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Characterization of trace organic contaminants in marine sediment from Yeongil Bay, Korea: 2. Dioxin-like and estrogenic activities

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In vitro bioassay responses observed for Yeongil Bay surficial sediment and sediment core extracts showed the greatest dioxin-like and estrogenic activities in the mid-polarity fraction containing PAHs as well as chlorinated dioxins and furans.

Abstract

This study employed mechanism-specific in vitro bioassays to help characterize the occurrence and distribution of dioxin-like and estrogenic contaminants in sediment from Yeongil Bay, Korea. Approximately 85% of the sediments tested induced significant dioxin-like activity in the H4IIE-luc bioassay, while approximately 50% induced significant estrogenic activity in the MVLN bioassay. Instrumentally-derived estimates of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and 17β-estradiol equivalents tended to underestimate the magnitude of response observed in the bioassays, suggesting that compounds detected by chemical analysis did not account for all the activity associated with Yeongil Bay sediments, or that non-additive interactions were occurring. The greatest dioxin-like and estrogenic activity was associated with the mid-polarity Florisil fractions (F2) expected to contain polycyclic aromatic hydrocarbons (PAHs) as well as chlorinated dioxins and furans. As in previous studies of Korean coastal sediment, more polar fractions (F3) generated more modest responses both in terms of magnitude and the number of samples responding.

Keywords: In vitro bioassay; Polycyclic aromatic hydrocarbons (PAHs); Mass balance; Persistent organic pollutants (POPs); Endocrine disrupting chemicals (EDCs)

1. Introduction

Yeongil Bay is located on the east coast of Korea. Due to its proximity to heavy industrial and commercial activity, Yeongil Bay is considered to be one of the most contaminated coastal areas in Korea (Koh et al., 2004, 2006). As part of a series of studies aimed at characterizing organic chemical contamination in Korean coastal areas (Khim et al., 1999a,b,c, 2001; Koh et al., 2002, 2004), this study examined concentrations and distribution of persistent organic pollutants (POPs), polycyclic aromatic hydrocarbons (PAHs), and selected endocrine disrupting compounds (EDCs) in Yeongil Bay sediment. In addition to the chemical analyses, presented elsewhere (Koh et al., 2006), in vitro bioassays were applied to facilitate a more complete understanding of the mixture of biologically active contaminants present in Yeongil Bay sediment.

Mechanism-based in vitro bioassays have several attributes that make them a useful complement to routine quantitative

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chemical analysis. First, in vitro bioassays are capable to detecting unknowns for which there are no established analytical methods, standards, etc. Second, in vitro bioassays target only those chemicals that contribute to an interaction with one or more biomolecules known to mediate a biological response through a defined mechanism of action. As a result, analyses are focused on biologically relevant compounds. Finally, bioassays integrate the response of complex chemical mixtures, providing an indication of the potential biological potency and/or relevance of an entire mixture. However, unlike instrumental analytical methods, bioassays generally cannot *identify* the chemical(s) causing the response, nor can they precisely *quantify* the concentrations of chemical(s) present, as bioassay response is a function of concentration, potency, and interactions. Thus, in vitro bioassays serve as a useful complement, although not a substitute, for chemical analysis.

Two mechanism specific in vitro bioassays were employed as part of this study. The first was the H4IIE-luc cell bioassay. H4IIE-luc cells are rat hepatoma cells, stably transfected with a luciferase reporter gene under control of dioxin-responsive enhancers (DREs) (Sanderson et al., 1996). They have been effectively used as a screen for detecting dioxin-like contaminants in extracts of sediment, surface waters, and animal tissues (Khim et al., 1999a,c, 2000, 2001; Koh et al., 2002; Tillitt et al., 1996). H4IIE-luc cells have been shown to sensitively detect a wide variety of compounds capable of interacting with the aryl hydrocarbon receptor (AhR) including certain polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), polycyclic aromatic hydrocarbons (PAHs), and polychlorinated naphthalenes (PCNs) (Sanderson et al., 1996; Villeneuve et al., 1998, 2000b, 2002). The H4IIE-luc bioassay was used in this study to help characterize the suite of dioxin-like contamination associated with Yeongil Bay sediment.

The second bioassay used for this study was the MVLN bioassay. MVLN cells are MCF-7 human breast carcinoma cells stably transfected with a luciferase reporter gene under control of estrogen response elements (EREs) of the *Xenopus laevis* vitellogenin A2 gene (Pons et al., 1990). Thus, the MVLN cell bioassay is a simple and sensitive screening tool able to detect estrogenic compounds which can modulate gene transcription through an estrogen receptor (ER)-mediated mechanism (Giesy et al., 2002). A variety of compounds including estradiol, ethynyl estradiol, coumestrol, bisphenol A (BPA), alkylphenols (APs), and certain PAHs have been previously shown to induce responses in the MVLN assay (Villeneuve et al., 1998, 2002). The MVLN bioassay was used in this study to help characterize the total load and potency of estrogenic chemicals associated with Yeongil Bay sediment.

Both the MVLN and H4IIE-luc cell bioassays have been applied in previous characterizations of organic contaminants in sediment from Korean coastal areas (Khim et al., 1999a,c; Koh et al., 2002). In the present study, the assays were used to address a number of specific research questions. First, used as a basic yes/no screening tool, the bioassays were used to determine whether there were AhR- and/or ER-active compounds present in extracts of Yeongil Bay sediments. Second, a basic mass-balance approach (Giesy et al., 2002) was used to determine whether the chemical composition and concentrations identified using instrumental analytical approaches could adequately account for the biological responses observed. This analysis would help determine whether future analytical monitoring should expand the suite of target analytes examined, and whether comprehensive bioassay-directed fractionation (Snyder et al., 2001) should be used to identify those additional analytes. Finally, limited fractionation was employed in order to broadly characterize the class of compounds responsible for an in vitro response and determine whether that pattern was similar to that observed for extracts of sediment from other Korean coastal areas. This would help determine whether any future efforts to identify additional target analytes, through bioassay-directed fractionation, should be site specific.

