Differing from E-screen and Ishikawa cell-ALP assays, YES assay and EIA method did not show the clear difference of E_2 level between sampling stations.

Keywords: estrogenic substances, seawater, *in vitro* assays

INTRODUCTION

About one hundred thousand of chemicals are distributed in our surroundings and many kinds of them are toxic to wild organisms and human kinds. Recently, some chemicals are reported to show the adverse effects on the endocrine and reproductive systems of various kinds of organisms in terrestrial and aquatic environments, and these are called endocrine disruptors or environmental estrogens. These hazardous chemicals such as DDT compounds and PCBs cause uterus occulsion in seals (Helle 1980) and decrease of egg shell thickness in birds of prey (Ratcliffe 1970). Alkylphenols being the metabolites of detergents cause vitellogenin synthesis for male and immature female fish in freshwater and estuarine waters, especially in the fish exposed to the effluent of sewage treatment plant (Sumpter et al. 1995). Imposex which is the symptoms of intersex in the females of neogastropods were caused by the exposure of antifouling paints containing tributyltinin in the coastal area of the world (Gibbs and Bryan 1986, Horiguchi 1998). Although various kinds of synthetic and naturally occurring chemicals have been demonstrated to show estrogenic activity to the animals including birds, amphibians, reptiles, fish, neogastropods and marine mammals, few informations are available on the potential and total or inclusive estrogenic substances in the river water, seawater and sediment, and also on the actual effect of them in the aquatic environment.

In this paper, three kinds of *in vitro* assays, namely, E-screen (human breast cancer cells) (Soto et al. 1995), Ishikawa cell-ALP (human endometrial cancer cells) (Nishida 1997) and YES (genetically recombinant yeast cells) (Klotz et al. 1996), were used for determining the estrogenic active substances in surface seawater collected from Otsuchi Bay, Yamada Bay and Ofunato Bay, Iwate Prefecture.

MATERIALS AND METHODS

Sampling of seawater: Surface seawater was collected from Otsuchi Bay and Yamada Bay in July, 2000, and from Otsuchi Bay and Ofunato Bay in July, 2001 (Fig. 1).

Extraction of estrogenic substances in seawater: 3.5 L of each seawater was extracted using Sep-PakC18 cartridge (Waters Co. Ltd). Lipophilic chemicals which dominate most part of endocrine disruptors in seawater are extractable by this cartridge. Elution of chemicals absorbed to the resin of cartridge was carried out with 7 ml of ethylalcohol followed by 7 ml of ethylether. Extracted solution was concentrated by a rotary evapolator, and was resolved in 1 ml of dimethylsulfoxide (DMSO) or ethylalcohol for *in vitro* assays.

E-screen assay: T-47D cells which are estrogen receptor-positive human breast cancer ones and proliferative in the presence of estrogenic substances (Soto et al. 1995) were grown in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% of fetal bovine serum in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C. Cells were trypsinized and plated in 24 well plates at an initial concentration of 5×10^4 cells/well. The cells were allowed to attach for 24 hr, then the medium was aspirated and replaced by 1 ml of experimental medium per well containing phenol red- free DMEM supplemented with

