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Article

Multiple Bioassays and Targeted and Nontargeted Analyses to Characterize Potential Toxicological Effects Associated with Sediments of Masan Bay: Focusing on AhR-Mediated Potency

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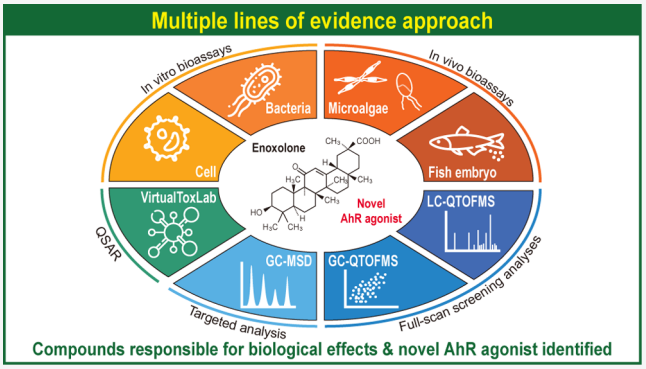
ABSTRACT: An enhanced, multiple lines of evidence approach was applied to assess potential toxicological effects associated with polluted sediments. Two in vitro bioassays (H4IIE-*luc* and *Vibrio fischeri*) and three in vivo bioassays (microalgae: *Isochrysis galbana* and *Phaeodactylum tricornutum*; zebrafish embryo: *Danio rerio*) were applied. To identify causative chemicals in samples, targeted analyses (polycyclic aromatic hydrocarbons (PAHs), styrene oligomers (SOs), and alkylphenols) and nontargeted full-scan screening analyses (FSA; GC- and LC-QTOFMS) were performed. First, great AhR-mediated potencies were observed in midpolar and polar fractions of sediment extracts, but known and previously characterized AhR agonists, including PAHs and SOs could not fully explain the total potencies of samples. Enoxolone was identified as a novel AhR agonist in a highly potent sediment fraction by use of FSA. Enoxolone has a relative potency of 0.13 compared to benzo[*a*]pyrene (1.0) in the H4IIE-*luc* bioassay. Nonylphenols associated with membrane damage that influenced the viability of the microalgae were also observed. Finally, inhibitions of bioluminescence of *V. fischeri* and lethality of *D. rerio* embryos were strongly related to nonpolar compounds. Overall, the present work addressed assay- and end point-specific variations and sensitivities for potential toxicities of mixture samples, warranting a significant utility of the "multiple lines of evidence" approach in ecological risk assessment.



INTRODUCTION

Various persistent toxic substances (PTSs) are introduced into coastal ecosystems from a myriad of (non)point sources, including natural processes and anthropogenic activities.[1,2] Coastal sediments are particularly vulnerable to pollution by chemicals since they are sinks of persistent and hydrophobic toxicants.[3] Furthermore, contaminated sediments potentially influence harmful effects on not only benthic animals but also pelagic organisms through sedimentary resuspension and bioturbation processes.[4] Thus, sediment quality is crucial to maintaining the environmental health of marine organisms, warranting ecological risk assessment.[5]

Assessing chemical contamination of sediments is complicated because chemicals occur as complex mixtures and undergo both biotic and abiotic transformations.[6] The development of highly sensitive target analyses allows precise and accurate quantification of chemical contaminations in sediments.[7] However, due to technical limitations, such analyses often provide little information about the toxicity of chemical mixtures and/or bioavailability.[8,9] More importantly, chemical concentrations are not sufficient to demonstrate biological effects, because they do not provide information about the potential adverse effects on aquatic organisms.[6,10,11] For a comprehensive assessment, bioassays combined with chemical analyses are needed to infer probable adverse biological effects.[1] However, it is not practical to identify the potential toxic effects of all chemicals, primarily due to mixture effects, in the environments of aquatic organisms. Accordingly, it is necessary to develop suitable and also ecologically relevant tools for assessing multiple or combined toxicological effects associated with sedimentary pollution.[12] Recently, environmental risk assessment has been challenged to adopt a multiple lines of evidence approach, by employing effects-based methods (EBMs) along with full-scan screening analysis (FSA).[8,13] EBMs are regarded as holistic approaches to complement in vitro bioassays and can

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include a battery of in vivo bioassays targeting some important pelagic communities of fish, invertebrates, and microalgae.[8]

With respect to diagnostic methods for identifying causative toxicants in complex environmental samples, such as sediments, effect-directed analysis (EDA) is regarded as one useful tool.[14] This analysis involves progressive fractionations, which reduce the complexity of the mixture, allowing focused chemical analysis on selected fractions exhibiting significant bioactivity.[6,14,15] EDA is a suitable tool for evaluating the ecological health and environmental management of contamination sources, supporting prioritization and regulation of environmental contaminants.[14,16] However, since it does not provide information on all bioactive fractions and/or end points-specific variations or sensitivity of applied bioassays, such targeted chemical analysis remains limited.[17,18] Alternatively, to fill the gaps, FSA is frequently practiced by use of high-resolution mass spectrometry, such as time-of-flight mass spectrometry (TOFMS) and Orbitrap ultrahigh-resolution mass spectrometry, which can detect untargeted compounds and potentially toxic substances in environmental mixture samples.[9,19−21] Furthermore, these techniques can provide accurate masses from which the formulas of chemicals can be determined, and putative structures can be derived by use of the MS/MS and libraries.

In the present study, EDA was conducted to identify the toxicity profile of contaminants in sediments by use of multiple bioassays. On the basis of the results of previous pollution studies in Korean coastal waters, Masan Bay was selected as a study area from which to obtain contaminated sediments. Severe pollution of sediments and associated significant toxic effects have been reported for Masan Bay since the 1990s (Figure S1 of the Supporting Information, SI).[10,18,22] Bioassays utilized in the present study cover three mechanisms of actions, including specific-, baseline-, and reactive-toxicity.[23] In vitro bioassays comprise receptor gene assays for measuring "specific toxicity" of AhR agonists, such as polycyclic aromatic hydrocarbons (PAHs) (H4IIE-*luc* bioassay), and the bacterial test (*Vibrio fischeri* assay), which is related to the energy metabolism of a bacterium, was used for "baseline toxicity" measurements.[23−25] In vitro bioassays are characterized by being rapid, cost-effective, sensitive, and reproducible assays.[26] However, despite the obvious advantages of in vitro assays for the determination of toxicological profiles of sediments, results of in vitro bioassays cannot directly predict biological responses in aquatic ecosystems.[27] Thus, in addition to in vitro bioassays, several in vivo toxicity tests, including microalgae and fish embryo, for "reactive toxicity" were conducted, which are well-suited for high-throughput analysis while keeping some advantages of in vitro assessment tools, such as the low cost, sensitivity, and shortened test period.[27] Inhibition of growth of microalgae and the fish embryo toxicity test (FET) were conducted to assess the acute toxicity with multiple end points on the primary producers and vertebrate species, respectively.[3] Each bioassay exhibits different sensitivities to chemical contaminations in sediments; therefore, the combination of a battery of bioassays provides a better assessment of the sediment contaminations.[8,28]

The present study aimed to (i) assess potential toxicological effects of polluted sediments using a battery of bioassays with various organisms and end points, (ii) measure concentrations and compositions of target PTSs (PAHs, styrene oligomers (SOs), and alkylphenols (APs)) in sediments using GC-MSD, (iii) identify untargeted AhR agonists in some potent fractions using GC-QTOFMS and LC-QTOFMS, (iv) evaluate relative potencies (RePs) of tentative AhR agonists using the H4IIE-*luc* bioassay, and finally (v) determine the relationships between chemical concentrations and/or compositions in sediments and observed in vitro and in vivo biological effects. A schematic representation of stepwise procedures of the present study is shown in Figure S2.

MATERIALS AND METHODS

Sampling and Sample Preparation. Sites S1 and S2 were located near Samho Creek and Nam Creek, respectively, which are two major rivers flowing into the Masan Bay (Figure S1). In March 2016, surface sediments (∼3 cm) were collected by use of a hand shovel. Sample preparation for bioassays and chemical analyses was conducted, with minor modifications to previously published methods.[29] In brief, sediments were freeze-dried, passed through a 1 mm sieve, and homogenized. Sediments (60 g) were extracted with 350 mL dichloromethane (DCM, J.T Baker, Phillipsburg, NJ) in a Soxhlet extractor for 16 h. Raw organic extracts were concentrated to 6 mL with a rotary evaporator and N_2 gas flow (∼10 g sediment equivalent (SEq) mL^{-1}). For bioassays, the aliquot of raw extracts was exchanged into dimethyl sulfoxide (DMSO, Sigma-Aldrich, Saint Louis, MO) to obtain a final concentration of 0.1% DMSO in the test solution (Table S1).

Raw organic extracts were fractionated in a two-step procedure (Figure S2). Step one fractionation was conducted with 4 mL raw extracts using 8 g activated silica gel (70−230 mesh, Sigma-Aldrich) in a packed glass column based on differences in polarity (F1 to F3).[29] The first fraction (F1) was collected by elution with 40 mL hexane. The second fraction (F2) was eluted with 50 mL of 20% DCM in hexane (v/v). The third fraction (F3) was eluted in 50 mL of 60% DCM in acetone (J.T Baker). If the primary fractions were significantly toxic, then a secondary fractionation step was applied using reverse-phase (RP)-HPLC (Agilent 1260 HPLC; Agilent Technologies, Santa Clara, CA). Instrumental conditions of RP-HPLC, including sampling times and log K_{ow} intervals, were presented (Table S2). Separation conditions of RP-HPLC were optimized previously through several tests with GC/MSD confirmation using 34 PCBs, 16 PAHs, 7 APs, and 5 phthalates (with log K_{ow} values).[25] Acceptable elution efficiency with all compounds was achieved (>85%).

In Vitro and In Vivo Bioassays. Two in vitro bioassays (H4IIE-*luc* and *V. fischeri*) and three in vivo bioassays (microalgae: *Isochrysis galbana* and *Phaeodactylum tricornutum*; zebrafish embryo: *Danio rerio*) were employed for testing potential toxicities of sediments. More details about the preparation of samples (or stocks), toxicity tests, and corresponding statistical analyses are presented in the SI (Table S1 and Figure S3).

Targeted Chemical Analyses. Thirty-one PTSs (including 15 PAHs, 10 SOs, and 6 APs) were analyzed in sediments, using the methods adapted from previous studies.[29,30] The full names of the target compounds and details of instrumental conditions are provided in Tables S3−S5.