2. Materials and methods

2.1. Samples and fractionation

Sediment samples were collected in March 2000 from 26 locations in Yeongil Bay, Korea (Fig. 1). Surface sediment (0–5 cm) samples were subsampled from triplicate grab samples (25 × 40 × 30 cm) then freeze dried and stored in pre-cleaned high-density polyethylene (HDPE) bottles at −20 °C until extraction. A sediment core was collected from Pohang Harbor near Posco Industrial Complex (IC), using a Haps corer (31.5 cm long and 13.6 cm i.d.; KC-Denmark, Silkeborg, Denmark). Once aboard ship, the core was sectioned immediately, at 2-cm intervals up to 10 cm depth, using a sediment ejector and clean stainless steel slicer. Each section was freeze dried and stored in a pre-cleaned HDPE bottle at −20 °C until extraction. Based on the vertical profiles of ^{210}Pb, the sedimentation rate was estimated to be approximately 0.21 ± 0.05 cm/yr.

Detailed descriptions of the sample extraction and fractionation procedures have been provided elsewhere (Khim et al., 1999a; Koh et al., 2006). Briefly, 40 g of sediment samples were Soxhlet extracted for 20 h using 400 ml of dichloromethane (DCM; Burdick and Jackson, Muskegon, MI, USA). Extracts were treated with activated copper granules to remove sulfur from the Soxhlet raw extracts (REs). REs concentrated to 2 ml were passed through 10 g of activated Florisil (60–100 mesh size; Sigma, St. Louis, MO, USA) packed in a glass column (10 mm i.d.) and eluted as three separate fractions (FEs). The first fraction (F1) eluted with 100 ml of hexane (Burdick and Jackson) contained PCBs, HCB and *p,p′*-DDE. Remaining OC pesticides and PAHs were eluted in the second fraction (F2) using 100 ml 20% DCM in hexane. Nonylphenol (NP), octylphenol (OP), butylphenol (BP) and BPA were eluted in the third fraction (F3) with 100 ml 50% DCM in methanol (Burdick and Jackson). Procedural blanks (PBs) generated from each set of six samples during fractionation and field blanks (FBs) obtained during field survey were concurrently analyzed. The instrumental analysis was completed using FEs and corresponding results have been provided elsewhere (Koh et al., 2006).

2.2. Bioassay

All of the RE, FE, PB and FB samples were screened by the H4IIE-luc and MVLN bioassays to determine dioxin-like and estrogenic activities, respectively. Cells were seeded into 96-well plates and exposed to 2.5 μl of extract for 72 h. A minimum of three solvent control and three blank wells were analyzed with each 96-well plate, and all samples were tested in triplicate. Luciferase activity was determined after 72 h of exposure using methods described previously (Villeneuve et al., 2002). Sample responses, expressed as relative luminescence units (RLUs), were converted to relative response units, expressed as a percentage of the maximum response observed for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; %-TCDD-max.) or 17β-estradiol (E2; %-E2-max.) standard curves generated on the same day.

Mass-balance analysis (Giesy et al., 2002) was used to estimate the proportion of the activity in the samples that had been accounted for by identified

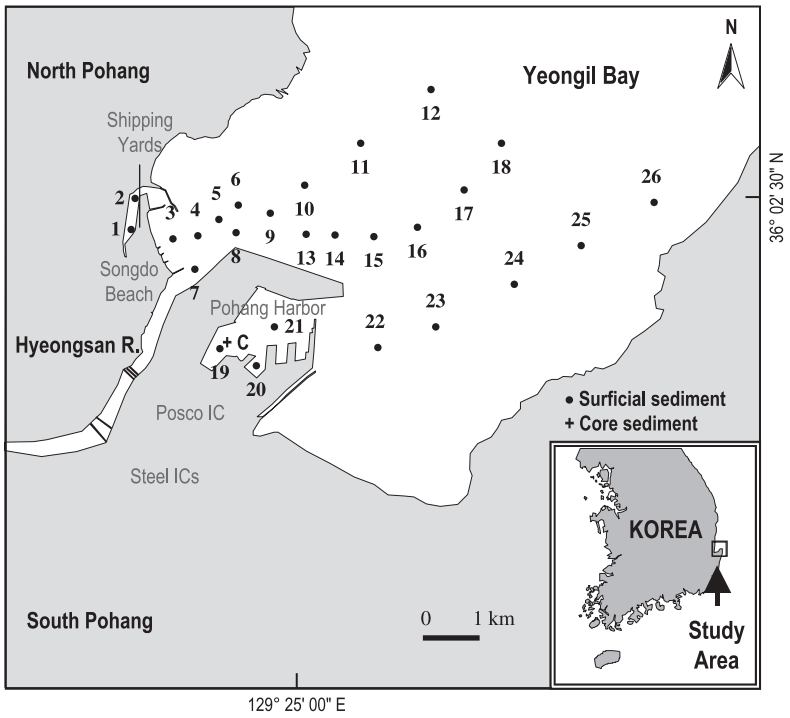

Fig. 1. Map of Yeongil Bay study area in Korea. Surficial sediment samples were collected at locations #1–26 and a sediment core (+) was obtained from the inner Pohang Harbor.