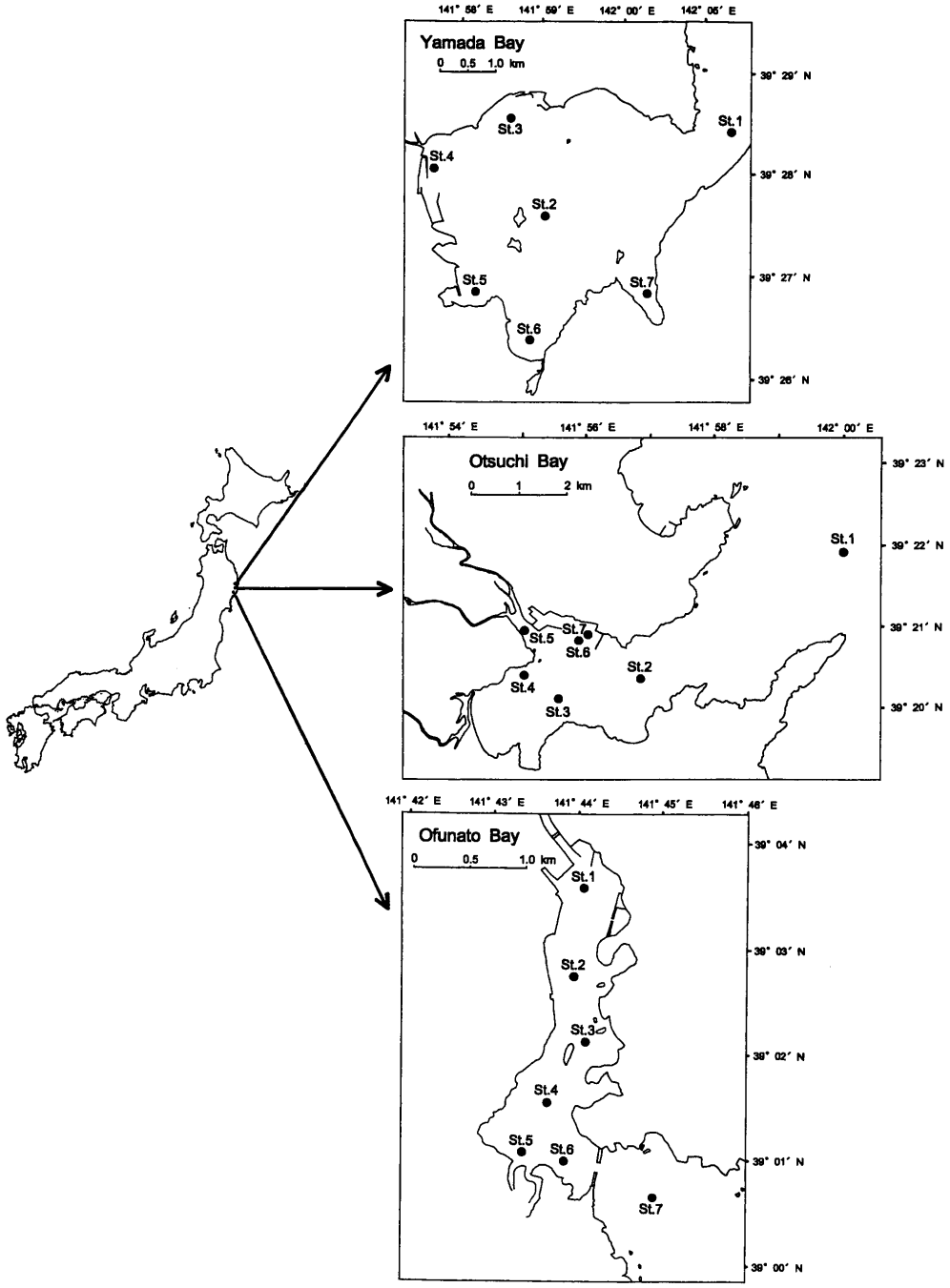

Fig. 1. Sampling stations in Yamada Bay, Otsuchi Bay and Ofunato Bay, Iwate prefecture.

5% charcoal dextran-treated fetal bovine serum (CD-FBS) and 5 μl of seawater extracts which is equivalent to 8.8 ml of seawater. Six days later, cells were fixed with 10% trichloroacetic acid and incubated at 4°C for 30 min. Then the cells were washed five times with distilled water and left to dry. The fixed cells were stained with 0.4% sulforhodamine-B (SRB) dissolved in 1% acetic acid for 20 min. Wells were washed with 1% acetic acid and air dried. Bound dye was solubilized with 10 mM Tris base (pH 10.5) in a shaker. The extinction of SRB at 492 nm is directly proportional to the cell number within a wide range. 17β-estradiol (E_2) in six concentrations of between 5×10^{-13} M (0.136 ng/l) and 5×10^{-8} M (13.6 μg/l) was the internal positive control in each assay. E_2 equivalent expressed as ng/L, which is the total amount of estrogenic substances in seawater, was calculated by calibration curve for E_2.

