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Effect-directed analysis and nontarget screening for identifying AhR-active substances in black-tailed gull eggs from South Korea

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ABSTRACT

This study identifies major aryl hydrocarbon receptor (AhR) agonists in the eggs of black-tailed gulls (*Larus crassirostris*) from South Korea using effect-directed analysis and nontarget screening (NTS). Significant AhR-mediated potency is observed in the mid-polar fraction (F2) of the egg extracts, as determined by the H4IIE-*luc* bioassay, with notable activity detected in subfractions F2.3 and F2.6–F2.9, corresponding to log K_{OW} ranges of 2.0–3.0 and 5.0–9.0, respectively. Fourteen targeted AhR agonists, including traditional polycyclic aromatic hydrocarbons (PAHs), emerging PAHs (e-PAHs), and styrene oligomers, account for 15–61% of the total AhR-mediated potency. Among them, e-PAHs such as 20-methylcholanthrene (14%), benzo[*b*]anthracene (8.8%), and 10-methylbenzo[*a*]pyrene (4.5%) contribute substantially to the overall AhR-mediated potency. NTS using GC-QTOFMS on F2.3 and F2.6–F2.9 identifies 24 AhR agonist candidates through a five-step selection process. Among the candidates with available standards, 1,4-dicyclohexylbenzene shows significant AhR-mediated activity in the H4IIE-*luc* bioassay. This compound is detected in egg extracts at concentrations ranging from 0.25 to 5.1 ng g^{-1} wet mass. However, due to its low relative potency value (7.0×10^{-4}) compared to benzo[*a*]pyrene, its contribution to overall AhR-mediated potency is only 0.01%, on average. The log K_{OW} and log K_{OA} values of the maternally transferred AhR agonists in the eggs range from 5.0 to 8.0 and 6.5 to 11.5, respectively. This study represents the first identification of maternally transferred AhR agonists in the eggs of black-tailed gulls, providing new insights into the ecological and toxicological risks associated with these contaminants.

1. Introduction

Persistent toxic substances (PTSs) are released from various industrial and anthropogenic activities, subsequently entering the marine environment through multiple pathways (Cha et al., 2023; Hu et al., 2023; Kim et al., 2024b). Once these PTSs enter the marine ecosystem, they can negatively affect aquatic life (Hu et al., 2023; Kim et al., 2024b). Due to their bioaccumulative and biomagnifying properties, these substances can reach concentrations in top predators that are exponentially higher than those in their surrounding environments and food sources (Cha et al., 2023). Black-tailed gulls (*Larus crassirostris*) in East Asia are top trophic-level consumers, feeding on large zooplankton, crustaceans, small fish, and cephalopods (Deguchi et al., 2004). They serve as key biological indicators, reflecting the contaminant levels present in their prey and, by extension, the broader marine environment (Cha et al., 2022; Choi et al., 2001; Hong et al., 2014).

The maternal transfer of essential nutrients to eggs is critical for the growth and development of seabird chicks (Jouanneau et al., 2022). However, this process also facilitates the accumulation of pollutants in eggs, as evidenced by studies detecting persistent organic pollutants

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(POPs) in seabird eggs (Jang et al., 2022; Jouanneau et al., 2022). Previous studies have reported significant positive correlations between pollutant concentrations in adult birds and their offspring, supporting the plausibility of maternal transfer (Bourgeon et al., 2013; Dauwe et al., 2006). For example, organochlorine concentrations in the blood of mother-chick pairs of great skuas (*Stercorarius skua*) were significantly correlated in the Northeastern Atlantic (Bourgeon et al., 2013), and POPs detected in 15-day-old chicks of great tits (*Parus major*) were shown to originate primarily from maternal sources (Dauwe et al., 2006). Additionally, trophic positions derived from stable nitrogen isotopes of amino acids in the eggs and mothers of black-tailed gulls in Korea ranged from 3.3 to 4.0 for eggs and 3.6 to 4.3 for mothers (Kim et al., 2024a). This indicates that black-tailed gull mothers, consuming prey across multiple trophic levels, accumulate organic matter and pollutants, which are subsequently transferred to their eggs (Jang et al., 2022). Consequently, the eggs can also reflect a certain level of trophic positions. The maternal transfer of pollutants poses potential risks to the growth and development of both eggs and chicks (Jouanneau et al., 2022).