compounds. Instrumentally derived TCDD equivalents (TEQ) or E2 equivalents (EEQs) were calculated by multiplying congener-specific chemical concentrations by published assay-specific relative potencies (RPs) or toxic equivalency factors (TEFs), and summing them for each sample (Khim et al., 1999c). Bioassay-derived TCDD or E2 equivalents (TCDD-EQ, E2-EQ) were derived by using the regression equation for the appropriate TCDD or E2 standard curve to calculate a concentration of TCDD or E2 (-EQs) that produced a bioassay response equal in magnitude to that induced by the sample in question. Conversion to pg TCDD-EQs or E2-EQs/g dry wt. was back-calculated based on the volume of extract assayed (i.e. 2.5 μl) and the degree of concentration during the extraction procedure (i.e. 40 g sediment concentrated into 2 ml of extract). This approach to estimating bioassay-derived -EQs was based on the principle of *equally effective doses*, which provides the theoretical basis for indirect bioassay approaches such as relative potency estimation (Finney, 1978; Putzrath, 1997; Villeneuve et al., 2000a). Indirect bioassay approaches are based on the assumption that the sample being analyzed responds as if it were a dilution (or more concentrated form) of the standard compound (i.e. sample concentration response is parallel to standard curve and maximum efficacy of sample and standard are equal; Finney, 1978). Given limited sample volumes, rigorous testing of these assumptions by analyzing concentration response curves was not feasible for this study. Previous studies suggest that environmental samples rarely conform to these assumptions (Villeneuve et al., 2000a). Nonetheless, when applied cautiously with limitations in mind, estimates based on indirect bioassay can still be broadly informative for comparisons based on multiple samples (Villeneuve et al., 2000a). Further details of cell culture condition, in vitro bioassay, and data analysis have been described extensively in earlier publications (Khim et al., 1999c; Villeneuve et al., 2000a). Statistical analyses including correlation analysis, paired *t*-tests, ANOVA, and multiple comparisons were conducted using SAS 9.0.

3. Results and discussion

3.1. Dioxin-like activity

H4IIE-luc-based screening of Yeongil Bay sediment extracts indicated that AhR-active compounds were present in most sediments collected from the Yeongil Bay study area (Fig. 2). Twenty-two of 26 REs tested induced significant dioxin-like responses in the H4IIE-luc bioassay (Fig. 2). Response magnitudes were uniformly less than the maximum response produced by a 1500 pM TCDD standard, and ranged from 0%- to 45%-TCDD-max (Fig. 2). Based on the limit of quantification (LOQ) for TCDD in the H4IIE-luc bioassay, one can infer that 22 of the 26 sediment samples contained greater than 1.55 pg TEQ/g dry wt. (Table 1). Thus, screening results suggest that Yeongil Bay sediments did contain dioxin-like compounds.

Mass (potency) balance analysis suggested that the chemical composition identified by instrumental analytical methods (Koh et al., 2006) does not adequately account for the magnitude of response observed in the H4IIE-luc bioassay. Based on the instrumental data, Yeongil Bay sediment contained some potent AhR agonists including certain PCBs, ranging in concentration from 0.22 to 3.74 ng/g dry wt., and AhR-active PAHs (Villeneuve et al., 2002; Koh et al., 2004). However, the estimated bioassay-derived TCDD-EQs for Yeongil Bay sediment REs exceeded instrumentally-derived TEQs for all but three (#1, 2, and 20) of the 26 samples analyzed (Table 1). For over 80% of the samples tested, bioassay-derived TCDD-EQs were at least an order of magnitude greater than instrumentally derived TEQ estimates (Table 1). Furthermore, based solely on instrumentally derived TEQs, only six samples were expected to produce a significant H4IIE-luc response (i.e. $\geq$1.55 pg/g dry wt.; Table 1). Thus, the results support the conclusion that at least some of the Yeongil Bay sediment samples likely contained dioxin-like compounds other than those directly identified and quantified by instrumental chemical analysis (Koh et al., 2006).

A previous study conducted by our group quantified polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) in sediment from the Hyeongsan River which flows into Yeongil Bay (Fig. 1; Koh et al., 2004). TEQ_{PCDD} and TEQ_{PCDF} for Hyeongsan River sediment ranged from 0.38–937 and 0.22–197 pg/g dry wt., respectively, and generally accounted for greater than 80% of the total TEQs detected in the samples (Koh et al., 2006). Considering the direct daily discharge of over 0.2 million ton of industrial and municipal wastewater via Hyeongsan River to Yeongil Bay, PCDDs and PCDFs residues likely persist in sediments and contribute significantly to total TCDD-EQs. Before launching an extensive bioassay-directed fractionation project aimed to toxicant identification, additional effort should be dedicated to quantifying concentrations of PCDDs and PCDFs in Yeongil Bay sediment.

To further examine the potential cause-effect relationships between known AhR agonists quantified and AhR-mediated bioassay response to be observed, Florisil fractions (F1, F2, and F3) of the Yeongil Bay sediment extracts were analyzed in the H4IIE-luc bioassay. Based on spike recovery studies, TEQ_{PCB} should have eluted in F1 (Khim et al., 1999b; Kannan et al., 2000). Two F1 samples (#1 and 2) contained TEQ_{PCB} concentrations that were greater than the LOQ (1.55 pg/g dry wt.). However, no F1 samples induced significant responses with associated TCDD-EQs that exceeded 1.55 pg/g dry wt. This suggests that either the relative potency of the PCBs was overestimated or that interactions among compounds present in F1 results in total dioxin-like activity that was lower than predicted. As a whole, the lack of F1 response was consistent with previous studies of dioxin-like activity in Korean sediments (Khim et al., 1999a,c, 2001; Koh et al., 2002).