Ishikawa cell-ALP assay: Ishikawa cells possessing estrogen receptor was derived from a well differentiated human endometrial adenocarcinoma, and was an established cell line. Alkaline phosphatase (ALP) activity in these cells is markedly stimulated by estrogenic substances in the medium (Holinka 1986). Cells grown in DMEM supplemented with 15% FBS in the same culture condition as that of E-screen described above were seeded into 24 well plates at a density of 1.5×10^5 cells/well. After 24 hr the medium was replaced by the medium containing phenol red-free DMEM supplemented with 15% CD-FBS and seawater extracts. Three days later, the medium was removed and washed with phosphate buffered saline (PBS, pH 7.4), and stored at −80°C for 1 hr. Afterward, 1 ml of substrate solution containing 5 mM *p*-nitrophenyl phosphate, 0.24 mM $MgCl_2$ and 1 M diethanolamine (pH 9.8) was added to each

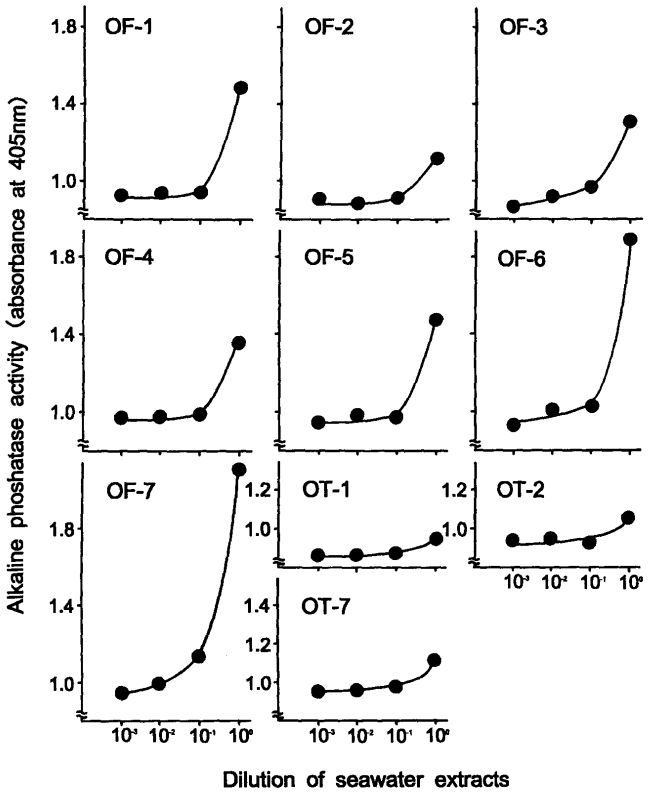

Fig. 2. Potential estrogenic substances in the seawater collected from each Sampling stations in Otsuchi Bay, Ofunato Bay using Ishikawa cell-ALP assay.
(OF: Ofunato Bay, OT: Otsuchi Bay)