Full-Scan Screening Analyses. FSA using GC-QTOFMS was performed on F2.7 and F2.8 of S2, on which the AhR-mediated potencies of the samples were great. Instrumental conditions and process of tuning are presented in Table S6. LC-QTOFMS analysis was performed with a Kintex Core-Shell C18 column to screen F3 subfractions (F3.6 and F3.7). Information on the instruments and analytical conditions are presented in Table S7. Peak View Software v.2.2 (AB SCIEX, Foster City,

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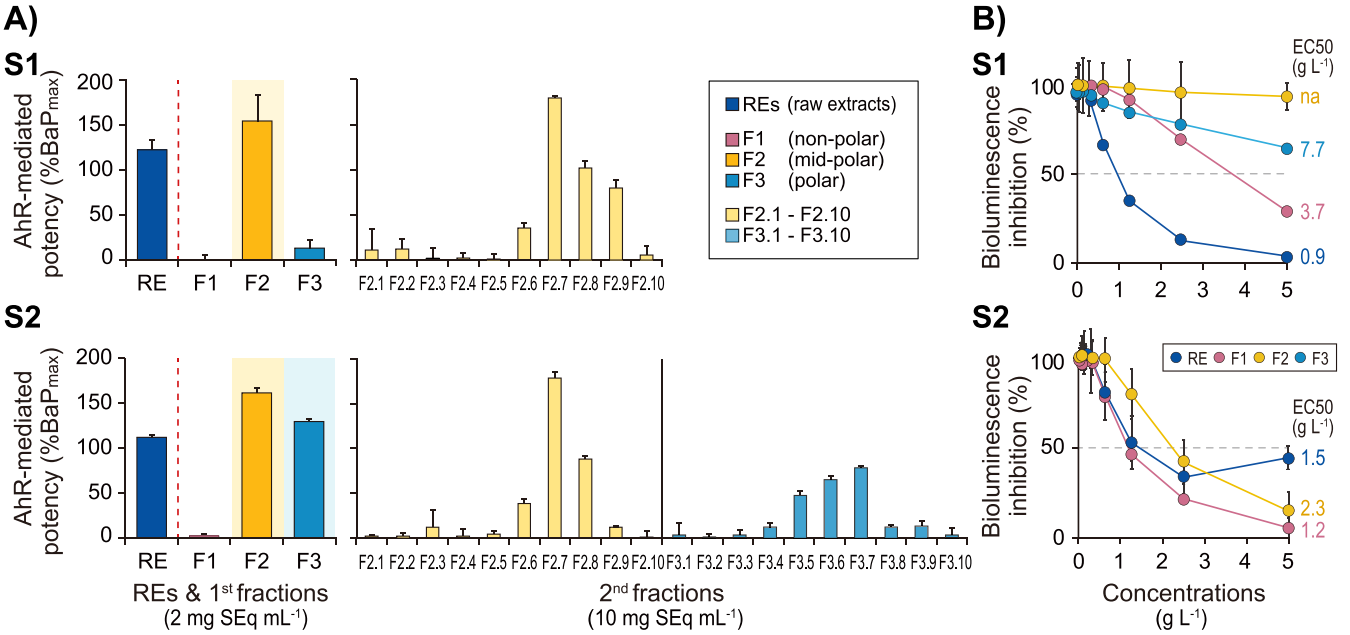

Figure 1. (A) AhR-mediated potencies of raw extracts (RE), silica gel fractions, and RP-HPLC fractions (F2.1-F2.10 and F3.1-F3.10; subfractions of F2 and F3, respectively) of S1 and S2 sediments from Masan Bay, Korea determined at 4 h exposures in the H4IIE-*luc* bioassay (Error bar: mean ± SD; $n = 3$). (B) Bioluminescence inhibition of *Vibrio fischeri* for the EC_{50} test of raw extracts and fraction samples in the sediments of Masan Bay over a 30 min period.

CA) was used to detect peaks, and peak lists were obtained from full-scan chromatograms of the samples, solvent, and processed blanks.[31] Five criteria were used to select candidates for AhR agonists based on the LC-QTOFMS analysis, and are described in the SI and Figure S4A. From the FSA, four commercially available compounds (such as 1,2-di(*p-tolyl*)ethane, flucofuron, niflumic acid, and enoxolone) were selected to confirm their AhR-mediated potencies using the H4IIE-*luc* bioassay (Figure S4B).

Toxicological Confirmation. The RePs for the AhR-mediated potencies of the four candidate compounds were determined. Each compound was prepared at six concentrations (viz., 100, 20, 4, 0.8, 0.16, and 0.032 μg mL^{-1}), and was analyzed using the method of in vitro bioassay that described above. RePs were measured based on the previous study with minor modifications.[32]

Potency Balance Analysis. Potency balance analysis was performed between instrument-derived BEQs and bioassay-derived BaP-EQs to determine how each compound contributed to total induced AhR-mediated potency. BEQ concentrations were calculated as the sum of products of measured concentrations of individual compounds multiplied by their RePs. The RePs of AhR-active PAHs are summarized in Table S4.[33,34]

VirtualToxLab In Silico Analysis. For compounds without reference compounds, tentative identification was completed using QSAR modeling. The AhR binding affinities of tentative compounds, which were identified from FSA, were simulated by VirtualToxLab. It combines automated, flexible docking with multidimensional QSAR to simulate and quantify the toxic potential and the binding of chemicals toward a series of currently implemented proteins that trigger adverse effects.[35] Details about statistical analyses are presented in the SI.

RESULTS AND DISCUSSION

AhR-Mediated Potencies. Raw extracts of sediments from both sites, S1 and S2, reached saturation efficiency (≥100% BaP_{max}) exhibited strong AhR-mediated potencies in the H4IIE-*luc* bioassay (Figure 1A). Among the three silica gel fractions (F1−F3) of raw extracts, AhR-mediated potencies were greater in F2 than those of F1 and F3. Typical AhR-active compounds in F2 reported include planar hydrophobic contaminants, such as PAHs. These chemicals have been repeatedly reported as major groups of sedimentary PTSs in the study area and elsewhere in the Korean coastal waters.[17,36−38] Although coeluting chemicals in F1 include some AhR agonists, such as hexachlorobenzene and *p,p′*-1,1-*dichloro*-2,2-bis(*p*-chlorophenyl)ethylene, these chemicals likely occur at small concentrations in the sediments of Masan Bay.[39,40]

The F2 of S1 and F2 and F3 of S2 exhibited greater AhR-mediated potencies compared to those of raw extracts. Thus, these F2 and F3 were further fractionated into 10 subfractions using RP-HPLC. Among the 10 RP-HPLC subfractions of F2 from both sampling sites, the greatest AhR-mediated potency was observed in the F2.7, followed by F2.8 and F2.6 (Figure 1A). The results indicated that these fractions contained major AhR active chemicals, which seemed to be aromatics with 5−8 log K_{ow} values. In addition, significant AhR-mediated potencies were shown in the superhydrophobic fractions such as F2.8−F2.10 of S1, of which fractions contained the 7−9 ring PAHs (≥C24-PAH).[41] Meanwhile, the S2 sediment had greater AhR-mediated potency of the F3 (Figure 1A), which indicated that polar AhR agonists (e.g., dinitro-, hydroxyl-PAHs, and N-heterocycles) were present.[9,42] For the 10 RP-HPLC subfractions of F3 from S2, considerable AhR-mediated potencies were evidenced in the F3.5−F3.7 fractions.

Inhibitions of Bioluminescence. In the screening test, nearly all fractions were acutely toxic to the bacterium, *V. fischeri* (Table S8). Only one sample, F3 of sediment extract of S2, showed a hormetic effect with a bacterial inhibition rate of −4%.

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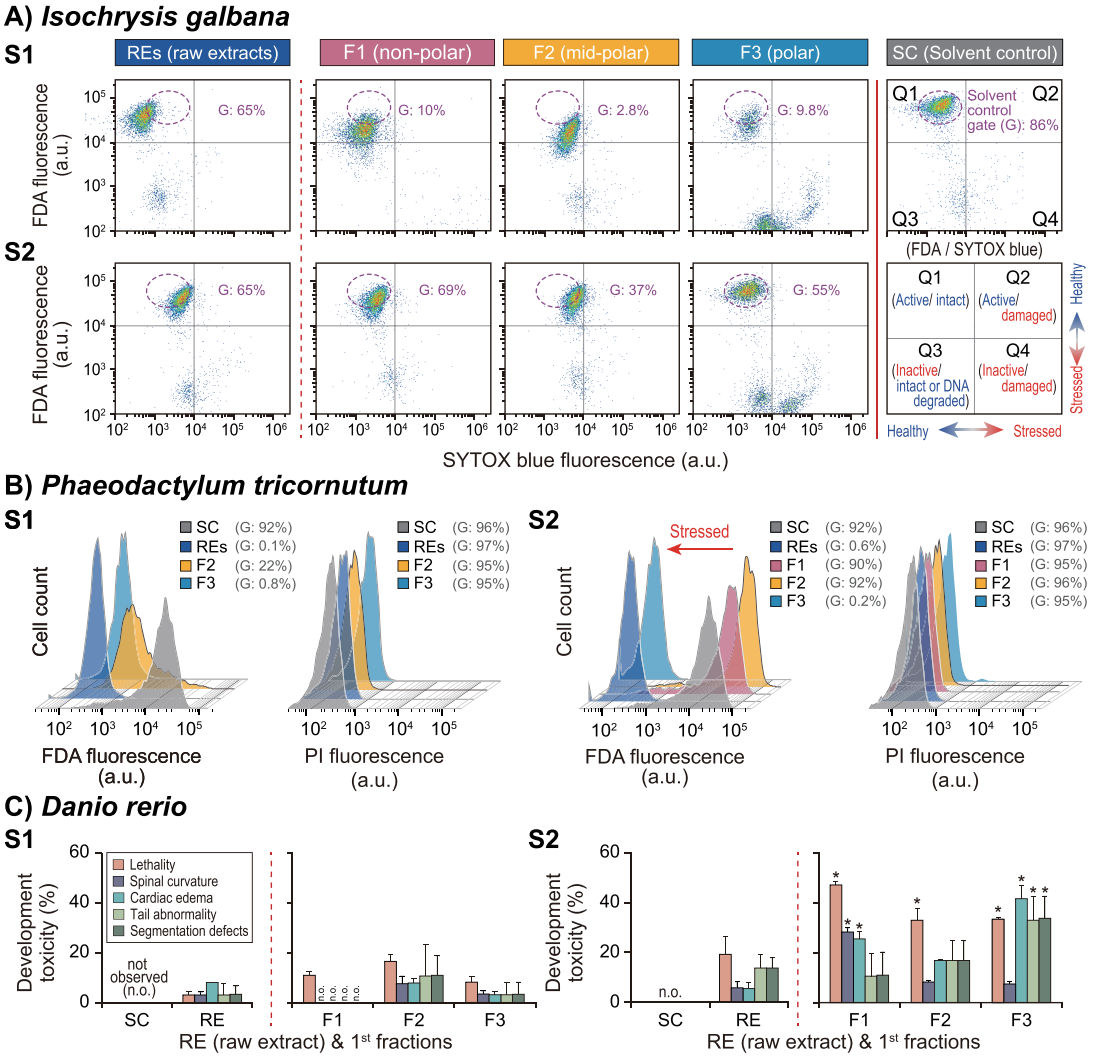

Figure 2. (A) Two-dimensional flow cytometry dot plots of *Isochrysis galbana* cell signals plotted as coordinates of FDA- and SYTOX blue-fluorescence intensity treated for 96 h with solvent control, raw extracts (REs), and silica gel fractions (F1−F3). Each dot represents one cell particle; colors denote relative particle density in each population. All cells were placed in four categories and were easily differentiated: viable FDA-unstained SYTOX blue cells (Q1; upper left quadrant, healthy cells), viable fluorescein diacetate (FDA)- stained SYTOX blue cells (Q2; upper right quadrant, membrane-damaged cells), unstained cells (Q3; lower left quadrant, inactive cells), and only stained with SYTOX blue (Q4; lower right quadrant, dead cells). (B) Fluorescence histograms of *Pheodactylum tricornutum* stained with FDA or PI following exposure to solvent control, raw extracts, and F1−F3. F1 fraction of Site 1 was not analyzed. Mean values were obtained in logarithmic scale and represented in arbitrary units (a.u.). Microalgae of solvent controls exposed gated. (C) Results of in vivo bioassay with zebrafish embryo (*Danio rerio*) exposed to solvent controls, raw extract, and fraction samples (Error bar: mean ± SD; $n = 3$). *t*-test was performed for difference between sites. Significance was determined at p values <0.05 (*).

Thus, the EC_{50} testing was performed, excluding F3 of S2. Bioluminescence of *V. fischeri* was inhibited by both raw extracts of S1 and S2; however, raw extracts from S1 (EC_{50} = 0.9 g SEq L^{-1}) showed greater inhibition compared to raw extracts from S2 (EC_{50} = 1.5 g SEq L^{-1}) (Figure 1B). Of the three silica gel fractions, the least EC_{50}s were observed in F1 for both sites. F2 of S2 also showed significantly acute toxicity (EC_{50} = 2.3 g SEq L^{-1}), while the F2 of S1 did not cause inhibition.