Effect-directed analysis (EDA) is a robust methodology for identifying major toxicants in environmental matrices (Hong et al., 2023; Kim et al., 2019; Liu et al., 2024). This approach integrates bioassays, multistep fractionation, and instrumental analysis (Hong et al., 2023; Liu et al., 2024). Toxicological effects of environmental matrices are assessed using cell-based *in vitro* and whole-organism *in vivo* bioassays (Neale et al., 2020). The complexity of environmental matrices is effectively reduced through targeted extraction and fractionation processes (Cha et al., 2022; Neale et al., 2020). Significant toxic fractions are identified through relevant bioassays, with the contributions of targeted chemicals quantified to assess their roles in bioassay outcomes (Cha et al., 2019; 2022; Lee et al., 2022). However, there are instances where the observed results from receptor-mediated bioassays cannot be fully explained by the targeted chemicals present in the sample (Cha et al., 2023). In such cases, unidentified toxicants can be identified through nontarget screening (NTS), followed by chemical and toxicological confirmation (Gwak et al., 2022). NTS characterizes compounds in highly potent fraction samples using chromatographic screening and library matching software, selecting potential toxicants based on empirical strategies (Black et al., 2021; Simon et al., 2013; Titaley et al., 2021). This approach enables effective verification of the contribution of individual compounds to bioassay results (Cha et al., 2022; Kim et al., 2019; Lee et al., 2022).

Studies employing EDA combined with NTS to identify unmonitored toxicants in marine organisms have been increasingly conducted. For instance, thyroid hormone-disrupting compounds, such as branched nonylphenols and mono- and dehydroxylated-octachlorinated biphenyl, have been identified in the blood plasma of polar bears in Svalbard, Norway, demonstrating the efficacy of this approach (Simon et al., 2013). Similarly, previous research has identified major aryl hydrocarbon receptor (AhR)-active compounds in the liver of black-tailed gulls along the southeastern coast of South Korea (Cha et al., 2022). Of particular note, polar AhR agonists derived from the use of pharmaceuticals and pesticides were identified in the liver of black-tailed gulls (Cha et al., 2022). While most prior studies have primarily focused on the accumulation of toxic substances in top predators within marine ecosystems, research on the maternal transfer of these compounds to eggs or offspring remains exceedingly rare.

AhR is a ligand-activated transcription factor that responds to a wide range of exogenous and endogenous ligands (Sanderson et al., 1996). Exogenous ligands include environmental contaminants such as polychlorinated biphenyls, polychlorinated dibenzo-*p*-dioxins and dibenzofurans, and polycyclic aromatic hydrocarbons (PAHs), whereas endogenous ligands comprise naturally occurring compounds such as flavonoids, stilbenes, carotenoids, and indoles (Busbee et al., 2013). Activation of AhR has been associated with adverse effects, including carcinogenicity, developmental toxicity, and reproductive toxicity (Kolluri et al., 2017), but also with beneficial functions such as immune modulation (Busbee et al., 2013). Thus, once these compounds enter organisms, they can trigger diverse toxicological and physiological responses (Kolluri et al., 2017).

The primary objective of this study was to identify major AhR agonists in the egg extracts of black-tailed gulls from the West Sea Coast of South Korea, utilizing EDA and NTS. The study design is depicted in Fig. S1. Specifically, this study aimed to: 1) investigate the major AhR-active fractions in egg extracts, 2) quantify the concentrations of targeted AhR agonists, 3) perform NTS to select AhR agonist candidates and identify novel AhR agonists, and 4) determine the contributions of AhR agonists to the overall bioassay response. In addition, the study evaluated the potential for maternal transfer and explored the predicted toxicities of both known and candidate AhR agonists in the egg extracts. To the best of our knowledge, this is the first study to apply EDA and NTS for the identification of major AhR agonists with potential maternal transfer in black-tailed gull eggs. This comprehensive approach is intended to enhance our understanding of the behavior of PTSs within marine ecosystems, thereby improving conservation strategies for marine wildlife affected by pollution.