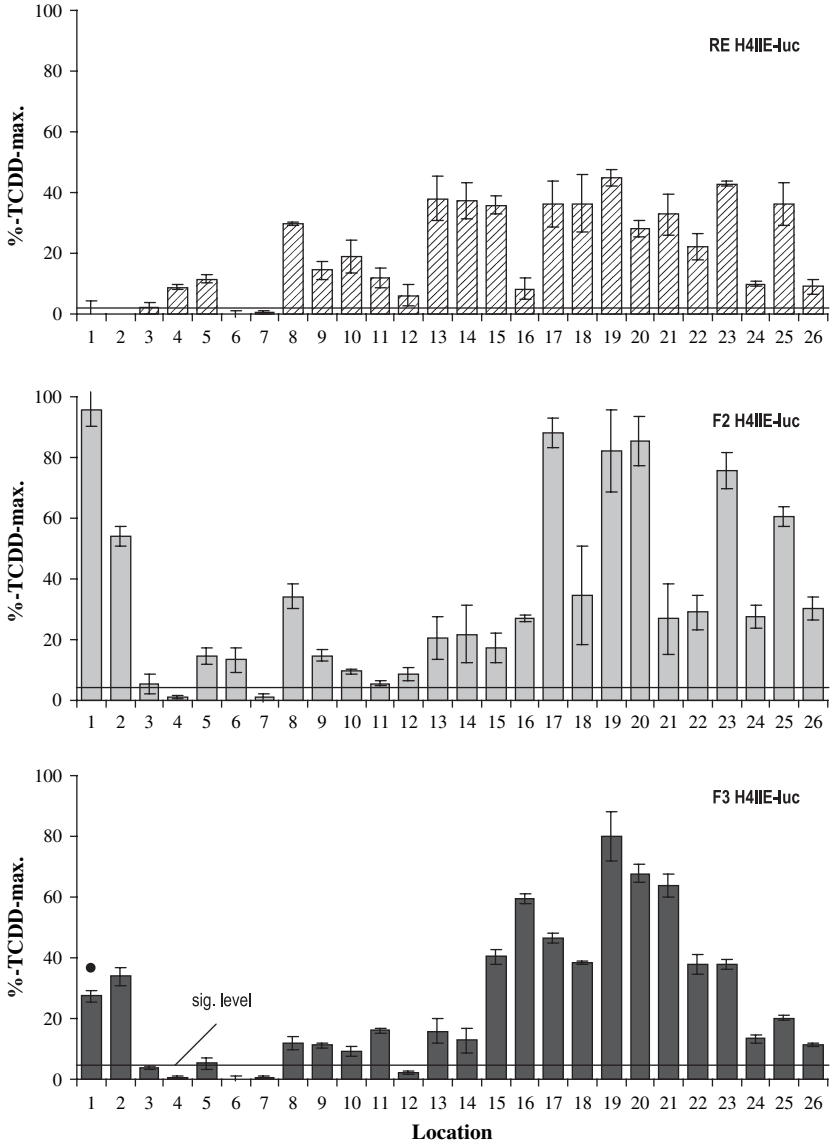

Fig. 2. Luciferase induction in the H4IIE-luc cell bioassay elicited by sediment raw extract (RE) and fractionated extracts (F2 and F3). Response magnitude presented as percentage of the maximum response observed for a 1500 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin standard (%-TCDD-max.). Horizontal line equals 3 standard deviations (expressed in %-TCDD-max.) above the mean solvent control response (set to 0%-TCDD-max.). ● indicates cells exhibited an altered or "stressed" morphology (cytotoxicity).

Table 1
Instrumentally derived dioxin equivalents of PCBs (TEQ_{PCB}), PAHs (TEQ_{PAH}), and total TEQs in Yeongil Bay sediment and bioassay-derived TCDD-EQ estimates (±95% confidence interval)

Location	TEQs (pg/g dry wt.)[a]			TCDD-EQ[b]		
	TEQ_{PCB}	TEQ_{PAH}	Total TEQs	RE	F2	F3
1	6.08	1.08	7.17	4.22 (1.24)	311 (92.4)	5.17 (0.56)
2	2.55	1.48	4.03	3.41 (0.57)	25.5 (4.50)	7.65 (1.33)
3	<0.01	0.002	<0.01	3.89 (0.17)	<1.55	<1.55
4	<0.01	<0.001	<0.01	5.49 (0.11)	<1.55	<1.55
5	<0.01	<0.001	<0.01	6.00 (1.18)	2.43 (0.37)	<1.55
6	<0.01	0.004	<0.01	3.77 (0.19)	2.30 (0.53)	<1.55
7	<0.01	<0.001	<0.01	4.30 (0.12)	1.08 (0.09)	<1.55
8	<0.01	1.38	1.38	14.3 (1.02)	7.91 (1.77)	2.08 (0.24)
9	<0.01	0.01	0.01	6.48 (0.64)	2.44 (0.27)	1.97 (0.07)
10	<0.01	0.01	0.01	6.96 (0.98)	1.79 (0.08)	1.77 (0.17)
11	<0.01	<0.001	<0.01	6.83 (0.76)	<1.55	2.66 (0.13)
12	<0.01	<0.001	<0.01	4.35 (0.38)	1.72 (0.24)	<1.55
13	<0.01	<0.001	<0.01	17.5 (6.88)	3.60 (1.57)	2.67 (0.63)
14	<0.01	0.21	0.21	14.3 (6.30)	4.08 (2.03)	2.21 (0.52)
15	<0.01	0.19	0.19	34.0 (6.41)	2.91 (0.91)	11.2 (1.64)
16	<0.01	0.03	0.03	6.85 (1.50)	5.03 (0.36)	35.0 (3.26)
17	<0.01	6.43	6.43	36.5 (17.8)	194 (54.2)	16.0 (1.62)
18	<0.01	0.03	0.03	12.7 (7.28)	10.1 (6.54)	9.92 (0.43)
19	0.40	1.11	1.51	17.8 (5.76)	165 (129)	126 (59.7)
20	0.60	25.4	26.3	21.5 (4.06)	174 (87.7)	57.2 (9.61)
21	<0.01	0.01	0.01	25.0 (3.06)	5.94 (4.22)	45.1 (9.80)
22	<0.01	0.54	0.54	11.6 (2.03)	5.93 (2.20)	9.72 (1.95)
23	0.67	10.8	11.5	50.2 (2.84)	95.5 (30.7)	9.59 (0.92)
24	<0.01	0.03	0.03	5.64 (0.86)	5.35 (1.21)	2.25 (0.18)
25	<0.01	3.12	3.12	16.4 (1.00)	37.5 (6.74)	3.36 (0.17)
26	<0.01	0.82	0.82	7.13 (1.17)	6.22 (1.38)	2.02 (0.06)

RE, raw extract. F1 did not induce a significant response in the bioassay. Estimated 2,3,7,8-TCDD equivalents (TCDD-EQ) for F1 samples <1.55 pg TCDD-EQ/g dry wt., in the H4IIE-luc bioassay.

