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Characterization and Distribution of Trace Organic Contaminants in Sediment from Masan Bay, Korea. 2. In Vitro Gene Expression Assays

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Extracts of sediment collected from Masan Bay, Korea were fractionated using Florisil columns. Fractions were screened for their ability to induce estrogen- and dioxinlike gene expression in vitro using MVLN and H4IIE-luc cells, respectively, both before and after acid treatment. Florisil fraction 1 (F1), which was shown to contain polychlorinated biphenyls, induced very little response in either assay. The midpolarity Florisil fraction (F2) was the most active fraction. Twenty-seven of 28 F2 samples induced significant estrogenic activity, and all 28 samples induced significant dioxinlike activity. Twelve of the F2 samples produced magnitudes of response in the dioxin-responsive H4IIE-luc cells similar to those induced by a 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard. Acid treatment did not markedly diminish the activity of F2 samples. These results suggested the presence of unidentified, acid stable, aryl hydrocarbon receptor (AhR) and estrogen receptor (ER) agonists in F2. Twenty-three of the 28 most polar florisil fractions (F3) were either cytotoxic or caused morphological changes in estrogen-responsive MVLN cells, while over half of the F3 samples caused similar effects in H4IIE-luc cells. Empirical evidence suggested that acid labile compounds contributed to both the estrogenic and cytotoxic responses of the MVLN cells. Mass balance suggested that known concentrations of alkylphenols and bisphenol A may account for a portion of the estrogenic response but were not great enough to account for the toxicity. Acid labile compounds also contributed substantially to the dioxinlike activity of F3 samples. This adds to a growing body of evidence which suggests the presence of unidentified, relatively polar, aryl hydrocarbon receptor agonists in sediment from some areas.

Introduction

In recent years, there has been increasing concern, worldwide, over compounds in the environment which may adversely affect reproduction, development, immune function, and cancer formation in humans and wildlife (*1–3*). A variety of organic contaminants including polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, halogenated aromatic hydrocarbons (HAHs) such as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs), and potential xenoestrogens such as nonylphenol (NP), octylphenol (OP), and bisphenol A (BPA) have been either directly linked to or have shown potential to cause such adverse effects (*4–17*). This study examined the concentrations and spatial distribution of such compounds in sediment collected from 28 locations on Masan Bay, Korea. Masan Bay, located on the south coast of Korea, is a long and narrow inlet of a semiclosed bay, which receives industrial and municipal wastewaters from Masan and Changwon cities. As part of this study, Florisil fractions of sediment extracts were analyzed for total organic carbon (TOC), NP, OP, BPA, organochlorine pesticides (hexachlorobenzene [HCB], hexachlorocyclohexanes [HCHs], chlordanes [CHLs], and DDTs), individual PCB congeners, and 16 PAHs using instrumental analyses (*18*).

In addition to instrumental analysis, two in vitro bioassays were employed to screen extracts for compounds able to elicit estrogen- or dioxinlike responses. The first used MVLN cells (*19, 20*) to screen extracts for compounds which can modulate gene transcription through an estrogen receptor (ER)-mediated mechanism. The second utilized H4IIE-luc cells (*21*) to screen for compounds capable of modulating aryl hydrocarbon receptor (AhR)-mediated gene expression. Aliquots of some Florisil fractions were treated with concentrated sulfuric acid (H_2SO_4), and bioassays were performed to compare responses before and after acid treatment. Association of bioassay responses with specific Florisil fractions and acid treated or nontreated aliquots was used to identify characteristics of the compounds associated with mechanism specific biological responses. Additionally, the estrogen- or dioxinlike potency of many of the target compounds analyzed in this study, relative to a 17-β-estradiol (E2) or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard, has been characterized previously (*21–25*). This permitted a mass balance analysis (*26*) to be conducted. Bioassay results were compared to predicted responses, based on published relative potencies for the target compounds and the analytical results (*18*). Together, the toxicity identification evaluation (TIE) and mass balance approaches were used to develop hypotheses regarding which specific compounds or classes of compounds were responsible for ER or AhR mediated activity observed. The use of bioassay-based TIE and mass balance approaches was important, since the sediment extracts may contain a myriad of potentially bioactive compounds, which were not analyzed for using instrumental methods.