The greater inhibition of *V. fischeri* in F1 was likely associated with some nonpolar compounds, such as *n*-alkanes (C11−C29), and 4,4′-dichlorodiphenylsulfide being eluted in the fraction (Table S9).[25] In addition, the toxicity of F1 was suspected to be caused by aliphatic hydrocarbons containing sulfur, which are known as major toxicants to *V. fischeri* in raw extracts of sediments.[43] Even if elemental sulfur is removed in the pretreatment procedure, the sulfide compounds in sediments might have been partially removed. In the previous study, toxicity to *V. fischeri* completely disappeared after removal of sulfur.[25] Some chinoidic PAH metabolites, which frequently occur in environmental samples, are suspected as contributors to the potential toxicity in F2 (Table S9). Possible toxicants contributing to the effects of F3 are polar compounds, such as endosulfan, dimethoate, atrazine, and *n*-tributyltin (Table S9).[25,26] In particular, the NPs of known toxic chemicals to *V. fischeri* present in the F3 can be suspected to be responsible at least for certain parts of the observed adverse effects shown in S1.[44]

Overall, *V. fischeri* bioassay showed another aspect of the acute in vitro response to environmental fraction samples, compared to the H4IIE-*luc* bioassay. That is, inhibition of bioluminescence was sensitive to the increasing chain length among nonpolar molecules, while insignificant acute toxicity was observed in the H4IIE-*luc* cells.[45] This is because the Gram-

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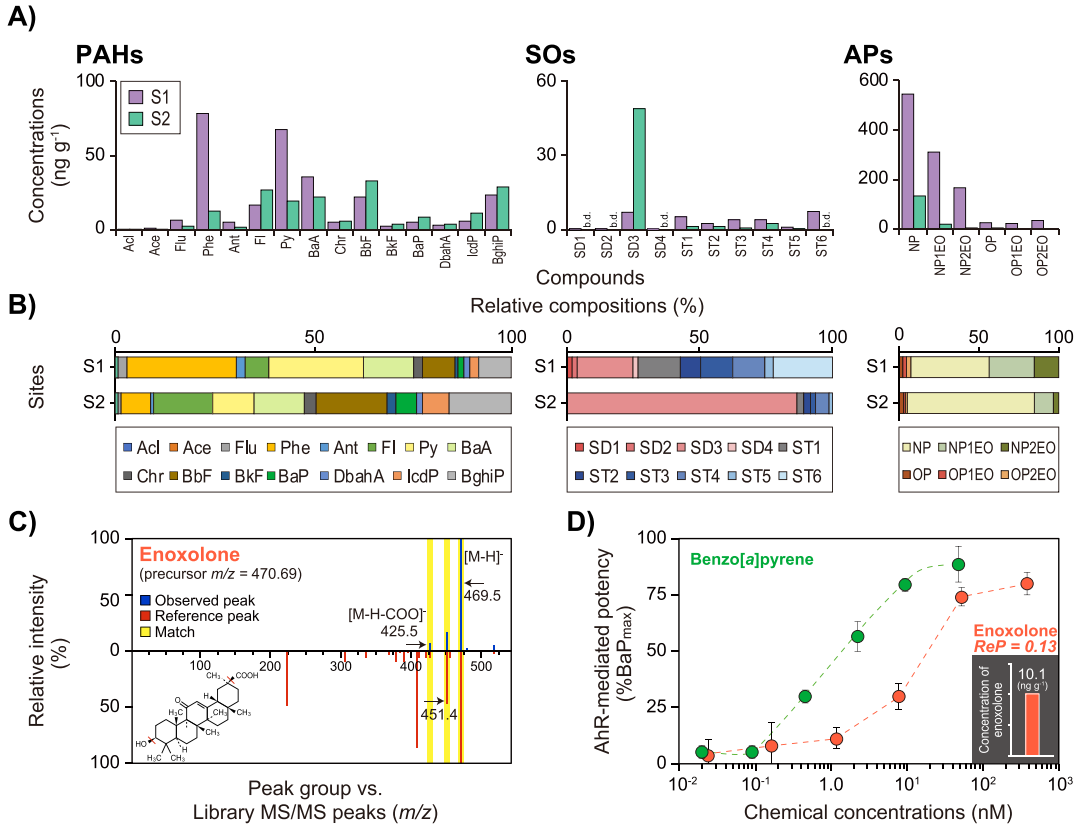

Figure 3. (A) Concentrations of targeted polycyclic aromatic hydrocarbons (PAHs), styrene oligomers (SOs), and alkylphenols and their ethoxylates (APs) in sediments from Masan Bay, Korea. Abbreviations of target compounds are shown in Tables S4 and S5. (B) Comparison of the relative composition of PAHs, SOs, and APs in sediments. (C) Molecular structure of tentative AhR agonist for toxicological confirmation and butterfly plot comparing the observed MS/MS annotated spectrum of enoxolone (m/z 469.5 → 425.5) to the library spectrum. (D) Dose−response relationships for AhR-mediated potency of enoxolne and benzo[a]pyrene in the H4IIE-*luc* bioassay (Error bar: mean ± SD; n = 3) and concentrations of enoxolone.

negative luminescent bacteria have a multilayered structure, and thus transmittance of polar molecules is likely small.[46]

Inhibition of Growth and Viability of Cells. The inhibition rates of growth of *I. galbana* were 68% and 39% in the F3 of S1 and S2, respectively (Figure S5). More toxicants that affected *I. galbana* were present in S1 compared to S2. In contrast, the numbers of algal cells of the F1 increased for both sites (Figure S5). This hormesis is a phenomenon that is defined as a stimulatory beneficial effect at low concentrations of toxic chemicals.[47] Rates of growth of *I. galbana* were significantly inhibited ($p < 0.05$), mainly by F3 from both sites, of which the fraction contained aromatic esters, such as alkylsulfonic acid phenylesters, phthalates, and aromatic amine such as *N*-phenyl-*b*-naphthalene amine.[25,48]

Previous studies analyzing toxicities of environmental samples to microalgae mostly focused on inhibition of growth.[6,12,48] In this study, the inherent properties and viability of microalgae exposed to polarity-based fraction samples were assessed by flow cytometry. *I. galbana* cultured in the presence of the F3 from S1 exhibited lesser forward scatter signals, which were related to a significant decrease in the sizes of cells ($p < 0.05$) (SI and Figure S5).[49] This sample also exhibited significantly ($p < 0.05$) less chlorophyll-*a* (Chl *a*) fluorescence, whereas the other samples exhibited 14% greater amounts of chl *a* fluorescence in comparison to control cultures. However, this second result was not statistically significant ($p > 0.05$) (Figure S5). Cell granularity responded similarly to chl *a* fluorescence.

In *I. galbana*, the least percentages of living cells (healthy cells; % solvent control gate) were observed in F2 for both sites (Figure 2A). For the cells exposed to F2 samples, the effects were observed for esterase activity rather than membrane integrity. This result was more pronounced in S1. In other words, compounds that existed in F2 caused toxicity to algae, affecting enzyme activity, but did not cause lethality. Variations in sensitivities of microalgaeenzyme activity between samples could be explained by chemical compositions since total concentrations of PAHs in S1 and S2 did not greatly differ, but corresponding homologues were different between these two samples. For example, the high proportion of phenanthrene (28%) and pyrene (24%) contributing to total concentrations of PAHs in S1 might result in significantly greater enzyme activity compared to that of S2 (both explained 17% to total PAHs) (Figure 2A,B and Table S4). This result is consistent with the previous finding that phenanthrene and pyrene adversely affect the growth and abundance of marine phytoplankton.[50] In particular, pyrene has strong phytotoxic effects on algae (average LC_{10} values for algae 2−6 $\mu g\ L^{-1}$),[51,52] affecting nutrient uptake (ammonium, nitrate, and silicate) and incorporation carbon by algal communities.[53]

Relatively high populations of *I. galbana* were observed in Q3/Q4 quadrants (by a vertical and a horizontal line based on the FDA- and SYTOX blue-fluorescence intensity from the cells) for F3 for both S1 and S2 (stressed and dead cell; details in SI) especially S1, which indicated the effect of F3 might be associated with nonylphenols (NPs). The concentration of NP

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was 5.4 μg L^{-1} in the sample exposed to microalgae. Although the exposed NP concentration was relatively low considering the effective NP concentration reported in the previous study (EC_{50} = 24.1 μg L^{-1}; calculated by growth inhibition), NP could be one potential environmental pollutant being related to membrane damage of *I. galbana* (Figure 2A). The results of previous studies have shown that NPs as well as *N*-phenyl-*b*-naphthalene amine, which elutes in F3, damaged cell membranes of algae and invertebrates, by causing oxidative stress (Table S9).[49−51] It is necessary to confirm the toxicity contributions of NPs by spiking toxicity test, which will provide a better understanding of the ecologically relevant predictions of NP's risk in the contaminated sediments.

For *P. tricornutum*, no significant effects were observed on growth, chl *a* fluorescence, cell size, and cell granularity for all of the samples (Figures 2B and S5). However, lesser esterase activity was identified by displacement of FDA fluorescence on the *x*-axis in the histogram (shift to the left) (Figure 2B). Strong inhibition of esterase activity was detected in F3 for both sites and in F2 from S1 (Figure 2B). The percentage of nonviable cells of *P. tricornutum* (measured using PI staining) was not significantly different from that of control cultures. Because *P. tricornutum* has a thicker cell layer than does *I. galbana*, effects on enzymatic activity were more pronounced than was damage to the cell membrane. In general, toxicological responses and sensitivities of Masan Bay sediments to the two species of microalgae, *P. tricornutum* and *I. galbana*, generally indicated both species- and end point-dependent toxic responses to environmental mixtures.[54]

Embryo Developmental Toxicity. Raw extracts from both sites caused significant lethality, after 96 h exposures, compared to the solvent control (Figure 2C). Exposures to fractions also caused significant developmental toxicities, with varying responses among samples and sites. Results of a previous study demonstrated significant toxic effects to this species are mainly associated with the presence of PAHs, e.g., naphthalene, phenanthrene, pyrene, and benzo[*a*]pyrene, all of which eluted in F2.[55] However, in contrast to the greater concentration of PAHs in S1, toxic effects were not significantly different between sampling sites (Figure 2C). Results indicated that the PAHs concentration in sediments from the Masan Bay is below the level that is sensitive to FET. Further study is necessary to clarify the evaluation of PAHs (dioxin-like compounds) in sediments, the analyses of CYP1A at the transcriptional and protein levels and use of a transgenic along with for developmental toxicity.[56]

However, F1 and F3 in S2 caused significantly greater lethality and developmental toxicities than those in S1. Specifically, among fractions of S2, the greatest lethality (47%) was observed in embryos exposed to F1, followed by similar levels for F3 (34%) and F2 (33%). The incidence of spinal curvature followed a similar trend to lethality; however, sublethal effects were greatest in embryos that exposed to F3. Many lipophilic neurotoxins accumulating in sediments primarily target membrane sodium channels and cause adverse effects on *D. rerio*.[57,58] In the previous study, brominated phenols and indoles showed a sensitivity of the *D. rerio* embryo test.[26] Thus, it is plausible that these chemicals, which are predominant in F3, contribute to the observed effects. Polar organic toxicants including NP and phthalates were detected in the FSA, and those are well-known to cause toxicity to fish embryos (Table S9). However, the NP concentration in S1 was greater than that of S2, indicating that NP might not actually be responsible for the observed effects (Figure 3A).