[a] Instrumentally derived 2,3,7,8-TCDD equivalents (TEQs) of PCBs (F1) and PAHs (F2) in sediment extract; total TEQs are sum of TEQ_{PCB} and TEQ_{PAH}.

[b] Estimate of bioassay-derived 2,3,7,8-TCDD equivalents (TCDD-EQ), based on regression against TCDD standard curve and the indirect bioassay principle of equally effective doses.

Among the three fractions tested, F2 samples induced the greatest magnitude of response in the H4IIE-luc assay with 24 out of 26 F2 samples inducing significant dioxin-like responses (Fig. 2). Magnitudes of induction as great as 96%-TCDD-max were observed (#1), and greater dioxin-like activities were generally observed at the inner locations. Laboratory spike-recovery tests have shown that PAHs and some PCDDs/DFs elute in F2 (Khim et al., 1999b; Kannan et al., 2000). Thus, the significant H4IIE-luc response caused by F2 samples may be attributable to PAHs, PCDDs/DFs, or unknowns of similar polarity. Based on the measured concentrations of some AhR-active PAHs such as benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[1,2,3-*cd*]pyrene, and dibenz[*a,h*]anthracene, four F2 samples had TEQ_{PAH} that were sufficient to induce a significant response (Table 1), and all four of those samples induced strong responses in the H4IIE-luc bioassay (Table 1, Fig. 2). There was also a significant positive correlation between TEQ_{PAH} and bioassay-derived TCDD-EQs for F2 samples ($r^2 = 0.489$, $p = 0.0112$). This suggests that the known concentrations of PAHs could account for at least a portion of the response observed for some locations. Without additional fractionation or chemical treatment to separate the effects of PAHs and PCDDs/DFs, it was not possible to determine the proportion of response contributed by PAHs versus PCDDs/DFs and unknowns. As a whole, the robust response of F2 samples was consistent with previous studies of Korean coastal sediment contamination (Khim et al., 1999a,c; Koh et al., 2002).

F3 samples also showed a robust response in the H4IIE-luc assay. Twenty-one out of the 26 F3 samples tested induced significant dioxin-like activity (Fig. 2). The magnitude of induction ranged up to 80%-TCDD-max and the spatial distributions was similar to that observed for F2 samples (Fig. 2). There was a significant positive correlation between F2 and F3 responses ($r^2 = 0.418$, $p = 0.033$). It was not clear from the results of this study whether the activity was due to PCDDs/DFs that carried over into F3, or whether the activity was caused by unidentified, relatively polar, compounds present in Yeongil Bay sediment. However, moderate to high dioxin-like responses in the H4IIE-luc bioassay, for F3 samples, has been consistently found in the earlier studies of Korean sediments (Khim et al., 1999a,c, 2001; Koh et al., 2002).

It was also worth noting that in a number of cases, most notably for samples #1 and 2 but others as well, F2 and F3 induced much greater dioxin-like activity than the corresponding RE (Fig. 2). This supports the hypothesis that interactions among the complex mixture of compounds present in the RE

may result in responses that are significantly lower than those that would be predicted based on the sum of its parts (i.e. using a TEQ-based additive model). In general, results of this study are consistent with the idea that compounds present in F1 may antagonize and/or mask the potency of dioxin-like compounds found in F2 and F3. However, at this time we cannot rule out the possibility that F2 and F3 components antagonize one another, or that alterations during the fractionation process itself changed the overall activity of the sample. The discrepancy observed between the RE and FE responses supports the need for complementary biological and instrumental analysis when characterizing sediment contamination and its potential biological potency, since instrumental analysis alone is unlikely to accurately account for relevant interactions that affect a biological response, while bioassays may underestimate the potential for exposure. Future experiments aimed at elucidating and understanding the interactions occurring between known components of the sediment extracts, unknowns, and potentially natural components of sediment, could aid the interpretation of results.

3.2. Estrogenic activity

MVLN-based screening of Yeongil Bay sediment extracts indicated that estrogenic compounds were present in some Yeongil Bay sediments (Fig. 3). Approximately half of the 26 REs elicited significant estrogenic responses in the MVLN bioassay (Fig. 3). The magnitude of response was generally less than 40%-E2-max and the greatest estrogenic activity was associated with sediment from the outer bay at location #26 (Fig. 3). Significant estrogenic responses were associated with various locations throughout the bay, both near-shore and outer- bay areas (Figs. 1 and 3). This result was in agreement with widespread distribution characteristics of known polar ER-active compounds such as APs in Yeongil Bay (Koh et al., 2004, 2006). In particular, the increasing pattern of estrogenic activity in the middle transect line from location near Pohang Harbor to outer bay was consistent with the gradient of NP concentrations (Koh et al., 2006).

However, mass balance analysis of the MVLN results did not support the hypothesis that NP or other known constituents detected in the Yeongil Bay sediment samples could adequately account for the bioassay responses observed. Estrogen equivalents (EEQs) calculated by multiplying the known concentrations of NP, OP, BPA, and certain ER-active PAHs (benzo[*a*]anthracene, and dibenz[*a,h*]anthracene) by their MVLN-specific relative potency were generally less than the LOQ of 5.85 pg EEQ/g dry wt. Only one RE sample (#1) was shown to contain sufficient EEQs (18.7 pg EEQ/g dry wt.) to elicit significant estrogenic response in the MVLN bioassay, but at least 11 other samples induced a significant MVLN response. Estimated concentrations of bioassay-derived E2-EQs ranged from <5.85 to 18.9 pg E2-EQ/g dry wt., with a mean of 6.0 ± 3.9 pg E2-EQ/g dry wt. Furthermore, there were no significant correlations between measured concentrations of NP, OP, BP, or pesticides and MVLN responses to RE samples ($r = -0.058$–0.078, $p = 0.904$–0.703). The results suggest that significant estrogenic responses associated with sediment from locations, other than location 1, were likely caused by unidentified ER-active compounds or synergistic interactions between known and/or unknown compounds present in REs.