Materials and Methods

Sample Fractionation. A detailed description of the sample collection, extraction, and fractionation procedure was provided previously (*18, 27*). Spike-recovery tests demonstrated that the target analytes for this study were separated into three distinct Florisil fractions (Table 1). Although nontarget compounds (unidentified components of the sediment extract) were expected to partition to the three Florisil fractions based on polarity, such separation could not be confirmed for nontarget compounds. Spike-recovery tests with PCDDs/DFs analyzed with high-resolution mass spectrometry (HRMS) suggested that they eluted in F2 and F3, despite their relative nonpolarity. PCDDs/DFs were not quantified for the samples analyzed in this study, however.

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TABLE 1. Target Analytes for Instrumental Analysis of Florisil Fractions of Masan Bay Sediment Extracts, Based on Spike Separation/Recovery Tests ($n = 3$)

F1	F2	F3
PCBs[a]	PAHs[c]	NP[f]
HCB[b]	p,p'-DDD, p,p'-DDT	OP[g]
p,p'-DDE	HCHs[d]	BPA[h]
	CHLs[e]	

[a] PCBs = polychlorinated biphenyls, 98 individual congeners. [b] HCB = hexachlorobenzene. [c] PAHs = polycyclic aromatic hydrocarbons, 16 priority components. [d] HCHs = α- + β- + γ-hexachlorocyclohexanes. [e] CHLs = α- + γ-chlordanes. [f] NP = nonylphenol. [g] OP = octylphenol. [h] BPA = bisphenol A.

Thus, to help address the potential contribution of PCDDs/DFs and other unknowns to bioassay responses, some F2 and F3 samples were treated with concentrated H_2SO_4 to separate the effects of acid stable compounds such as PCDDs/DFs from those of acid labile compounds such as PAHs and alkylphenols (APs). Selected F2 and F3 samples were mixed with concentrated H_2SO_4 (1:1 sample:acid) for 1 h. The solvent layer was then rinsed with Nanopure water (1:1 water:sample) to remove H_2SO_4 prior to bioassay analysis. The samples selected represent those for which sample volumes sufficient for acid-treament and reanalysis were available.

Cell Culture and Bioassay. H4IIE-luc cells are rat hepatoma cells which were stably transfected with a luciferase reporter gene under control of dioxin-responsive elements (DREs) (*21*). 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* vitellogenin A2 gene (*19, 20*). Culturing conditions for both cell lines have been described previously (*27*). Cells for bioassay were plated into the 60 interior wells of 96-well culture plates (250 μL per well) at a density of approximately 18 000 cells per well. The 36 exterior wells of each plate were filled with 250 μL culture media. Cells were incubated overnight, prior to dosing. Test and control wells were dosed with 2.5 μL of the appropriate extract or solvent. Blank wells received no dose. A minimum of three control wells and three blank wells were tested on each plate. Samples were also tested using three replicate wells. Luciferase and protein assays (*28*) were conducted after 72 h of exposure. Detailed methods for the H4IIE-luc and MVLN in vitro bioassays have been described elsewhere (*21, 27, 29*).

Bioassay Data Analysis. Protein content per well was calculated by regression against a bovine serum albumin standard curve. Total protein in the wells was used as an index of cell number to detect outliers that were not apparent by simple visual inspection. Relative luminescence units (RLU) were not adjusted for protein.