Occurrence, Distribution, and Sources of Target Analytes. All of the targeted chemicals including PAHs, SOs, and APs were detected in sediments from both sites in Masan Bay. Concentrations of PAHs were moderate to low with 280 and 180 ng g^{-1} dry mass (dm) at S1 and S2, respectively (Table S4). Phenanthrene was the most dominant PAH at S1, followed by pyrene and benzo[*g,h,i*]perylene (Figure 3A and Table S4). Phenanthrene and pyrene are widely used in industry, including the manufacture of resins, pesticides, and pigments,[59−61] some of which are highly developed in the Masan industrial region. Concentrations of PAHs in sediments from both sites did not exceed the interim sediment quality guidelines (ISQGs) suggested by the Canadian Council of Ministers of the Environment.[62] Results of recent studies have shown that concentrations of PAHs have been decreasing in sediments of Masan Bay for the past decade. This is in response to control of pollution by implementation of the Total Pollution Load Management System; thus the lesser concentrations of PAHs observed in 2016 samples were reasonable.[63]

Concentrations of SOs were also low with 35 and 56 ng g^{-1} dm at S1 and S2, respectively (Table S5). It was recently reported the historical pollution of SOs in Masan Bay sediments by analysis of 1998 archived and 2014 collected samples.[10] A drastic decrease in sedimentary concentrations of 10 SOs in the region from 1998 (mean = 4940 ng g^{-1} dm; n = 7) to 2014 (mean = 128 ng g^{-1} dm; n = 7). SOs found in the present study reflected the relatively low concentrations of recently reported SOs in Masan Bay, thus continuing input would be minimal for this group of emerging chemicals of concern at the moment.

Concentrations of APs in sediments from S2 (180 ng g^{-1} dm) were relatively low, but an elevated concentration of APs was found in the upper creek site of S1 (1100 ng g^{-1} dm), which exceeded the ISQG (Figure 3A and Table S5).[62] Indeed, the Masan Bay area has long been contaminated by APs with maximum reported concentrations of 4070 ng g^{-1} dm (NPs and octylphenols) in the lower reach of Samho Creek in 1998.[64] Thus, the relatively great concentration of APs detected in the S1 sediment reflected the continuing input of land-driven municipal sources such as surfactants into the bay.

Compositional Characteristics of Target Analytes. Concentrations of PAHs did not greatly differ between S1 and S2, but S1 sample showed relatively greater concentration (280 ng g^{-1} dm) compared to that of S2 (180 ng g^{-1} dm). While, proportions of PAHs with 4−6 rings (higher molecular mass PAHs), which include relatively strong AhR agonists, prevailed in S2. These compositional characteristics of PAHs apparently influenced great BEQs in S2 than that in S1; however, targeted PAHs can explain as much as 0.1% of total AhR-mediated potency in corresponding samples and fractions. Collectively, these results indicated presence of more unknown AhR agonists in S2. Thus, FSA was further conducted on fraction samples of S2.

There was a difference in SOs composition between S1 and S2. Among SOs, styrene trimers (STs) mainly dominated in S1, while styrene dimers (SDs), especially 2,4-diphenyl-1-butene (SD3), dominated in S2 (Figure 3B). However, concentrations of SOs were relatively small compared to other targeted chemicals. Thus, interpretation of direct association to varied responses of multiple bioassays was limited. APs showed both great variations in concentrations and compositions between S1 and S2 samples. Among 6 APs, NP dominated concentrations in sediments at both sites, but other APs showed varied proportion to total APs. Nonylphenol ethoxylates (NPEOs) dominated in

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Table 1. List of Candidates for AhR-Active Compounds in the Fraction Samples (F2.7, F2.8, F3.6, and F3.7) of Organic Extracts of S2 Sediment Using GC-QTOFMS (F2.7 and F2.8 fractions) and LC-QTOFMS (F3.6 and F3.7 Fractions) and Binding Affinity to AhR Estimated by VirtualToxLab

fraction and compounds	CAS number	molecular formula	molecular weight (g mol^{-1})	matching factor/ mass error[a]	AhR binding affinity[b]
F2.7 fraction					
2,5-dimethyl-8-(*propan-2-yl*)-1,2,3,4,4a,7,8,8a-octahydronaphthalen-2-ol	254182[c]	$C_{15}H_{26}O$	222.37	76.5	not binding
1,2-di(*p-tolyl*)ethane[d]	61558[c]	$C_{16}H_{18}$	210.31	71.3	binding
methyl 1-(*2,3,4-trifluorobenzoyl*)prolinate	462819[c]	$C_{13}H_{12}F_3NO_3$	287.23	72.5	not binding
2-(*4-biphenylyl*)-2-propanol	34352-74-4	$C_{15}H_{16}O$	212.29	72.8	binding
4-*tert*-butylbiphenyl	1625-92-9	$C_{16}H_{18}$	210.31	74.0	binding
F2.8 fraction					
2,6-diisopropylnaphthalene	24157-81-1	$C_{16}H_{20}$	212.33	83.3	binding
cis-calamenene	72937−55−4	$C_{15}H_{22}$	202.33	72.5	binding
Naproxen	22204−53−1	$C_{14}H_{14}O_3$	230.26	75.0	binding
2-[(*5-methylpyridin-2-yl*)amino]-1-phenylethanol	14140[c]	$C_{14}H_{16}N_2O$	228.29	71.9	not binding
5,7-Difluoro-3,4-dihydro-2(*1H*)-napthalenone	280105[c]	$C_{10}H_8F_2O$	182.17	71.0	binding
F3.6 fraction					
Flucofuron[d]	370-50-3	$C_{15}H_8Cl_2F_6N_2O$	417.13	1.996	failed
Niflumic acid[d]	4394-00-7	$C_{13}H_9F_3N_2O_2$	282.22	1.248	not binding
Enoxolone[d]	471-53-4	$C_{30}H_{46}O_4$	470.69	0.507	not binding
F3.7 fraction					
Scytalone	49598-85-8	$C_{10}H_{10}O_4$	194.18	0.611	not binding

[a]Mass error expressed in ppm for LC-based results. [b]Data from VirtualToxLab. [c]Chemspider ID. [d]These chemicals were confirmed for AhR-mediated potency by use of H4IIE-*luc* bioassay.

S1 compared to S2 (Figure 3B). Differences in the sensitivity for the APs enriched fraction sample, viz., F3 from S1, to in vivo bioassays were observed for both inhibition of growth of algae and embryo developmental toxicity. Differences in homologue compositions within a group of targeted analytes justified the use of multiple bioassays and end points in risk assessment.

Full-Scan Screening Analyses. In order to address possible contributions of unidentified AhR agonists in samples to the observed potencies as determined by the H4IIE-*luc* bioassay, FSA with GC-QTOFMS was performed on some of the most active fractions; F2.7 and F2.8 of raw extracts of S2 (Figure 1A). First, formulas derived from accurate mass were compared to those in the NIST library.[65] Following two-step fractionations of sediment raw extracts, 142 and 264 compounds were detected in F2.7 and F2.8, respectively. Overall, 34 and 118 compounds in F2.7 and F2.8 had matching factor scores of >70.[66] Next, 11 and 28 compounds that were aromatic were selected from F2.7 and F2.8, respectively.[67] Of these chemicals, no compounds with more than three rings, which is the main feature of AhR agonists, were detected.[9] However, five compounds with more than two benzene rings were detected in F2.7 and F2.8. These chemicals were selected as tentative AhR-active compounds (Table 1).

From F3 subfractions, F3.6 and F3.7 of raw extracts of S2 sediment exhibited significant AhR-mediated potencies (Figure 1A). Thus, we also focused on characterizing the AhR agonists in these two fractions using LC-QTOFMS. After peaks were detected, elemental compositions were matched exhaustively using accurate m/z.[68] Overall, 1732 and 2402 compounds were detected in F3.6 and F3.7, respectively. Second, after removing noise peaks, 1229 and 1888 compounds were retained.[69] On the basis of accurate masses and isotope distribution scores, flucofuron, niflumic acid, and enoxolone in F3.6 and scytalone in F3.7 were finally identified as tentative AhR active compounds (Figures 3C and S4B and Table 1).

Overall, through nontarget analyses, 14 candidate compounds were identified including xenobiotics, antibiotics, and insecticides.[69,70] Among these chemicals, flucofuron is an insecticide and known to be highly toxic to fish and invertebrates.[71] Enoxolone is a pentacyclic triterpenoid derivative of the β-amyrin type obtained from the hydrolysis of glycyrrhizic acid, and has some additional pharmacological properties, with possible antiviral, antifungal, antiprotozoal, and antibacterial activity.[72,73]

Toxicological Confirmation. During biological characterization, AhR-mediated potencies of four tentative compounds, including 1,2-di(*p-tolyl*)ethane, flucofuron, niflumic acid, and enoxolone, for which authentic standards were available, were assessed by use of the H4IIE-*luc* bioassay (Table 1). Among these chemicals, only enoxolone (18β-glycyrrhetinic acid) showed significant AhR-mediated potency. Results of previous studies suggested that enoxolone increases CYP gene activity, but, to our knowledge, none have been identified as an AhR agonist.[74,75] The ReP value of enoxolone for the AhR-mediated potency compared to that of BaP was determined for the first time during this study, by use of the dose−response relationship obtained with the H4IIE-*luc* bioassay. AhR-mediated potency of the identified AhR agonist was sufficiently great (4−80% BaP_{max}), and the calculated ReP value was 0.13 (Figure 3D). The enoxolone peak was confirmed in the LC-QTOFMS by use of authentic standards and was detected in F3.6 from S2 (∼10 ng g^{-1} dm) (Figure 3D).

Binding affinities with AhR and other potential toxicities (mutagenicity, carcinogenicity, developmental toxicity, and estrogen activity) of 14 candidates, including 10 unavailable standard materials, were tested by use of in silico method such as VirtualToxLab. Among 14 candidates, seven compounds were found to be able to bind to the AhR (Table 1). Interestingly, it was found that enoxolone, which showed AhR potencies by in vitro assay (H4IIE-*luc*), did not bind to the AhR in the

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VirtualToxLab. Because VirtualToxLab is based solely on thermodynamic considerations, i.e., ignoring mechanisms influencing the availability of a compound at the site of action, there could be many antiempirical examples that are not consistent with toxicity results.[76,77] This result shows that the use of QSAR models also has a certain limitation and thus, prediction requires careful consideration combined with empirical confirmation through exercising multiple bioassays.

In the present study, the toxicological potential of sediment extracts from Masan Bay, Korea was analyzed with five bioassays and 13 end points involving a 2-step fractionation procedure. In addition, to improve the bioassay-based strategies (both in vitro and in vivo) combined with target analysis and nontarget analyses to identify major AhR agonists. Such a multiple lines of evidence approach would enhance the accuracy in evaluation of the potential sediment toxicity in sediments. In parallel, quantification of selected PTSs was undertaken, thus allowing one to investigate the correlation between identified substances and observed biological effects. The target chemicals partly caused the effects, but mostly they were caused by a wide spectrum of untargeted substances present in the environmental samples. These results provided a better understanding of the toxicological signatures of contaminant mixtures that commonly exist in Masan Bay and elsewhere, showing different patterns of potential toxicity to the sediment raw extracts and fraction samples.

Data on co-occurrences of several toxicological end points generated by the same sample represent a clearer structure when reduced by a comprehensive understanding using statistical tools. The canonical analysis of principal coordinates (CAP) for raw extracts and fraction samples of both sites and all end points, such as AhR-mediated potency, bioluminescence inhibition, microalgae cell viability of *I. galbana*, development toxicity, and concentrations of PAHs and APs, identified that each end point could be grouped with ecotoxicologically relevant fractions (Figure 4).