2. Materials and methods

2.1. Sample collection and preparation

Black-tailed gulls inhabit regions across Japan, southern Primorye, and Korea, with breeding sites in Korea located at Dokdo in the East Sea, Hongdo in the South Sea, and Baengnyeongdo, Nando, and Gyeongyeolbi-yeoldo in the West Sea (Kim et al., 2024a). To date, most studies investigating regional contamination in black-tailed gull eggs have focused on sites such as Dokdo, Hongdo, and Baengnyeongdo, while relatively few have examined eggs collected from Nando and Gyeongyeolbi-yeoldo (Jang et al., 2022; Kim et al., 2024a; Lee et al., 2024). In this study, black-tailed gull eggs were collected from Nando ($n = 6$) and Gyeongyeolbi-yeoldo ($n = 4$) on the West Sea Coast of Korea in April 2019 (Fig. S2). These islands, located about 50 km off the west coast of the Korean mainland, are influenced by extensive industrialization and urbanization (Thang et al., 2019). They also serve as breeding grounds for black-tailed gulls, whose eggs provide suitable matrices for biomonitoring contaminants from anthropogenic activities (Jang et al., 2022). The black-tailed gull eggs analyzed in this study were not collected for research purposes, but were confiscated by the Korean Coast Guard following illegal collection from protected breeding sites. Black-tailed gulls typically incubate their eggs for approximately 24–25 days until hatching (Lee et al., 2013). However, the eggs used in this study had not been incubated and showed no evidence of embryonic development. The collected eggs were stored at –20 ℃ until analysis. Detailed information on the collected samples is provided in Table S1. More details about the sample preparation methods and fractionation methods are presented in the Supplementary Materials (Hong et al., 2016a; Lee et al., 2020).

2.2. H4IIE-luc in vitro bioassays

The H4IIE-*luc* cell line, a recombinant rat hepatoma line stably transfected with a luciferase gene regulated by a dioxin-sensitive factor (Sanderson et al., 1996), was used to measure AhR-mediated potencies of raw organic extracts (REs), silica gel fractions, and RP-HPLC fractions from black-tailed gull eggs. This reporter gene construct in the H4IIE-*luc* cell line is regulated by xenobiotic response element (XREs), allowing sensitive detection of AhR activation (Sanderson et al., 1996). Upon binding of an AhR agonist to the cytosolic AhR complex, the receptor undergoes a conformational change and translocates into the nucleus (Sanderson et al., 1996). In the nucleus, AhR forms a heterodimer with the aryl hydrocarbon receptor nuclear translocator (ARNT), creating a functional transcription factor that binds to XREs located in the