Sample responses, expressed as mean RLU (three replicate wells), were converted to a percentage of the mean maximum response observed for standard curves generated on the same day (%-E2-max. and %-TCDD-max. for 17-β-estradiol and TCDD standards, respectively). This was done to normalize responses for day-to-day variability in response magnitude. The mean solvent control response (RLU) was subtracted from both sample and standard responses (RLU) on a plate-by-plate basis, prior to conversion to a percentage, to scale values from 0 to 100%-standard-max. Significant responses were defined as those outside the range defined by three times the standard deviation (expressed in %-standard-max.) of the mean solvent control response (0%-standard-max.).

Mass Balance Analysis. Most samples analyzed in this study yielded submaximal responses (relative to TCDD or E2 standards) at the greatest dose which could be tested (2.5 μL nondiluted extract per well). As a result, a complete dose–response relationship, covering a range of responses from maximal activity to baseline, could not be generated for most of the sediment extracts. This is a common limitation for environmental samples due to lack of control over sample composition, limited sample size, and the need to conserve sample volume. Thus, mass balance analysis was based on predicted magnitude of sample response, rather than predicted potency. 17-β-Estradiol or TCDD equivalents (EEq or TEQ, respectively) per g sediment were calculated by multiplying chemical concentrations by published relative potencies (RP) or toxic equivalency factors (TEFs) and summing them for each sample. Regression against the linear portion of an E2 or TCDD standard curve (RLU plotted as a function of log-dose) was used to estimate the response (converted to %-E2-max. or %-TCDD- max.) expected. Observed responses were then compared to predicted responses.

Results and Discussion

Limitations of the Mass Balance Analysis. The mass balance analysis conducted as part of this study provided useful insights into the mechanism specific biological activity of Masan Bay sediment extracts but should be interpreted carefully. Mass balance does not provide conclusive evidence for a cause-effect relationship. EEq or TEQ estimates generated are only as good as relative potency or TEF values upon which they are based. Although consensus values for the relative potencies of the most active PCDD/DFs and PCBs have been established (*30*), the database of relative potency values for PAHs and estrogenic compounds is currently limited. Thus, some of the mass balance analyses conducted as part of this study were based on a relatively limited number of compounds. Furthermore, at present, the representiveness and reliability of published relative potencies for PAHs, APs, and BPA is uncertain. Additional characterization of these compounds using a wide variety of bioassay systems is needed to evaluate and/or confirm the values currently in the literature. The mass balance analyses conducted as part of this study assumed that the complex mixtures tested would respond as if they were simply a dilution of the standard compound (equal efficacy and slope). This is rarely the case, however. Thus, violation of this assumption may produce some error in estimates (*31, 32*). Mass balance analysis also assumes additivity. Nonadditive interactions between both known and unknown components of the samples could lead to erroneous conclusions. Finally, the variability of predicted and observed responses was not considered. Therefore, the mass balance analyses conducted as part of this study should be considered qualitative. It served as a useful basis for discussion and hypothesis formulation, but conclusions based soley on mass balance should be considered tentative until supported or rejected using appropriate experimental methods.

Estrogenlike Activity. Florisil fractions F1, F2, and F3 from each of the 28 sediment extracts were screened for their ability to promote ERE-mediated gene expression in MVLN cells (Figure 1). Only two of the 28 F1 samples tested elicited a significant increase in luciferase expression (Figure 1), and all responses were less than 25%-E2-max. No target xenoestrogens were expected to elute in F1 (Table 1). Thus, the lack of estrogenic response in most F1 samples was expected and consistent with the polarity of estrogen agonists.

F2 samples were much more active than F1. F2 samples from all locations except location 2 caused a significant increase in ERE-mediated luciferase expression (Figure 1). Response magnitudes up to 47%-E2-max. were observed. Acid treated F2 samples generally elicited a magnitude of response similar to that obtained for the untreated sample (Figure 2a). This suggested that acid stable compounds probably accounted for the majority of the activity observed.