Of the five bioassays used as detectors for sediment toxicity in the raw extracts and fractions, the inhibition of bioluminescence

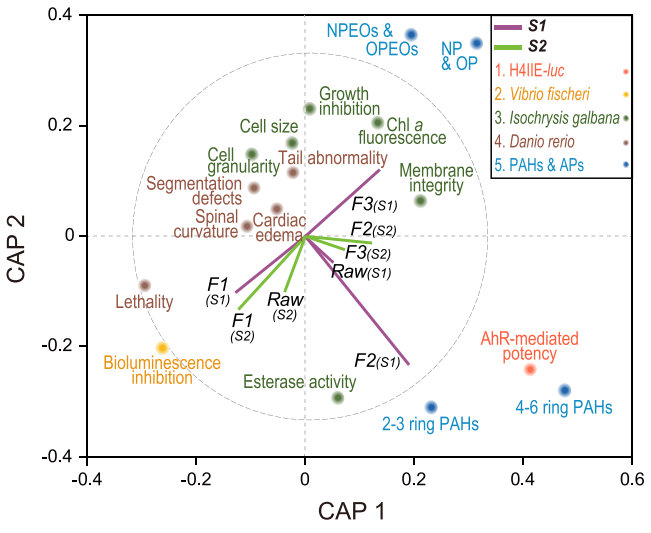

Figure 4. Scatter diagram of canonical analysis of principal coordinates (CAP) between the bioassay data and treated samples. CAP was significant with $p = 0.05$. Purple and green vectors (Spearman pairwise correlations) point in the direction of the increased values for any given variable.

of *V. fischeri* and lethality of FET showed remarkable sensitivities to nonpolar fraction of F1. The AhR-mediated potency was associated mainly with the midpolar fraction of F2. Overall, the effect of compounds eluting in the most polar fraction of F3 was reflected by the membrane integrity of the microalgae cell viability, and not by cell size or granularity. Strict regulations on releases of NPs have been implemented, so concentrations of NPs in F3 are lower. NPs were banned in kitchen cleaners in 2002, and then NPs were designated as restricted-use chemicals and subsequently prohibited for domestic applications in 2007. In Korea, use of NPs in paints and ink binders has been banned since 2010.[78] However, they are still used for industrial cleaning agents, textiles, and leather processing and found in the Korean coastal waters and elsewhere.[79] Thus, more restrict-regulations and monitoring of NP should be of immediate concern. Of note, some bioassays detecting NP exposure in the sediments was evidenced in the present work; thus, rapid biological monitoring could be utilized for the screening of NPs in the given coastal environment.

In all bioassays (except luminescent bacteria), the biological effects associated with fractions were greater than those in the raw extracts. For the sediments of Masan Bay investigated here, antagonistic reaction in mixture raw extracts seems to play an important role as highlighted by the previous study.[26] Such antagonistic effects might be the result of false-negative results in bioassays exposed to raw extracts.[11,15] For example, the presence of polymeric PAHs, such as dibenz[*a,j*]anthracene, dibenz[*a,c*]-anthracene, and picene inhibited BaP-induced AhR toxic potency.[80] Another possible explanation for antagonism lies in the natural environment, compounds might be mixed; resulting in a delayed uptake as well as a reduced accumulation.[48,80] Future studies are necessary to confirm this effect, such as testing the remixing of the separated fractions.[81]

In further studies, it is suggested to use an approach for the weight of multiple bioassays such as effect-based trigger values that represents the acceptable risk for complex mixtures as they occur.[82] In particular, ignoring the bioavailability in EDA may cause a biased and even erroneous identification of causative toxicants in a mixture sample. Considering the bioavailability in the EDA approach is important to improve the accurate identification of key toxicants in the environment. Recently, various approaches were introduced to overcome these limitations and to improve the integration of bioavailability into the EDA of abiotic samples, such as bioaccessibility-directed extraction and adverse outcomes pathway-guided bioassays and their combined application.[83−85] Overall, the selected end points offered comprehensive toxicological properties of complex environmental mixtures, and with FSA, we found a novel AhR agonist "enoxolone" in sediments from Masan Bay, Korea.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.9b07390.

Details on materials and experimental methods, including sampling sites, in vitro and in vivo bioassays, LC-QTOFMS analysis, and statistical analyses; additional details regarding the experimental design for the bioassays (Table S1); instrumental conditions and results for target analyses (Tables S2−S5); instrumental conditions for full-scan screening analyses (Tables S6−S7); results of

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bioluminescence inhibition test (Table S8); mini review on application of bioassay for toxicity assessment of chemicals (Table S9); map of sampling sites (Figure S1); workflow overview of this study (Figure S2); QA/QC results of microalgae bioassays (Figure S3); candidates selection procedures for AhR agonists (Figure S4); and results for cell viability test of microalgae assays (Figure S5) (PDF)

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Supporting Information for

Multiple bioassays and targeted and non-targeted analyses to characterize potential toxicological effects associated with sediments of Masan Bay: Focusing on AhR-mediated potency

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Supporting Methods

Sampling sites. Masan Bay, located on the southeastern coast of South Korea, is a semi-enclosed bay with restricted water exchange. It is subject to numerous anthropogenic impacts, including urbanization, industrialization, and intensive shipping activity (Figure S1). In particular, over the past few decades, riverine and bay sediments from the study area are reported to be contaminated with various environmental pollutants.[1-3]

H4IIE-*luc In Vitro* Bioassay. To measure AhR-mediated potencies in REs, silica-gel fractions, and RP-HPLC fractions, a panel of established *in vitro* reporter gene cell lines (H4IIE-*luc*) were used.[4] The bioassay methods were previously described in detail (Table S1).[5] Luminescence of luciferase was quantified using a Victor X3 multi-label plate reader (PerkinElmer, Waltham, MA). The responses were converted to the percentage of the maximum response according to 50 nM BaP, viz., % BaP_{max}.

***Vibrio fischeri In Vitro* Bioassay.** A bioluminescence test with the marine bacterium *Vibrio fischeri* (NRRL B-11177) was conducted by use of the luminescent bacteria toxicity measurement apparatus (N-TOX model 200; NeoEnBiz Inc., Bucheon, Korea), following standard methods of the Ministry of Maritime Affairs and Fisheries of South Korea[6] and Lee et al.[7] The screening test was performed following the methods described in Table S1. When the inhibition of luminescence was detected in the screening test, a serial dilution test was performed. Each experiment consisted of four controls and four replicates. A basic test to maintain the validity of the test method was also conducted with the reference standard using zinc sulfate solution for each fresh vial of bacteria that was opened.[8]

***Isochrysis galbana* and *Pheodactylum tricornutum In Vivo* Bioassays.** To investigate possible adverse effects of sediments on primary producers, inhibition of growth of algae was investigated, with some modifications to ISO[9] and Lee et al.[10] (Table S1). The marine algal strains *Isochrysis galbana* and *Pheodactylum tricornutum* were obtained from the Korea Marine Microalgae Culture Center (KMMCC). After 96 h of culture, inhibition growth of microalgae was determined by counting the number of cells in each treatment by use of a disposable hemocytometer (In CYTO, Chungcheongnam-do, Korea) under a light microscope (Optical

Microscope: CHS, Olympus, Japan). Growth rate (μ) and percentage of inhibition were calculated using ISO.[9] The viability of the microalgae population was assessed by flow cytometry, which is described in the Figure S2. Sub-samples (1 mL) for the flow cytometry (FCM) analysis of microalgae (*Isochrysis galbana* and *Phaeodactylum tricornutum*) were taken after 96 h from the same culture flasks as those measured microscopically. FCM analysis of microalgae was performed on a BD FACS Canto II flow cytometer equipped with 405 nm laser exciting SYTOX blue, 488 nm laser exciting fluorescein diacetate (FDA) and propidium iodide (PI), and 633 nm laser exciting chlorophyll *a* (Chl *a*). The microalgal population was quantified as the Pacific Blue (450/50), FITC (530/30) and APC (660/20) filters related to fluorescence from SYTOX blue, FDA, and Chl *a*, respectively.[11] Samples were measured in triplicate using the counting cells function of the flow cytometer. In addition, cell size and granularity (intracellular complexity) were measured by forward scatter detector and side scatter (SS), respectively.[12,13]

The viability of the microalgal population after treatment was analyzed with FDA (Sigma), SYTOX blue (Thermo Fisher scientific), and PI (Invitrogen, Ltd. UK). For *I. galbana*, dual staining was performed with FDA and SYTOX blue. FDA, which is taken up by live cells and converted to its fluorescent derivative fluorescein by cellular esterase, was applied at a final concentration of 5 μM mL^{-1} (15 min, room temperature and darkness). After FDA staining, samples were stained with SYTOX blue at a final concentration of 2 μM mL^{-1} and incubated in darkness for 10 min at room temperature. Membrane integrity was assessed by SYTOX Blue, which penetrates damaged membrane cells, becoming stuck to nucleic acid structures.[14] The FCM signals from *I. galbana*, dual stained with FDA and SYTOX blue were presented as dot plots and separated into quadrants (Q1 to Q4) (Figure S2). For *P. tricornutum*, staining was conducted with FDA (30 μM mL^{-1}) and PI (5 μM mL^{-1}).[15] Thermal shock dead cells were obtained using a 60 ℃ water bath for 1 h heating, and were used as a positive control. Flow cytometry dot plots of *I. galbana* and *P. tricornutum* cell signals were plotted as coordinates of FDA, SYTOX blue, and PI fluorescence intensity for unstained, stained, and boiled cells, respectively (Figure S2). Cell viability was expressed as the percentage of metabolically active cells with respect to the cells of solvent control treatment (Figure S2). The data were analyzed with the software FlowJo™ ver.10.0, which is independent of the flow cytometer used.

***Danio rerio In Vivo* Bioassay.** Maintenance and breeding of zebrafish, *Danio rerio*, followed the previously described method.[16] The fish embryo testing (FET) was performed according to the OECD Test No. 236[17] using a wild-type zebrafish strain. In brief, five freshly fertilized zebrafish embryos were randomly placed in 24-well plates, with one embryo per well in 2 mL of test media (i.e., solvent control or sample extract).[18] Three replicates were tested per treatment (total n = 15 embryos per treatment) (Table S1). The 24-well plates were then covered with self-adhesive foil and incubated at 26 ± 1 °C. Lethal and sub-lethal effects were monitored every 24 h after the start of exposure until the end of the test, at 96 h.

Selection criteria of candidates for AhR agonists by LC-QTOFMS. The first filtering step involved elemental composition matching of the compounds with a mass tolerance of 20.0 ppm to the NIST library (ver. 2017) (Figure S3).[19] The matching score (mass accuracy score) indicated that how close the observed mass is to the theoretically expected mass. It is a function of the user's specific Parent Mass Tolerance. Intermediate matches are scored based on the following linear equation (Equation 1).

$$\text{Matching Score (mass accuracy score)} = \frac{\text{-0.5 Delta Mass (AMU)}}{\text{Precursor Mass Tolerance (AMU)}} + 1 \qquad (1)$$

The second step involved removing noise peaks. If blank samples showed the intensity, they were removed.[20] The number of candidates was greatly reduced for most peaks by using a cutoff value of the MS/MS matching score (in all cases ≥ 90) in the third step.[21] The fourth step involved selecting only compounds with a score of ≥ 90 by isotopic distribution.[20] Finally, we choose the chemicals that had an aromatic ring because a previous study showed that compounds with aromatic ring structures had AhR binding affinity.

Statistical analyses. Univariate analyses were carried out using IBM SPSS software (version 23.0; SPSS Inc., Chicago, IL, USA). The difference among treatments and control was analyzed by Kruskal–Wallis test, followed by Dunnett's test for microalgae bioassay. And the difference between sites of the response of treatments was analyzed by *t*-test for *Danio rerio* bioassay. In all statistical analyses, *p* values less than 0.05 were considered to be statistically significant. Multivariate data analyses were performed using the PRIMER 6 statistical software

(PRIMER-E Ltd, Plymouth, UK) with the PERMANOVA+ add-on package. The data for each endpoint (for microalgae cell viability is only data from *I. galbana* and except for SOs because of less concentration) were standardized and were used to construct a Bray-Curtis similarity matrix. This matrix was then subjected to canonical analysis of principal coordinates (CAP) and principal component analysis (PCA) to visualize the similarity between fractionation and bioassays.[22]

Supplementary Tables

Table S1. Description of the experimental design for the bioassays examined in this study.