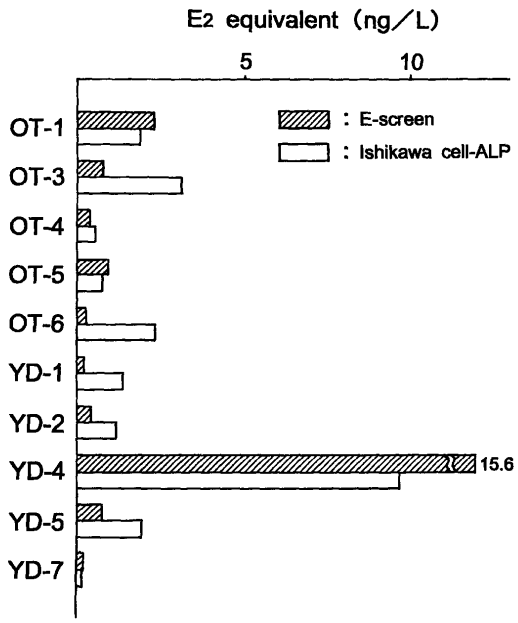

Fig. 3. E_2 equivalent (ng/L) in seawater collected from each sampling Station in Otsuchi Bay and Yamada Bay using E-screen and Ishikawa cell-ALP assay.
(OT: Otsuchi Bay, YD: Yamada Bay)

well, and were incubated for 1 hr at 30°C in a shaker. Yellow color due to the formation of *p*-nitrophenol was measured spectrophotometrically at 405 nm. E_2 equivalent (ng/L) was obtained by the calibration curve for E_2 in six concentrations in the same range as E-screen described above.

YES assay: The yeast estrogen screen (YES) was created

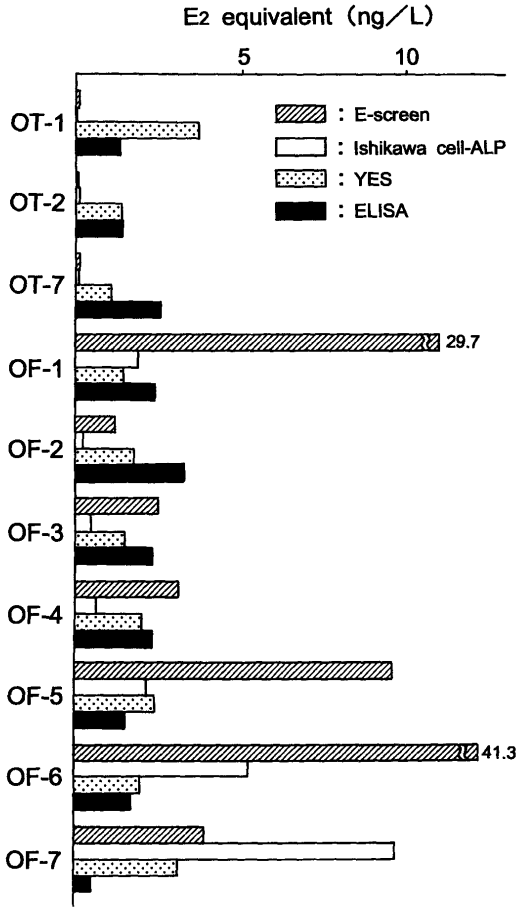

Fig. 4. E_2 equivalent (ng/L) in seawater collected from each sampling station in Otsuchi Bay and Ofunato Bay using E-screen, Ishikawa cell-ALP assay, YES and ELIZA method.
(OT: Otsuchi Bay, OF: Ofunato Bay)

by expressing human estrogen receptor and two estrogen elements linked to the LacZ gene. The β-galactosidase activity of the YES system is significantly increased after treatment with E_2 or other naturally occurring, synthetic estrogens and xenoestrogens (Coldham 1997). Briefly, yeast cells were grown in the enrichment medium for 24 hr at 32°C. Seawater extracts dissolved in ethylalcohol was added into 96 well plate. After ethylalcohol was evapolated at room temperature, assay medium containing *o*-nitrophenyl β-D-galactopyranoside (ONPG) as substrate and yeast cells were added into each 96 well, and were incubated for 84 hr at 30°C. *o*-Nitrophenol released from ONPG was measured at 540 nm, and E_2 equivalent (ng/L) was calculated from the sigmoid curve for E_2 standard.

Determination of E_2 using a commercial assay kit: E_2 concentration in seawater extract was measured using the Correlate-EIA E_2 kit (Assay Designs, Inc. Mich. USA). This kit is an application of the enzyme immuno assay and is called Enzyme-Linked Immunosorbent Assay (ELISA).