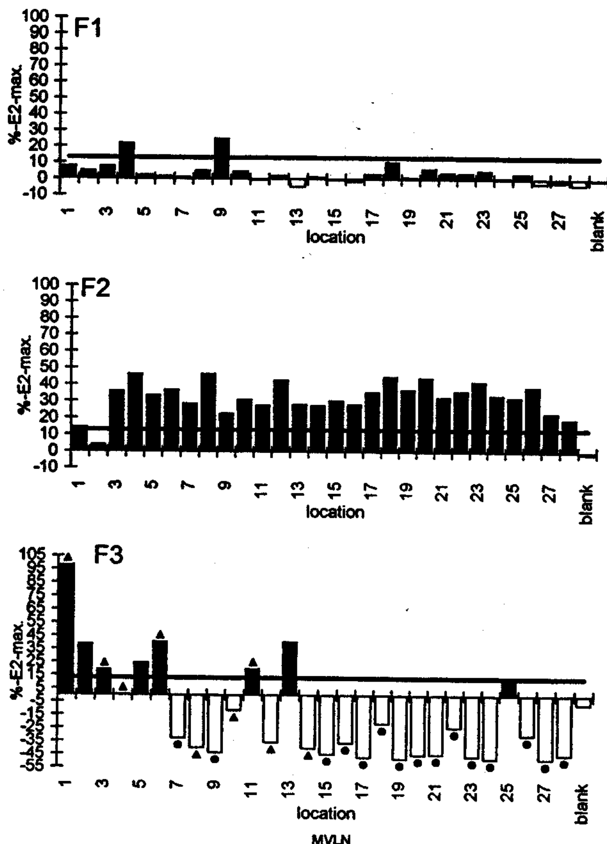

FIGURE 1. Luciferase induction in the MVLN cell bioassay (estrogen responsive) elicited by Masan Bay sediment extract fractions 1, 2, and 3 (F1, F2, F3) and procedural blank. Response magnitude presented as percentage of the maximum response observed for a 1000 pM 17-β-estradiol standard (%-E2-max.). Horizontal line equals 3 SD (expressed in %-E2-max.) above the mean solvent control response (set to 0%-E2-max.). ▲ indicates cells exhibited an altered or "stressed" morphology. ● indicates the sample was toxic to the cells.

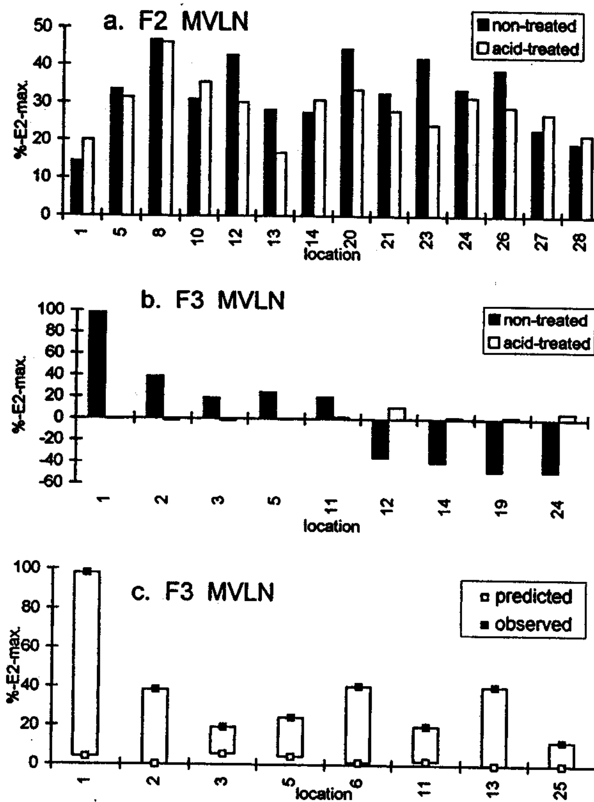

FIGURE 2. a, b. Comparison of observed MVLN cell bioassay (estrogen responsive) responses to sulfuric acid-treated and nontreated aliquots of Masan Bay sediment extract fractions 2 and 3 (F2, F3). c. Comparison of observed luciferase induction in the MVLN bioassay and predicted responses based on concentrations of target estrogenic compounds detected in F3 samples (*18*). F3 target compounds were nonylphenol (NP), octylphenol (OP), and bisphenol A (BPA). Response magnitude presented as percentage of the maximum response observed for a 1000 pM 17-β-estradiol standard (%-E2-max.).