Bioassays	***In vitro* assays**		***In vivo* assays**	
Test organism	**H4IIE-*luc***	***Vibrio fischeri***	***Isochrysis galbana* /*Pheodactylum tricornutum***	***Danio rerio***
Specific purpose	Measurement of AhR-mediated potencies	Evaluation of inhibition of bioluminescence	Reproduction inhibition of microalgae	Measurement of developmental toxicity
Test samples	Raw, F1-F3, F2.1-F2.10 for S1 and S2 F3.1-F3.10 for S2	S.T[a]: Raw, F1-F3 E.T[b]: Raw, F1-F3 for S1 Raw, F1-F2 for S2	Raw, F1-F3	Raw, F1-F3
Experimental conditions				
Test chamber	96-well plates	96-well plates	250 mL culture flasks	24-well plates
Solvent carrier	0.1% DMSO	0.1% DMSO	0.1% DMSO	0.1% DMSO
Temperature (°C)	37	15	15	26 ± 1
Test duration (hours)	4	0.5	96	96
Initial concentrations	7.0 x 10^4 cells mL^{-1}	S.T: 200 µL of samples with 25 µL of bacterial solution E.T: 100 µL of samples[c] with 100 µL of bacterial solution	6.0 x 10^4 cells mL^{-1} / 3.0 x 10^4 cells mL^{-1}	5 organisms
Replicates	3	4	3	3
Positive control	Benzo[*a*]pyrene	Zinc sulfate solution	-	-
Endpoint	AhR-mediated potency	Bioluminescence inhibition	Growth inhibition Cell size Cell granularity Chlorophyll *a* Esterase activity Membrane integrity	Lethality Spinal curvature Cardiac edema Tail abnormality Segmentation defects
Data presented in	Figure 2A	Figure 2B and Table S7	Figures 3A, S2, and S4	Figure 3B

[a] S.T: Screening test
[b] E.T: EC_{50} test
[c] Tested with eight concentrations of 50% serial dilution.

Table S2. Instrumental conditions of reverse phase-HPLC for fractionation of organic raw extracts. Retention times of various organic chemicals (test standards, n = 62) given as a function of the log K_{ow} values of chemicals.

RP-HPLC system	Agilent 1260 HPLC system (Preparative scale)
Detector	1260 Multiple wavelength detector
Column	PrepHT XDB-C18 reverse phase column (250 mm × 21.2 mm × 7 µm)
Mobile phase	Water (A):MeoH (B) (40:60, v/v), Isocratic elution
Injection volume	1 mL
Flow rate	10 mL min^{-1}

Gradient conditions [a]

Time (min)	Solvent A	Solvent B
0	40	60
40	0	100
65	0	100
66	0	60
70	40	60

Test standards [a]

34 polychlorinated biphenyls
16 polycyclic aromatic hydrocarbons
7 alkylphenols
5 phthalates

Retention time (min)
$y = 6.48 x + 1.81$ ($r^2 = 0.987$)

Fractions collected [a]

RP-HPLC Sub-fraction	Starting – End sampling time (min)	Log K_{ow}
1	1.81 – 8.30	< 1
2	8.30 – 14.78	1 – 2
3	14.78 – 21.27	2 – 3
4	21.27 – 27.75	3 – 4
5	27.75 – 34.24	4 – 5
6	34.24 – 40.73	5 – 6
7	40.73 – 47.21	6 – 7
8	47.21 – 53.70	7 – 8
9	53.70 – 60.18	8 – 9
10	60.18 – 66.67	> 9

[a] Hong et al., 2016.[5]

Table S3. Instrumental conditions of GC-MSD for determining PAHs, SOs, and APs.

GC/MSD system	Agilent 7890A GC and 5975C MSD	
Column	DB-5MS (30 m long × 0.25 mm i.d. × 0.25 μm film thickness)	
Carrier gas	He	
Gas flow	1.0 mL min^{-1}	
Inlet temperature	300 °C	
Injection mode	Splitless	
Ionization mode	EI mode (70 eV)	
Ion source temperature	230 °C	
Acquisition mode	SIM mode	
Injection volume	2 μL	1 μL
Oven temperature	1. 60 °C hold 2 min 2. Increase 6 °C/min to 300 °C 3. 300 °C hold 13 min	1. 60 °C hold 5 min 2. Increase 10 °C/min to 100 °C 3. Increase 20 °C/min to 300 ° C 4. 300 °C hold 6 min
Target Compounds	PAHs and SOs	APs

Table S4. Concentrations and relative potency of AhR-mediated activity for PAHs reported previously and potency balance between instrument-derived BEQs and bioassay-derived BaP-EQs in the Soxhlet fraction (F2) of sediment samples from Masan Bay, South Korea.

Target compounds	Abbreviations	ReP [a]	Sites	
PAHs concentrations (ng g^{-1} dm)			**S1**	**S2**
Acenaphthene	Ace		0.5	0.3
Acenaphthylene	Acl		1.4	0.7
Fluorene	Flu		6.3	2.4
Phenanthrene	Phe		78	13
Antracene	Ant		5.0	1.6
Fluoranthene	Fl		17	27
Pyrene	Py		67	19
Benzo[*a*]anthracene	BaA	3.2×10^{-1}	36	22
Chrysene	Chr	8.5×10^{-1}	5.3	6.0
Benzo[*b*]fluoranthene	BbF	5.0×10^{-1}	22	33
Benzo[*k*]fluoranthene	BkF	4.8×10^{-1}	2.3	4.0
Benzo[*a*]pyrene	BaP	1.0	5.4	8.7
Dibenz[*a,h*]anthracene	DbahA	6.6×10^{-1}	3.3	3.6
Indeno[*1,2,3-cd*]pyrene	IcdP	5.8×10^{-1}	5.9	11
Benzo[*g,h,i*]perylene	BghiP		23	29
Sum of PAHs	Σ PAHs		280	180
BEQ concentrations (Instrument-derived equivalents, ng BEQ g^{-1} dm)	ΣBEQs		39	48
Magnitude-based BaP-EQ concentrations [b] (Bioassay-derived equivalents, ng BaP-EQ g^{-1} dm)			3.3×10^4	4.5×10^4
Potency balance analysis (BEQ/BaP-EQ × 100 (%))			0.12	0.11

[a] Kim et al.[23]

[b] Bioassay-derived BaP-EQs were calculated as percentage of the maximum response observed for a 50 nM BaP standard elicited by 100% sediment organic extracts.

Table S5. Concentrations of styrene oligomers (SOs) and alkylphenols (APs) in the sediments of Masan Bay.

Compounds	**Abb.**[a]	**Concentrations (ng g^{-1} dm)**		**Detection limit (ng g^{-1} dm, n = 7)**
Sites		**S1**	**S2**	
Styrene Oligomers				
1,3-Diphenylpropane	SD1	0.6	0.0	0.34
cis-*1,2*-Diphenylcyclobutane	SD2	0.7	0.0	0.65
2,4-Diphenyl-1-butene	SD3	7.3	49	0.94
trans-*1,2*-Diphenylcyclobutane	SD4	0.7	0.0	0.28
4,6-Triphenyl-1-hexene	ST1	5.4	1.6	0.57
1e-Phenyl-4e-(1-phenylethyl)-tetralin	ST2	2.7	1.5	0.53
1a-Phenyl-4e-(1-phenylethyl)-tetralin	ST3	4.2	1.0	0.30
1a-Phenyl-4a-(1-phenylethyl)-tetralin	ST4	4.3	2.8	0.49
1e-Phenyl-4a-(1-phenylethyl)-tetralin	ST5	1.1	0.7	0.32
1,3,5-Triphenylcyclohexane (isomer mix)	ST6	7.6	0.0	0.34
Sum of SOs		35	56	
Alkylphenols				
4-*tert*-Octylphenol	OP	30	5.5	0.09
Nonylphenols	NP	540	140	0.97
4-*tert*-Octylphenol-monoethoxylate	OP1EO	20	1.2	0.10
Nonylphenol-monoethoxylates	NP1EO	310	21	0.49
4-*tert*-Octylphenol-diethoxylate	OP2EO	40	2.3	0.10
Nonylphenol-diethoxylates	NP2EO	170	5.4	0.88
Sum of APs		1100	180	

[a] Abb.: Abbreviations.

Table S6. Instrumental conditions of GC-QTOFMS for full-scan screening analysis.

Instrument	GC: Agilent Technologies 7890B QTOFMS: Agilent Technologies 7200
Samples	F2.7 and F2.8 RP-HPLC fractions from Site 2
Column	DB-5MS UI (30 m × 0.25 mm i.d. × 0.25 μm film)
Carrier gas	He
Flow rate	1.2 mL min^{-1}
Injection volume	2 μL
Mass range	50 - 600 *m/z*
Ion source temperature	230 °C
Ionization mode	EI mode (70 eV)
Tuning condition	- Instruments are tuned prior to use each day batches are initiated. - Tuned with a compound of known mass spectrum; perfluorotributyl-amine (PFTBA) - Ions from the PFTBA spectrum for its tuning: *m/z* 68.9947, 130.9915, 218.9851, 413.9770, 463.9738 and 501.9706 - Mass accuracy and correct mass errors to within 5 ppm
Software	Qualitative analysis B.08.01 MassHunter Quantitative analysis Unknown analysis B.08.01 NIST Library (ver. 2014)
Criteria of candidate AhR compounds	Minimum number of ion peaks >5 Peak shape quality > 60% Matching factor scores of >70 - Matching factor score (the accurate mass pattern match score) = Contribution of Mass Abundance Score, Mass Accuracy Score, Mass Spacing Score - Mass Abundance Score records how well the abundance pattern of the measured isotope cluster compared with values predicted from the proposed formula - Mass Accuracy Score records how well the measured mass (or *m/z*) compared with the value predicted from the proposed formula - Mass Spacing Score records how the *m/z* spacing between the lowest *m/z* ion and the A+1 and A+2 ions compared with the values predicted from the proposed formula

Table S7. Instrumental conditions of LC-QTOFMS for full-scan screening analysis.

Instrument	LC: Thermo Scientific Ultimate 3000 QTOFMS: Triple time-of-flight (TripleTOF®) 5600 mass spectrometer (Sciex, Foster City, CA, USA)
Samples	F3.6 and F3.7 RP-HPLC fractions from Site 2
Analytical column	Phenomenex Kinetex XB-C18 column (2.1 mm × 100 mm i.d. × 1.7 µm film)
Column temperature	40 °C
Injection volume	3 µL
Flow rate	0.4 mL min^{-1}
Mobile phase	A: 0.1% Formic acid and 10 mM ammonium formate in water, B: 0.1% Formic acid in acetonitrile
Mobile phase gradient	Time (min) / Solvent A / Solvent B 0 / 90 / 10 1 / 90 / 10 15 / 0 / 100 24 / 0 / 100 25 / 90 / 10 30 / 90 / 10
Ionization mode	Electro spray ionization (ESI) Positive and Negative mode
Mass scan type	Full scan and Information Dependent Acquisition (IDA) Scanning
Mass scan range	50-2000 *m/z*
MS/MS scan range	50-2000 *m/z*
Nebulizing gas	50 psi
Heating gas	50 psi
Curtain gas	25 psi
Desolation temperature	500 °C
Ion spray voltage	Positive: 5.5 kV, Negative: 4.5 kV
Collision voltage	Positive: 35 ± 15 eV, Negative: -35 ± 15 eV
Collision gas	Nitrogen
Software	PeakView 2.2 Scafford Elements version 2.2.1 NIST Library (ver. 2017 for Elements) HMDB Library MoNA export LC-MS, MS-MS Library

Table S8. Bioluminescence inhibition of *Vibrio fischeri* for the screening test and EC_{50} test of raw extracts and fraction samples in the sediments of Masan Bay over a 30 min period.