Measurement of water quality of seawater: Chemical Oxygen Demand (COD) in alkaline condition, NH_4-N, PO_4-P and suspended solid (SS) were measured according to the method JIS K 0102.

RESULTS AND DISCUSSION

Potential estrogenic substances in seawater: Figure 2 shows the estrogenic substances in seawater extracts in

Table 1. Water quality of seawater collected from Otsuchi Bay and Ofunato Bay.

Station	COD	PO_4-P	NH_4-N	SS
Otsuchi St-1	0	ND	0	6
St-2	0.36	0.01	0	22
St-7	0	ND	0	28
Ofunato St-1	0.44	0.02	0	36
St-2	0.24	0.01	0	11
St-3	0.04	ND	0	17
St-4	0.12	0.01	0	4
St-5	0	0.01	0	31
St-6	0	0.01	0	20
St-7	0	ND	0	11

ND: Not detected. (<0.01 mg/L) (unit: mg/L)

Otsuchi Bay and Ofunato Bay in July, 2001 expressed as ALP activity in optical density at 405 nm using Ishikawa cell. Estrogenic substances were observed in all samples taken from both bays. The original extract which was not diluted and was expressed as 10^0 in horizontal axis generally showed high activity in Ofunato Bay, and higher estrogenic activities were noticed near the mouth of harbor (Sts. 6 and 7) compared to the stations located in the inner part of the bay, while the activities in the samples taken from Otsuchi Bay (Sts. 1, 2 and 7) were relatively low.

The results obtained from E-screen assay were similar to those of Ishikawa cell-ALP assay mentioned above (Figs. 3 and 4). The reason why the higher estrogenic activity was noticed near the mouth of harbor remains uncertain.

E_2 equivalents in seawater of Otsuchi Bay and Yamada Bay: E_2 equivalents calculated from E-screen and Ishikawa cell-ALP assay was shown in Fig. 3. The values in Yamada Bay were almost similar levels compared to those in Otsuchi Bay, except in St. 4 of Yamada Bay located in the estuary area of a small stream. E_2 equivalent in St. 4 of Yamada Bay reached to about 10 ng/L or more in both E-screen and Ishikawa cell-ALP assay. Domestic sewage containing some estrogenic substances might flow into the estuary.

E_2 equivalents in seawater of Ofunato Bay and Otsuchi Bay: Figure 4 shows the E_2 equivalent obtained from three *in vitro* assays and from ELISA method in the seawater of Ofunato Bay and Otsuchi Bay taken in July, 2001. The values by both E-screen and Ishikawa cell-ALP in Otsuchi Bay were between 0.03 and 0.06 ng/L and were lower than those in July, 2000 ranging from 0.5 to 3 ng/L. On the other hand, E_2 equivalent in Ofunato Bay were higher near the mouth of harbor (Sts. 6 and 7), though the water quality of the stations, as shown in Table 1, were considered to be clear due to the water circulation. Although the results of YES assay showed no clear differences between sampling stations in Otsuchi Bay and Ofunato Bay, rather high E_2 equivalent was noticed St. 1 of Otsuchi Bay and St. 7 in Ofunato Bay.

E_2 concentration determined by ELISA method were considerably higher at the stations situated in the inner area of both bays. The difference of E_2 equivalents between three *in vitro* assays seems to depend on the difference in the endpoint of each assay. Namely, E-screen is based on the proliferation rate of breast cancer cells, T-47D, under the presence of estrogenic substances, and Ishikawa cell-ALP assay is based on the expression of alkaline phosphatase activity of the cells. The culture period, which is 6 days in E-screen, 3 days in Ishikawa cell-ALP assay and YES, may also affect the response of the cells to estrogenic substances in seawater extracts. Furthermore, yeast has cell walls differing from mammalian cells, and this is considered to disturb the permeability of chemicals from the assay medium into yeast cells. Anyway, the discussion as to the consistency of results obtained from three *in vitro* assays is necessary.