Due to an unidentified procedural error, results from the 96-well plates used to test F1 samples did not meet quality control standards. Coefficients of variation were generally in excess of 100% and both blank and SC responses well outside normal range. The F1 results generated were unusable and, unfortunately, it was not possible to reanalyze the F1 samples for this study. Therefore, F1 responses associated with Yeongil Bay sediments could not be assessed. However, no target xenoestrogens were expected to elute in F1, which contains non-polar compounds (Koh et al., 2006) and in previous studies F1 samples have shown little or no estrogenic activity (Khim et al., 1999a,c, 2001).

MVLN analysis of F2 and F3 samples yielded a pattern that was consistent with other studies of Korean coastal sediment contamination. F2 samples caused greatest responses in the MVLN bioassay both in terms of magnitude and the number of samples responding (Fig. 3). Response magnitudes as great as 50.4 ± 3.6%-E2-max were observed. Nearly all the F2 samples elicited significant estrogenic activities (Fig. 3), although this was partially caused by a slight increase in assay sensitivity on the day the F2 and F3 samples were run (LOQ = 4.0 pg EEQ/g dry wt.). Relative to RE responses, six F2 samples (#1, 2, 6, 10, 13, 21) induced responses that were statistically greater (paired *t*-test, $p < 0.05$) than corresponding RE responses, while three F2 samples (#9, 23, 26) induced responses that were statistically less than corresponding RE responses (Fig. 3). The significant differences observed suggest that some non-additive interactions were likely occurring between components of the various fractions. Nonetheless, the vast majority of ER-active compounds present in the Yeongil Bay sediment extracts appeared to partition to F2.

OC pesticides such as toxaphene, chlordecone, endosulfan, and *p,p′*-DDT, which would have eluted in F2, have been reported to cause weak in vitro estrogenic responses when concentrations exceed 1 μg/g (Soto et al., 1994). F2 responses were significantly correlated with the measured total concentration of OC pesticides ($r^2 = 0.703$, $p < 0.0001$). However, OC pesticide concentrations in F2 of Yeongil Bay sediment extracts were all less than 10 ng/g and were unlikely to account for the estrogenic activity of F2. Some ER-active PAHs such as benzo[*a*]anthracene and dibenz[*a,h*]anthracene have been reported to elicit estrogenic activity in the MVLN bioassay (Villeneuve et al., 2002). However, the EEQ_{PAH}, calculated from concentrations of two ER-active PAHs and their MVLN specific relative potency, reached a maximum of 0.07 pg EEQ/g. Based on the LOQ for the MVLN assay, PAHs alone did not provide a sufficient concentration of EEQ to induce a significant MVLN response (i.e. ≥ 4.04 pg EEQ/g). Thus, the likely responsible ER-active compounds for F2 responses caused by extracts of Yeongil Bay sediments were unknown and probably not due to OC pesticides and/or PAHs unless one assumes synergistic interactions.