Sites	Samples	Screening test			EC_{50} test
		Control (%)	Treatment (%)	Inhibition rate (%)	(g L^{-1})
S1	Raw extract	100	29.8	70.2	0.9
	F1	95	31.5	63.5	3.7
	F2	95.8	42.3	53.5	n.c.[a]
	F3	95.3	63.0	32.3	7.7
S2	Raw extract	100	29.0	71	1.5
	F1	95.0	27.5	67.5	1.2
	F2	95.8	21.8	74.0	2.3
	F3	95.3	99.3	n.c.	n.a.[b]

[a] n.c.: Not calculated.

[b] n.a.: Not analyzed.

Table S9. Mini review on application of bioassay for toxicity assessment of various chemicals.

Assay	Endpoint	Compounds	Uses/origins	Ref.
***In vitro* assay**				
H4IIE-*luc*	AhR-mediated potency	1,2-benzanthracene	Urban particulate matters and tobacco smoke	24
		1,2-benzofluorene	Tar and naturally synthesized	24
		2-(methylthio)benzothiazole (MTBT)	Rubber additive	25
		2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)	Waste incineration, metal production, and fossil-fuel and wood combustion	4
		2-mercaptobenzothiazole (MBT)	Rubber additive	25
		4,5-methanochrysene	Urban particulate matters and tobacco smoke	26
		5-methylbenz[*a*]anthracene (5MBA)	Petrogenic origin	23
		11H-benzo[*a*]fluorene	Gasoline engines and tobacco smoke	26
		11*H*-benzo[*b*]fluorene (11BF)	Petrogenic origin	23
		Alkylbenzenes	Derivatives of benzene	27
		Benzothiazole	Rubber additive	25
		Benz[*b*]anthracene	Film layer of OFETs and OLEDs	26
		Dinaphthofurans (DNFs)		28
		Halogenated aromatic hydrocarbons (HAHs)	Emissions from diesel vehicles, coal burning stoves, wood burning	29
		Methylated PAHs [methylated benzo[*a*]pyrenes, 1-methylchrysene (1MC), 3-methylchrysene (3MC), 3-methylphenanthrene (3MP)]	Petrogenic origin	23,30
		Nitro-PAHs	Emissions from diesel vehicles, coal burning stoves, wood burning	31

Table S9. (continued).

Assay	Endpoint	Compounds	Uses/origins	Ref.
H4IIE-*luc*	AhR-mediated potency	Organochlorine pesticides (OCPs)	Agricultural/ Produced in the high temperature environment of forest fires	32
		Polychlorinated biphenyl (PCBs) and Polychlorinated naphthalenes (PCNs)	Electrical apparatus, carbonless copy paper and in heat transfer fluids	25,33,34
		Polychlorinated dibenzo-*p*-dioxins (PCDD/F)	By-products in the manufacture of some organochlorides	4,25, 32-34
		Polycyclic aromatic hydrocarbons (PAHs)	Anthropogenic origin (gas work and coke	29, 32-36
		β-naphthoflavone	Putative chemopreventive agent.	35
Vibiro fischeri	Bioluminescence inhibition	((-) -(Z) 2,6-dimethylocta-5,7-diene-2,3-diol	Plant-derived pesticides	36
		4-nonylphenol (4-NP)	Non-ionic surfactants, / wastewater disposal	37
		Atrazine	Agriculture practices, golf courses	38
		Azaarene	Anthropogenic origin (gas work and coke manufacturing sites, timber and asphalt treatment facilities)	39
		Carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol, propranolol, and caffeine	Pharmaceutical products	40
		Chinoidic PAHs, brominated phenols and indoles	Combustion and natural origin	41
		Dexketoprofen	Pharmaceutical compounds	42
		Dimethoate	Organophosphate insecticide and acaricide	43
		Endosulfan	Chlorinated insecticide	44
		Heavy metals	Fishing, mining, industry, wastewaters, transport and/or recreational activities	45,46
		Imidazolium ionic liquids (ILs) with alkyl chain from C4 to C10	Accidental spills, containers washing operations, leaching from waste disposal sites	47,48
		n-alkanes, n-tributyltin, sulfur, 4,4´-dichlorodiphenylsulphide	Antifouling agents in paints for boats, ships, and docks (n-tributyltin)	49
		Phenolic pollutants	Natural substance degradation, industrial activities (pulp and paper industry, petrochemical works)	50
		Polycyclic aromatic hydrocarbons (PAHs)	Combustion	51

Table S9. (continued).

Assay	Endpoint	Compounds	Uses/origins	Ref.
		Simazine	Nonselective weed control in industrial areas	38
		Triclosan	Antimicrobial agent; Soaps, toothpaste, mouthwash, and cosmetics as well as in textiles	52
***In vivo* assay**				
Isochrysis galbana	Growth inhibition	4-nonylphenol (4-NP)	Non-ionic surfactants/wastewater disposal	53
		Aniline	Polyurethane and other industrial chemicals	54
		Bisphenol A (BPA)	Polycarbonate and epoxy	53
		Dibromochloromethane	Disinfection byproducts	55
		Ethylhexyl dimethyl p-aminobenzoic acid		56
		Octocrylene (OC)	Ingredient in sunscreens and cosmetics	56
		Oil (tar mat and MC252 crude oil)	Accidental spills and industry	57
		Pentachlorophenol	Organochlorine biocide	58
		PAH (pyrene)	Thermal decomposition of organic matter	59
		PAHs (benz[*a*]anthracene and fluoranthene)	Thermal decomposition of organic matter	60
		Petroleum hydrocarbon	Accidental spills and industry	61
		Propranolol carbamazepine, ibuprofen, fluoxetine, 17α-ethynylestradiol	Pharmaceutical products	40
		Triclosan	Antimicrobial agent; Soaps, toothpaste, mouthwash, and cosmetics as well as in textiles	53
Danio rerio	Embryo toxicity	2,3-benzofuran	Component of coal tar	62
		4-nonylphenol (4-NP)	Detergents, paints, pesticides, personal care products, and plastics	63
		BPA	Polycarbonate and epoxy	62
		Chlorpyrifos	Pesticides and insecticide	62
		Heterocyclic aromatic compounds (Acridine and carbazole)	Acridine dye, pesticides, and atebrin	64

Table S9. (continued).

Assay	Endpoint	Compounds	Uses/origins	Ref.
		Hexachlorobenzene	Fungicide	65
		Metals	Construction and home appliances.	66
Danio rerio	Embryo toxicity	Methyl mercury chloride	Inorganic mercury	62
		PAHs	Combustion	65,67
		Paraoxon-methyl	Insecticide parathion	62
		PCBs	Heat transfer agent	68
		Phthalate	Plasticizers	69
		Quinoline	Alkaloid and quinoline dye	62
	Embryo toxicity Gene expression	Polychlorinated dibenzo-p-dioxins (PCDD/F)	By-products in the manufacture of some organochlorides	64,70
	Embryo toxicity EROD activity Gene expression Teratogenicity	β-naphthoflavone	Drug products	67

Supplementary Figures

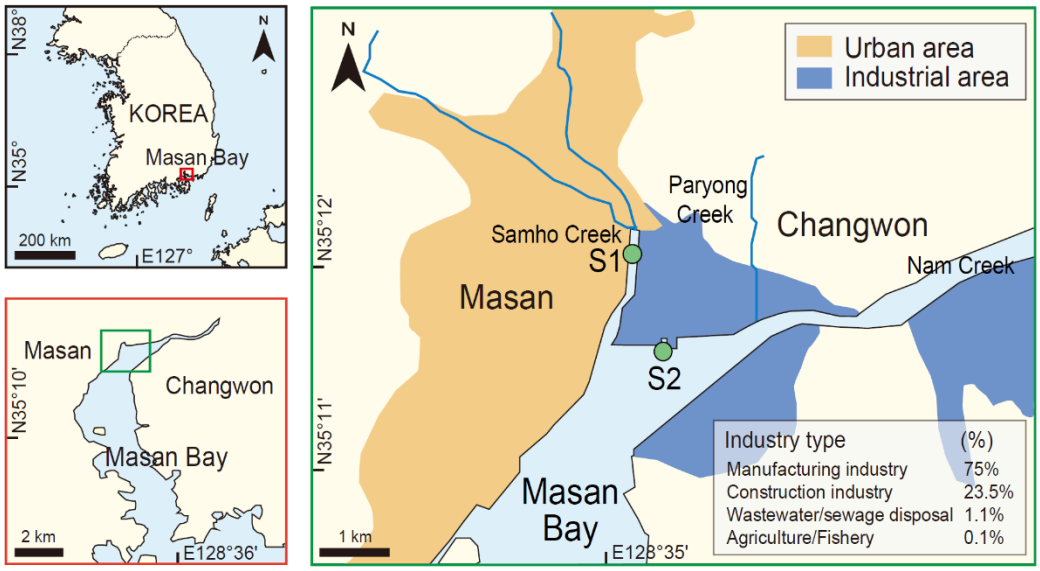

Figure S1. Sampling sites (S1 and S2) of surface sediments from the vicinity of inland creeks of Masan Bay, Korea (March 2016). The type of business in the industrial complex was referred from the website of Changwon City <https://www.changwon.go.kr>.

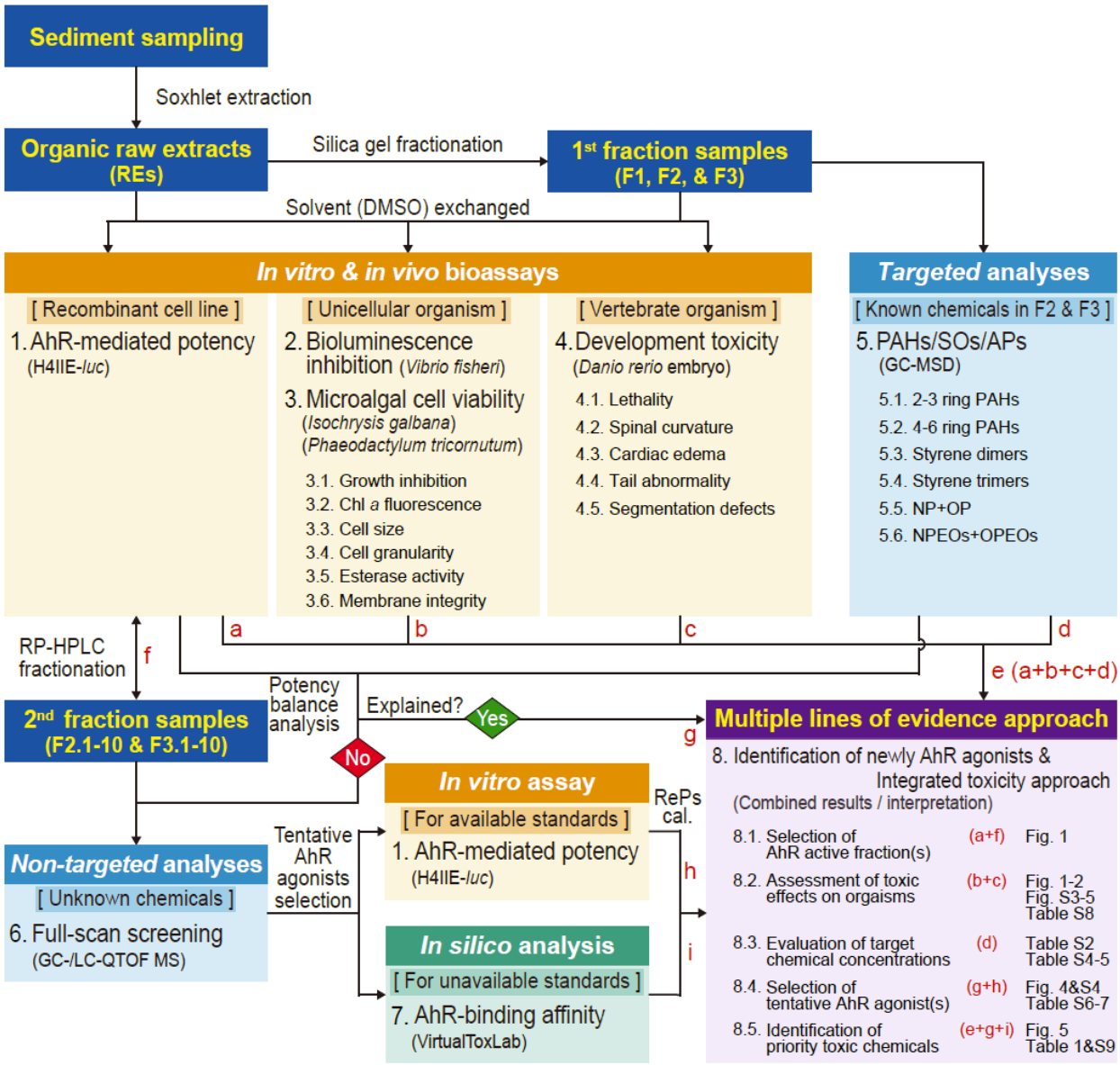

Figure S2. Workflow overview of the fractionation strategy, bioassays, and chemical analyses used to identify priority substances in the sediments from Masan Bay, Korea.