Organochlorine (OC) pesticides including HCHs, DDTs, and CHLs were detected in F2 samples (*18*). Pesticides such as toxaphene, chlordecone, endosulfan, and *p,p'*-DDT, which should have eluted in F2 if present, have been reported to cause weak estrogenic responses in vitro (*33*). Such responses were reported at concentrations generally exceeding 1 μg/g (*33*). The concentrations of target OC pesticides (Table 1) in F2 of Masan Bay sediment extracts were all less than 15 ng/g (*18*), and they were not correlated with the magnitude of induction (R^2 = 0.0842). Furthermore, of the pesticides mentioned above, only HCHs, DDTs, CHLs, and toxaphene are stable in acid. These results suggest that the target OC pesticides and known estrogenic pesticides, with the possible exception of toxaphene, did not contribute to the estogenic responses observed for F2.

There is some evidence to suggest that PAHs may have contributed to the estrogenic activity observed. Three PAHs, chrysene (CH), benz[*a*]anthracene (BaA), and benzo[*a*]pyrene (BaP), have been shown to elicit estrogenlike responses in an in vitro gene expression assay (*23*). All three of these PAH congeners were detected in F2 samples (*18*). EEq estimates for F2 samples, based on published relative potency estimates (*23*) for CH, BaA, and BaP ranged from 3 to 170 pg EEq/g dry wt (0.56–31 fmol in well). Based on regression against an E2 standard curve, such concentrations of EEq would be great enough to elicit response magnitudes as great as 70%-E2-max. PAHs would be destroyed by acid treatment, however. Thus, although active PAHs may have contributed to the responses observed, the empirical evidence does not support the hypothesis that they alone account for the estrogenic responses elicited by F2 samples.

F3 samples were expected to have the greatest estrogenic activity. All the target xenoestrogens analyzed in this study were shown to elute in F3 (Table 1). Only 25% of the F3 samples significantly increased luciferase expression, however (Figure 1). Seventeen of the 28 F3 samples elicited luciferase expression which was significantly lower than control levels (Figure 1). In all cases, however, the reduction in response corresponded to visual observations of cytotoxicity or altered (stressed) morphology relative to the control (Figure 1). Four of the seven active samples also exhibited altered (stressed) morphology or partial cytotoxicity.

Acid treatment eliminated the estrogenic activity of previously active F3 samples (Figure 2b). Furthermore, acid treatment appeared to alleviate the cytotoxic effect of the F3 extracts as evidenced by return of assay response to baseline (Figure 2b) and a complete lack of visual signs of stress or toxicity for cells exposed to acid treated F3 extracts. These results suggest that both the ER agonists and cytotoxic compounds present in F3 were acid labile.

In previous studies, concentrations greater than 50 μM (which would equate to $\approx$55μg/g dry wt) NP or 10 μM OP ($\approx$10 μg/g dry wt) were shown to be toxic to MVLN cells (Villeneuve et al. unpublished data). BPA concentrations up to 50 μM were not cytotoxic to MVLN cells (Villeneuve et al. unpublished data). Based on that data, APs and BPA alone were not present at sufficient concentrations to account for the cytotoxicity observed. This suggests that other, acid labile,