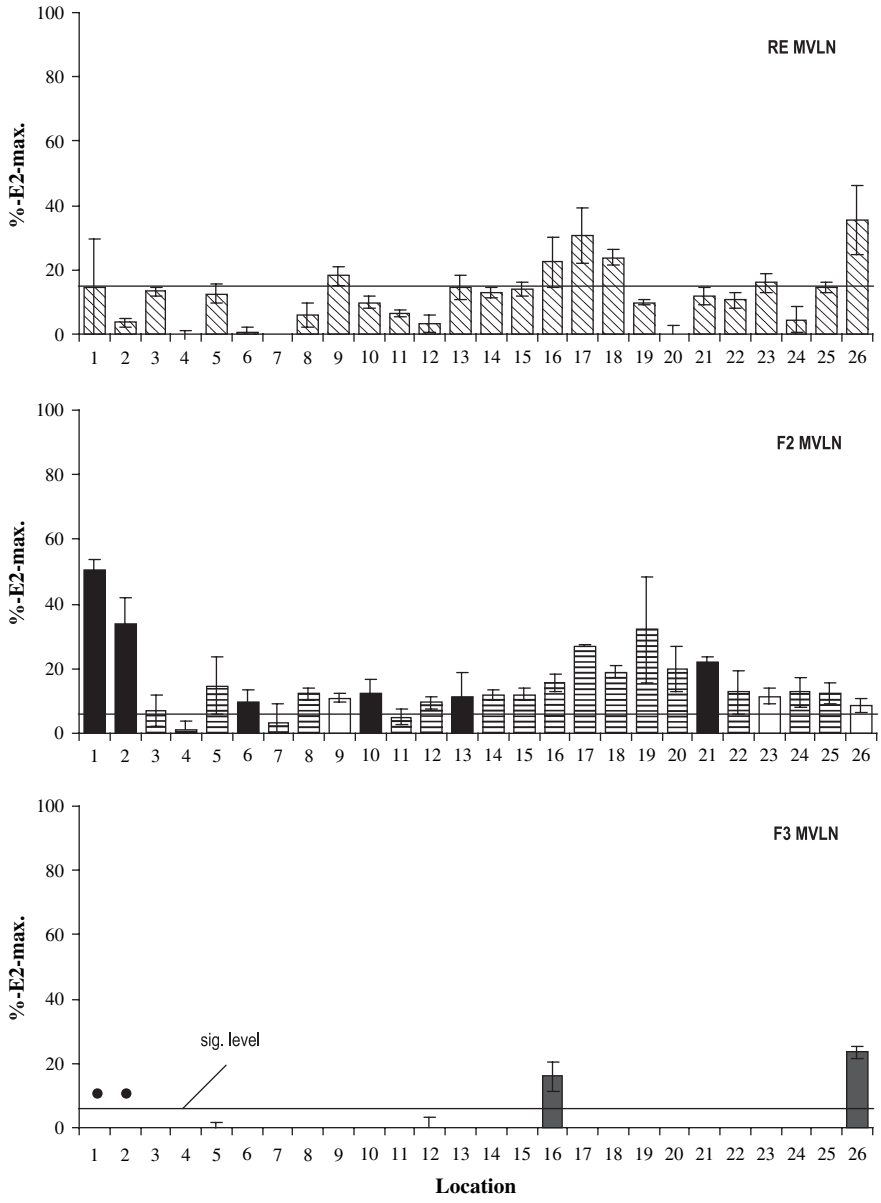

Fig. 3. Luciferase induction in the MVLN cell bioassay elicited by sediment raw extract (RE) and fractionated extracts (F2 and F3). Response magnitude presented as percentage of the maximum response observed for a 1000 pM 17β-estradiol (%-E2-max.). Horizontal line equals 3 standard deviations (expressed in %-E2-max.) above the mean solvent control response (set to 0%-E2-max.). ● indicates cells exhibited an altered or "stressed" morphology (cytotoxicity). For F2, black bars indicate responses significantly greater than RE response, white bars indicate responses significantly less than RE response, and dashed bars indicate no significant difference between F2 and RE response (significance defined as $p < 0.05$).

Interestingly, unlike MVLN responses for RE samples, F2 responses were significantly correlated with measured concentrations of total APs, NP, OP, and BP ($r^2 = 0.667–0.741$, $p < 0.0003$). However, based on spike recovery, F2 samples were not expected to contain significant concentrations of APs. The significant positive correlation between F2 response and concentrations of APs suggests that either these compounds partitioned unexpectedly into F2 or that the concentrations of the ER-active compounds present in F2 samples correlate with those of pesticides and APs. Even assuming that all APs detected partitioned to F2 rather than F3, only one sample (#1) was calculated to contain enough EEQs (>4.04 pg EEQ/g dry wt.) to induce a significant MVLN response. Although sample 1 did induce a positive F2 response, concentrations of AP-derived EEQ cannot account for the other positive responses observed. Thus, the latter explanation, that concentrations of unidentified ER-agonists associated with F2 are correlated with concentrations of APs and OC pesticides, appears more likely. The results suggest that a more comprehensive bioassay-directed fractionation aimed at identifying the ER-agonists present in F2 would provide a major contribution to our understanding of estrogenic contaminants associated with Korean coastal sediment.

One might expect F3 samples to have the greatest estrogenic activity since, based on previous spike recovery studies (Khim et al., 1999b), all the target xenoestrogens analyzed in this

study were expected to elute in F3. However, as in previous studies, F3 responses were more modest than those observed for F2 (Khim et al., 1999a,c). In this study, only 2 out of 26 F3 samples (#16 and 26) elicited significant estrogenic activities (Fig. 3). Based on relative potency values previously reported for NP, OP, and BP (Villeneuve et al., 2000b), only one sample (#1) contained enough EEQs to induce an F3 response (i.e. ≥4.04 pg/g dry wt.), but sample #1 did not induce a significant F3 response. The general lack of F3 response supports the conclusion that compounds other than NP, OP, and BP, and likely in the polarity range associated with F2, were the most potent ER-active compounds present in Yeongil Bay sediment.

In previous studies, cytotoxicity and/or stressed cell morphologies have often been associated with exposure to F3 samples (Khim et al., 1999c, 2001). In this study, indications of cytotoxicity were noted for only two samples (#1, 2). This may account for the apparent lack of F3 response for sample #1. In general, however, F3 samples analyzed as part of this project were less cytotoxic than most F3 samples of Korean coastal sediment extracts.

3.3. Vertical profiles

Sediment core samples collected from the inner Pohang Harbor, near Posco IC, were screened for their ability to induce AhR- or ER-mediated gene expression in the H4IIE-luc and MVLN cell bioassay, respectively. REs and three FEs (F1, F2, and F3) from each section of 0–2, 2–4, 4–6, 6–8, and 8–10 cm sediment were tested using equal volume of extracts from equal amount of sediment extracted. The vertical profile of H4IIE-luc responses sediment core REs showed significantly less activity at the 6–8 and 8–10 cm depths (ca. 1951–1970) than for the more surficial core sections (Fig. 4). This may be associated with breakdown of dioxin-like sediment contaminants over time, or an indication that inputs of dioxin-like contaminants have increased since ca. 1971. H4IIE-luc responses to sediment core F3 samples showed a trend toward decreasing dioxin-like activity associated with F3 at greater depths (i.e. older sediment; Fig. 4). Although some differences were observed with depth for F2 samples, the trend was less prominent. The results suggest that dioxin-like contaminants associated with F2 were more