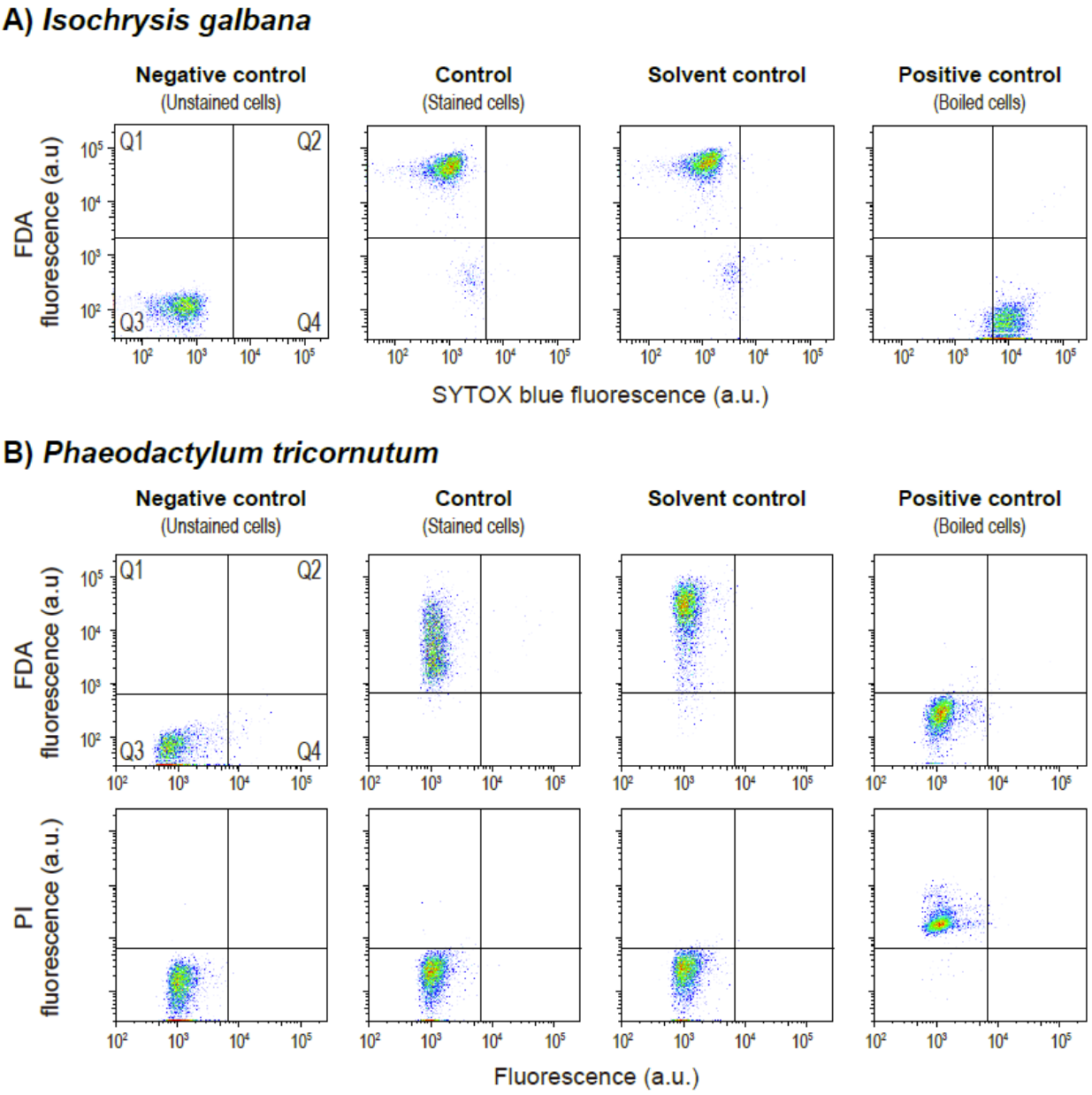

Figure S3. Dot plots of flow cytometry analysis of *Isochrysis galbana* and **B)** *Phaeodactylum tricornutum.* Mean values were obtained in logarithmic scale and represented in arbitrary units (a.u.).

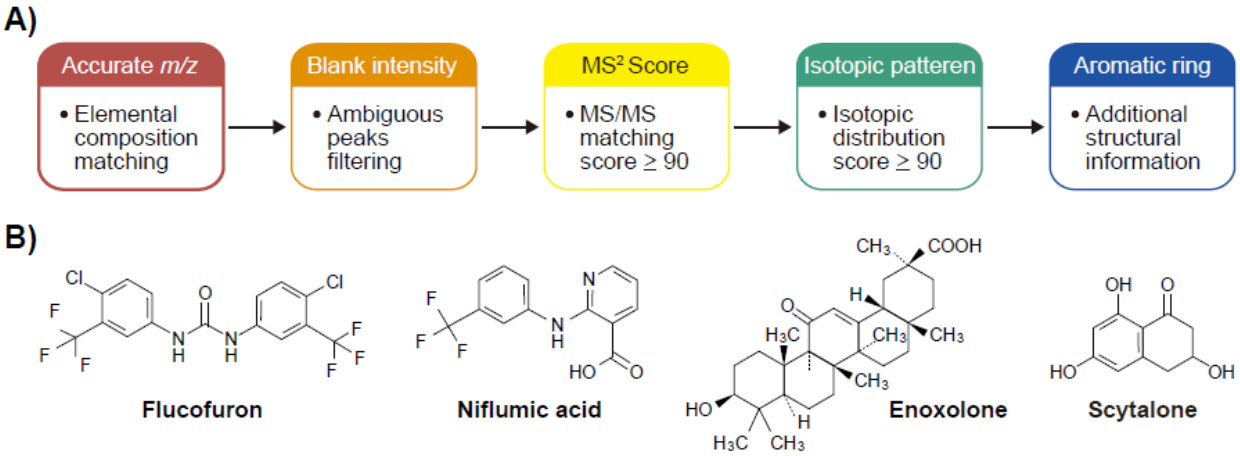

Figure S4. A) Stepwise approach of LC-QTOFMS data analysis to select AhR agonist candidates and **B)** the molecular structure of tentative AhR agonists.

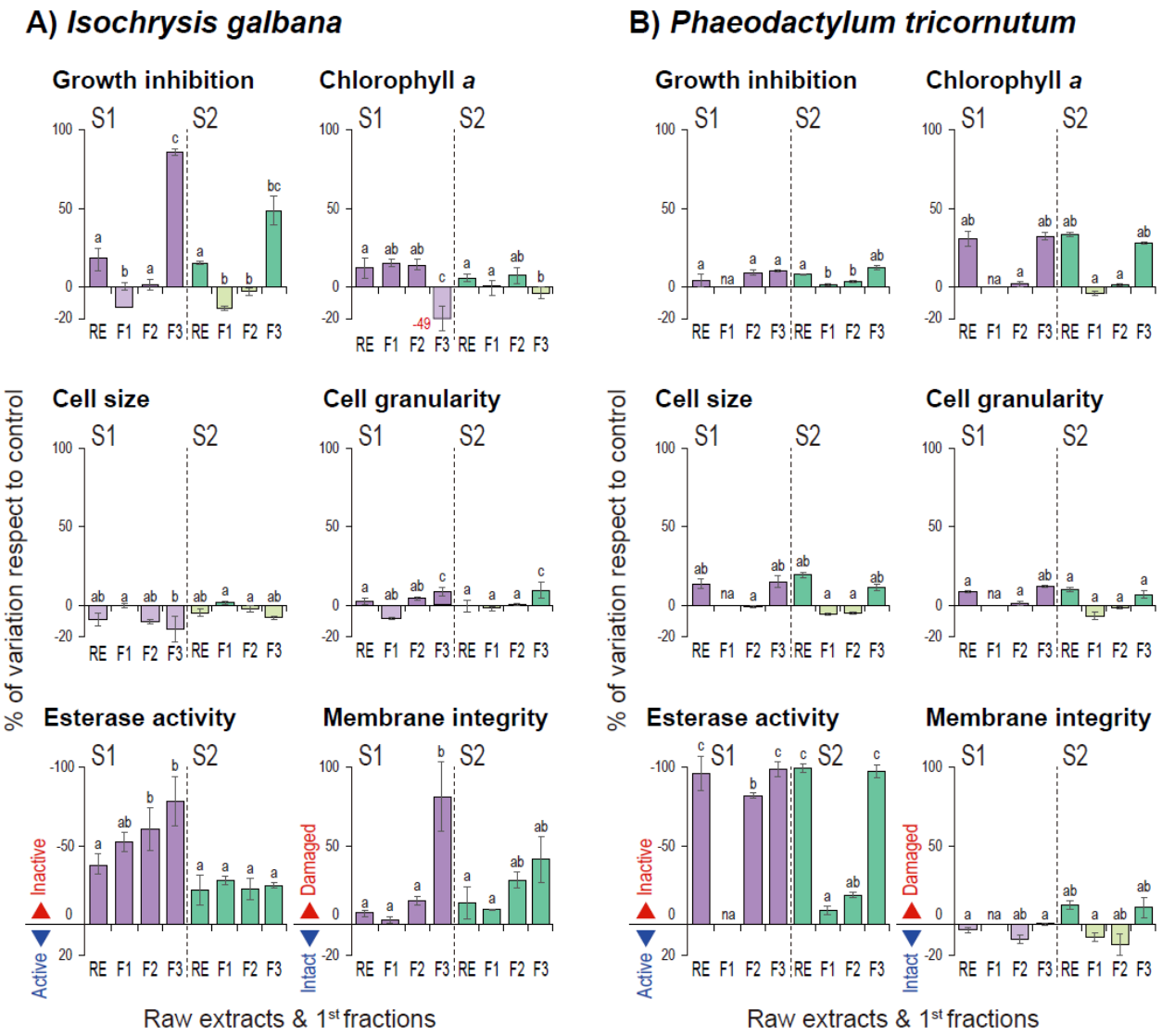

Figure S5. Variation in growth inhibition, inherent cell properties (chlorophyll *a*; autofluorescence, cell size, and cell granularity), and cell viability (esterase activity and membrane integrity) of **A)** *Isochrysis galbana* and **B)** *Phaeodactylum tricornutum* exposed to raw extracts (RE) and fraction samples (F1 to F3). Significant differences with respect to control are shown using lower case letters (Error bar: mean ± SD; n = 3).

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