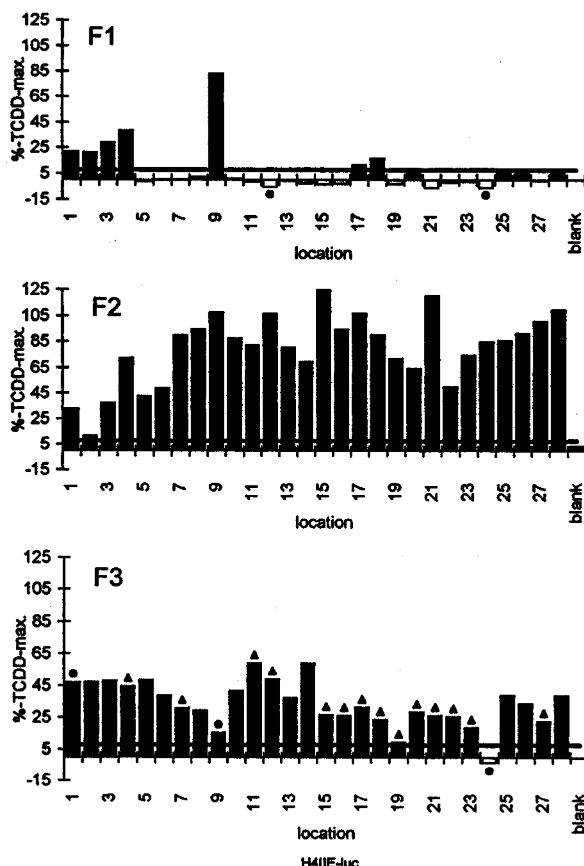

FIGURE 3. Luciferase induction in the H4IIE-luc cell bioassay (dioxin responsive) elicited by Masan Bay sediment extract fractions 1, 2, and 3 (F1, F2, F3) and procedural blank. Response magnitude presented as percentage of the maximum response observed for a 2000 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin standard (%-TCDD-max.). Horizontal line equals 3 SD (expressed in %-TCDD-max.) above the mean solvent control response (set to 0%-TCDD-max.). ▲ indicates cells exhibited an altered or "stressed" morphology. ● indicates the sample was toxic to the cells.

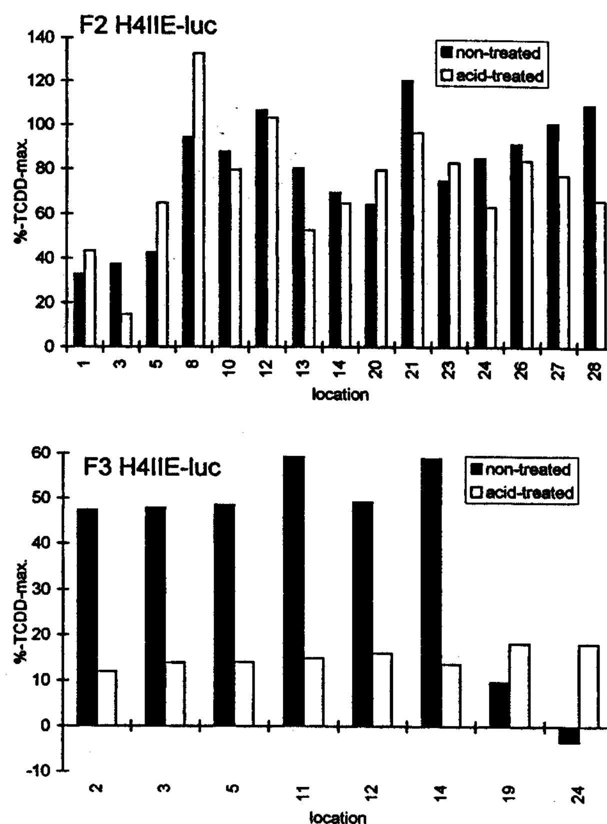

FIGURE 4. Comparison of observed H4IIE-luc cell bioassay (dioxin responsive) responses to sulfuric acid-treated and nontreated aliquots of Masan Bay sediment extract fractions 2 and 3 (F2, F3). Response magnitude presented as percentage of the maximum response observed for a 2000 pM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin standard (%-TCDD-max.).

relatively polar cytotoxic compounds were present in F3 extracts from most locations.