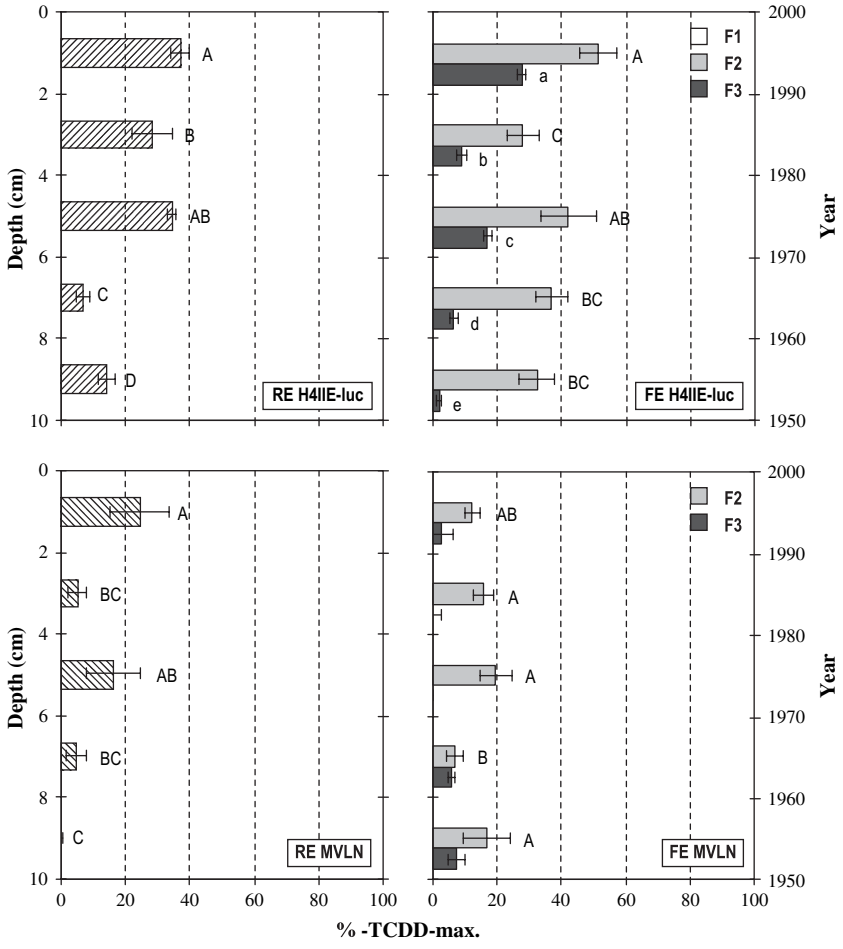

Fig. 4. Vertical profiles (0–10 cm) of bioassay response in the H4IIE-luc and MVLN cell bioassay elicited by sediment raw extract (RE) and fractionated extracts (F1, F2, and F3). Time interval (right *Y* axis: Year) represented is given for corresponding depth layer based on the sedimentation rate of 0.21 cm/yr at the sampling location. Different letters indicate a statistically significant difference ($p < 0.05$).

resistant to breakdown over time than those associated with F3 and/or that compounds associated with F2 had fairly consistent loading to Pohang Harbor since ca. 1951 while loading of compounds associated with F3 has increased.

H4IIE-luc responses to sediment core extracts and/or fractions were not significantly correlated with the concentrations of target analytes measured in those extracts (Koh et al., 2006). Based on mass-balance analysis, vertical profiles of TEQs (sum of TEQ_{PCB} and TEQ_{PAH}) were unlikely to account for the magnitude of bioassay response observed. Considering the great concentrations of $TEQ_{PCDD/DF,}$ up to 1040 pg $TEQ_{PCDD/DF}$/g dry wt., in the upper Hyeongsan River area (Koh et al., 2006), more potent AhR-active compounds such as PCDDs/DFs may be responsible for the significant dioxin-like activity associated with the sediment core, extracts, particularly for F2.

Only two of the five core section REs (0–2 cm; 4–6 cm) induced significant estrogenic responses in the MVLN assay (Fig. 4). Mean RE responses were not significantly correlated with concentrations of APs or PAHs detected in the core sections, and the historical trend or lack there of could not be readily explained. Following fractionation, F2 and some F3 samples caused significant MVLN induction (Fig. 4). As for the other sediment samples, F1 results for the sediment core did not meet quality control criteria and were unusable. However, as observed for surficial sediments, F2 samples induced the greatest estrogenic responses (Fig. 4). No clear trend with sediment depth was apparent, although compounds associated with F2 of the 6–8 cm core section (ca. 1961–1970) induced significantly less estrogenic activity than those associated with the other sections. Although responses were rather weak, older sediments appeared to have greater concentrations of estrogenic compounds associated with F3 than the more recently deposited sediment (Fig. 4).

Overall, the profile of responses to fractionated sediment core extracts was similar to the profiles observed for surficial sediments in most Korean coastal areas studied to date. F1 produced no significant dioxin-like responses, while F2 samples induced the greatest response, followed by F3. This result suggests a consistent trend both in space, comparing different Korean coastal locations (Khim et al., 1999c, 2001; Koh et al., 2002), and in time, as indicated by the core results. Furthermore, the vertical profile of the dioxin-like and estrogenic activities in the sediment core was similar to those associated with a Tokyo Bay sediment core in terms of depth profile and greatest contribution of F2 to total REs response both in H4IIE-luc and MVLN cell bioassay (Kannan et al., 2000). Given this consistent trend, it seems reasonable to focus future bioassay-directed fractionation work aimed at dioxin-like and/or estrogenic toxicant identification on finer fractionation of F2 and possibly F3.

4. Conclusions

Results of this study contribute to an ongoing effort to characterize organic contaminations in Korean coastal areas using instrumental analysis and mechanism-specific in vitro bioassays. Based on the initial screening of the REs, most of the sediment samples showed significant dioxin-like activity in H4IIE-luc bioassay. Analysis of Florisil fractions indicated that compounds present in F2 and F3 fractions were responsible for the significant reporter gene expression in H4IIE-luc bioassay. About half of the REs showed significant estrogenic activity in the MVLN bioassay and most F2 samples induced significant responses, while F1 and F3 samples appeared less efficacious. Although some samples contained sufficient instrumentally derived TEQs or EEQs to elicit a significant response in the bioassay, measured concentrations of target organic compounds did not account for all the activities of REs or FEs associated with Yeongil Bay sediments. A qualitative mass balance analysis suggested the presence of other unidentified dioxin-like and/or estrogenic compounds in F2 or F3. However, bioassay responses of REs and three FEs analyzed in this study showed a pattern that was consistent with other studies of Korean coastal sediment contamination, suggesting a similarity in chemical composition of organic contamination in the Korean coastal environment. Overall, the results suggest that more elaborate characterization of compounds associated with F2 should improve future understanding and monitoring of dioxin-like and estrogenic contamination associated with Korean coastal sediments.

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