E_2 concentration in river water was reported ND (Not detected) to 27 ng/L in Japan (Tanaka 1999). Estron (E_1) is more commonly distributed at higher levels than E_2 in the aquatic environment. In addition, E_1 is sensitive to these *in vitro* assays used in this study. Therefore, E_1 or some other estrogenic substances that are overlooked in water might be also responsible for the E_2 equivalents.

There are many papers concerning the synthesis of vitellogenin, a yolk-precursor protein normally found in the blood plasma of sexually mature female teleosts and other egg-laying vertebrates, in male fish caused by the exposure to the effluent of sewage treatment plant containing natural estrogens.

Recently, E_2 equivalent concentration was reported in the effluent from municipal and industrial sewage treatment plant in south Germany, and the levels were between 0.2 and 7.8 ng/L using E-screen (Körner 2001). Although round 10 ng/L of E_2 was demonstrated to cause the vitellogenesis in male fish such as rainbow trout and carp (Purdom 1994), it is not clear what extent of vitellogenesis is critical for the reproductive activity in adult male fish. Therefore, the effects of E_2 equivalent level in coastal seawater of Iwate Prefecture on the aquatic lives including fish and other organisms is not clear now.

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岩手県大槌湾，山田湾および大船渡湾で採取した表層海水中のエストロゲン様物質

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近年，各種動物に対して内分泌系の機能を撹乱する物質の存在が注目されており，実際に数種の野生生物の生殖機能に障害を与える物質が明らかにされている．しかし，内分泌撹乱現象に関して因果関係が明瞭になっているケースはむしろ稀であり，解明すべき課題は多い．本報では，3種の *in vitro* のアッセイ法を用いて水中のエストロゲン様物質をトータルに把握することを試みた．試料は岩手県三陸海岸の山田湾，大槌湾および大船渡湾の表層海水である．2000年7月（大槌湾，山田湾）と2001年7月（大槌湾，大船渡湾）に各湾の数地点で海水を採取し，実験室に持ち帰り，ろ過により懸濁物を除去後，SeppakC18カートリッジに通水して海水中のエストロゲン様物質を固相抽出した．抽出物はジメチルスルホキシドまたはエタノールに溶解して *in vitro* のアッセイに供した．3種の *in vitro* のアッセイは，ヒトの乳がん由来のT-47D細胞を用い，その増殖能を指標とするE-screen，ヒトの子宮内膜がん由来のIshikawa cell 3-H-12を用い、アルカリフォスファターゼ (ALP) 活性を測定する方法およびヒトのエストロゲンレセプターを導入した酵母の β-ガラクトシダーゼ活性を測定するYESアッセイである．また，市販の E_2（17β-エストラジオール）測定キットも併用し，*in vitro* アッセイにより求めた E_2 当量値と比較した．大槌湾では．2000年7月の調査時に，湾口に位置する地点でもかなり高い E_2 当量値を示したが，2001年7月には大槌湾内におけるエストロゲン様物質はほとんど検出されなかった．一方，山田湾では小河川が流入する地点の付近でE-screenやIshikawa cell-ALPアッセイのいずれもが高いエストロゲン様物質の存在を示した．2001年7月に実施した大船渡湾の調査では，湾内のいずれの地点でもエストロゲン様物質が検出されたが，湾口に近い地点でむしろ高い E_2 当量値が得られた．E-screenやIshikawa cell-ALPアッセイと異なり，YES法や E_2 キットから求めた E_2 濃度は地点間の差が明瞭ではなかった．このように，アッセイ法の違いによって，海水中のエストロゲン様物質濃度は異なる．これは各アッセイのエンドポイントが微妙に異なることに起因していると考えられるが，結果の整合性については今後の重要な検討課題である．

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