Estrogenic potencies, relative to an E2 standard, for luciferase expression in MVLN cells have been reported to be 1.25×10^{-5}, 1.9×10^{-5}, and 1.6×10^{-6} for NP, OP, and BPA, respectively (*15*). Based on these relative potencies, EEq contributed by NP, OP, and BPA ranged from 1.6 to 52 pg EEq/g dry wt. Based on regression against an E2 standard curve, 52 pg EEq/g dry wt was predicted to yield a response equivalent to 47%-E2-max. This suggested that the known concentrations of NP, OP, and BPA may be able to account for the significant positive responses observed. All samples which yielded a significant positive response (locations 1, 2, 3, 5, 6, 11, 13, 25) elicited a response that was greater than predicted (Figure 2c). Furthermore, there was no positive correlation between %-E2-max. and the concentration of NP, OP, and BPA for the F3 samples that gave a positive response ($R^2 = -0.215$). This suggests that, although NP, OP, and BPA may have been present in some samples in sufficient quantity to elicit a significant estrogenic response, other unidentified acid labile components of the complex mixture may have been modulating the activity as well.

Dioxinlike Activity. Florisil fractions F1, F2, and F3 of each of the 28 sediment extracts were screened for their ability to promote DRE-mediated gene expression in H4IIE-luc cells (Figure 3). Highly nonpolar HAHs such as PCDDs/DFs and PCBs are known to be potent AhR agonists (*4, 6, 30*). Spike recovery confirmed that PCBs eluted in F1 (Table 1). Little dioxinlike activity was observed for F1 samples, however. Only seven of the 28 F1 samples elicited a significant increase in luciferase expression (Figure 3). F1 of sample 9 yielded a response magnitude of 83%-TCDD-max. All other F1 samples yielded less than 40%-TCDD-max. F1 samples 12 and 24 were toxic to the H4IIE-luc cells. These bioassay results lend to a hypothesis that concentrations of AhR-active PCBs in Masan Bay sediments were relatively low.

Congener specific PCB analysis found total concentration of PCBs to be as great as 148 ng/g dry wt (*18*). TEQ_{PCB} were calculated for the F1 samples using congener specific PCB concentrations and previously reported H4IIE-luc-specific relative potencies (RPs) for PCBs 77, 81, 105, 118, 126, and 169 (*21*) and H4IIE-wild-type RPs for PCBs 156 and 167 (*22*). TEQ_{PCB} estimates ranged from 0.22 to 1.4 pg TEQ_{PCB}/g dry wt. Based on regression against a TCDD standard curve, the TEQ_{PCB} would not have been sufficient to induce a significant response in the H4IIE-luc assay (Figure 4). This supports the empirical results which suggested that PCBs were probably not responsible for the dioxinlike responses elicited by F1 samples.

All F2 samples, except the blank, elicited significant DRE-mediated luciferase expression (Figure 3). Magnitudes of induction were as high as 125%-TCDD-max., and 12 samples exceeded 90%-TCDD-max. Several PAHs, including benzo[*k*]fluoranthene (BkF), BaP, benzo[*b*]fluoranthene (BbF), CH, anthracene (A), and dibenz[a,h]anthracene (DBahA), have been shown to upregulate AhR-mediated gene expression and/or induce cytochrome P4501A1 activity in vitro (*23–25, 34*). F2 of Masan Bay sediment extracts contained detectable levels of these AhR-active PAHs (*18*), and H4IIE-luc responses were strongly correlated with log total PAH concentration

($R^2 = 0.837$). Relative potency values reported by Clemons et al. (*23*) were used to estimate TEQ contributed to F2 samples by PAHs (TEQ_{PAH}). TEQ_{PAH} estimates for F2 ranged from 240 to 3860 pg TEQ_{PAH}/g dry wt. Based on regression against a TCDD standard curve responses ranging from 70%- to 110%-TCCD-max. were predicted. This, mass balance evidence supported the hypothesis that concentrations of AhR-active PAHs present in F2 samples could account for the magnitude of response observed.

H4IIE-luc bioassay of acid treated F2 extracts contradict this hypothesis, however. The response magnitudes induced by acid-treated extracts were very similar to those induced by nontreated (Figure 4). This suggests that the contribution of PAHs to the activity of F2 was probably minor to negligible. None of the known constituents of F2 (target analytes) are known to be acid stable AhR-agonists. Thus, the empirical results suggest that PCDDs/DFs or other acid stable AhR-agonists in F2 accounted for the majority of the activity observed.

Previous studies have detected PCDDs/DFs in Masan Bay sediment (*35*). Total PCDDs ranged from 59 to 1190 pg/g dry wt, while total PCDFs ranged from 43 to 5673 pg/g dry wt (*35*). Using International TEFs (*4*), the samples were found to contain between 1 and 76 pg international TEQs (I-TEQ)/g dry wt (*35*). Although I-TEFs are not specific for the H4IIE-luc assay, they do not differ markedly from the H4IIE-wild type and H4IIE-luc-specific RPs reported for various PCDD/DF congeners (*21, 22*). Thus, the I-TEQ estimates were reasonable for qualitative mass balance purposes. Assuming this range was relevant for the spatial and temporal context of this study, PCDD/DF concentrations may have been great enough to elicit a response as great as 66%-TCDD-max. This supports the hypothesis that PCDD/DF contamination in sediment at some locations in Masan Bay may explain the significant H4IIE-luc induction observed. It does not rule out the possiblity that unidentified acid-stable AhR-agonists in F2 also contributed to the responses, however.

Nearly all F3 samples elicited significant luciferase activity in H4IIE-luc cells (Figure 3), but the magnitude of induction was less than that elicited by F2 samples. Based on regression against the TCDD standard curve, F3 samples were estimated to contain 0.7–48 pg bioassay derived TCDD equivalents (TCDD-EQ) per g dry wt. None of the target compounds detected in F3 (Table 1) have been shown to be AhR-active compounds. Thus, the known composition of F3 does not explain the dioxinlike responses. Furthermore, F3 samples treated with acid generally showed a decrease in their magnitude of dioxinlike activity (Figure 4). Acid treated F3 samples were estimated to contain 4–6 pg TCDD-EQ per gram dry wt. This suggests that acid labile, rather than acid stable compounds such as PCDDs/DFs, probably account for much of the activity observed. Thus, the H4IIE-luc results for F3 suggested the presence of unidentified, relatively polar, acid labile AhR-agonists in the Masan Bay sediment extracts.

Recent studies have suggested that the AhR may be capable of binding a wider range of structures than previously suspected (*36*). The responses observed in this study were not without precedent. Similar magnitudes of dioxinlike activity were elicited by F3 of extracts of sediment from Lake Shihwa, Korea (*27*). These observations add to a growing body of evidence which suggests that there may be unidentified, relatively polar, AhR-active compounds in sediment from some areas.

Over half of the F3 samples caused visible stress or toxicity to the H4IIE-luc cells (Figure 3). The toxicity of NP, OP, and BPA to H4IIE-luc cells has not been determined but should not differ markedly from the toxicity of these compounds to MVLN. H4IIE-luc cells are generally more tolerant than MVLN. The H4IIE-luc results support the MVLN results which suggested the presence of unidentified, relatively polar, cytotoxic compounds present in F3 extracts from most Masan Bay locations.

Role of Bioassays. The results of this study support the utility of in vitro bioassays in characterizing the occurrence and distribution of potentially adverse compounds in the environment. Empirical bioassay results and mass balance analyses suggested that the target compounds quantitated by instrumental analysis accounted for only a portion of the mechanism specific biological activity of Masan Bay sediment extracts. Risk assessment based soley on the instrumental results may underestimate the potential hazard of Masan Bay sediment contamination. Although in vitro bioassay results cannot be directly extrapolated to determine the risk for adverse effects on Masan Bay biota, they point out additional sources of uncertainty which should be considered.

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