promoter regions of target genes. This interaction initiates transcription and ultimately leads to the expression of reporter proteins such as luciferase, which can be quantitatively measured via luminescence to assess AhR-mediated activity (Sanderson et al., 1996). Luciferase assays are rapid, highly reproducible, and have been widely applied for assessing AhR activity in various environmental samples (He et al., 2011). To ensure non-cytotoxicity, an MTT assay was conducted prior to the bioassays, confirming >80% cell viability at the test concentrations. Test samples were exposed at a concentration of approximately 11.1 g biota equivalent (BEq) mL^{-1}, corresponding to the concentration of the sample extracts. Briefly, H4IIE-*luc* cells (74,000 cells mL^{-1}) were seeded into a 96-well plate (250 μL per well) and incubated in a humidified atmosphere at 37 ℃ with 5% CO_2 for 24 h. Test wells were treated with positive controls (benzo[*a*]pyrene, BaP), solvent control (0.1% DMSO), negative control (blank), and test samples, all at a 0.1% DMSO concentration. The BaP positive control was used for 4-h exposure assays targeting substances like PAHs, which are easily metabolized (Cha et al., 2021). BaP was serially diluted from an initial concentration of 50 nM (=100 $\%BaP_{max}$). Luminescence was measured using a Victor multilabel plate reader (PerkinElmer, Waltham, MA), and responses were calculated as a percentage of BaP positive control values. The impact of DMSO was minimized by adjusting relative luminescence units (RLU) based on the RLU of 0.1% DMSO. No significant AhR-mediated potencies were observed in the negative control, confirming the absence of background contamination from the sample preparation process. The reliability of the assay was confirmed by comparing the dose–response relationship, slope, coefficient of determination, and effective concentrations (ECs) of BaP with previous studies, demonstrating consistent results (Table S2) (Cha et al., 2021; 2022; 2023). The dose–response relationship was evaluated using a four-parameter log-logistic function, which describes the sigmoidal shape of the AhR-mediated potency curve. Additionally, recombined extracts of silica gel fractions were prepared, and their AhR-mediated potencies were measured and compared with those of the REs. Notably, surrogate standards were not added during the extraction and fractionation in this study, as these compounds could potentially interfere with AhR-mediated responses in the H4IIE-*luc* bioassay.

2.3. Targeted analysis

The concentrations of 15 traditional PAHs (t-PAHs), 14 emerging PAHs (e-PAHs), and 10 styrene oligomers (SOs) in the mid-polar fraction (F2) of black-tailed gull egg extracts were quantified using an Agilent 7890B gas chromatography (GC) coupled with a 5977B mass selective detector (MSD, Agilent Technologies, Santa Clara, CA) (Table S3). In the targeted analysis, hexane was used as a blank sample and included in each analytical batch. None of the target compounds was detected in the blanks, confirming the absence of cross-contamination or background interference. 2-Fluorobiphenyl was used as an internal standard (IS) for the analysis of target compounds, serving to correct for instrumental sensitivity. Given the comparable polarity and log K_{OW} values of the compounds detected in F2, a single IS was considered sufficient to represent all mid-compounds. The IS abundances in both calibration series for PAHs and SOs, as well as in five eggs, remained stable, with relative standard deviations (RSDs) of 19% and 5.9%, respectively (Table S4). The 14 e-PAHs, previously identified as AhR-active substances, were detected in sediments from industrial areas in Korea (Cha et al., 2019; Gwak et al., 2022; Kim et al., 2019). Detailed GC-MSD conditions are provided in Table S5. The 29 PAHs, including t-PAHs and e-PAHs, were purchased from ChemService (West Chester, PA) and Sigma-Aldrich (Saint Louis, MO). The 10 SOs were obtained from Waka Pure Chemical Ind. (Osaka, Japan) and Hayashi Pure Chemical Ind. (Osaka, Japan). Each PAH and SO compound was quantified using its authentic standard. All target compounds showed linear calibration curves, with R^2 values exceeding 0.99 (Table S3). In our previous study using black-tailed gull liver samples under the same extraction and analysis conditions, recovery rates for surrogate standards were generally acceptable, ranging from 47 to 78% for acenaphthene-d10, 52 to 115% for phenanthrene-d10, 70 to 153% for chrysene-d10, and 74 to 152% for perylene-d10 (Cha et al., 2022). Instrument performance and method accuracy were validated using the standard reference material, NIST SRM 1944, which yielded PAH recoveries ranging from 45% to 104% (mean = 75%), values that fall within the acceptable range for complex environmental matrices. The limit of detection (LOD) was calculated by multiplying 3.143 by the standard deviation of the lowest calibration standards (n = 7), corresponding to a 98% confidence level. Targeted AhR agonists and their relative potency values (RePs) for AhR efficacy are listed in Table S6 (Cha et al., 2019; Gwak et al., 2022; Kim et al., 2019).

2.4. Nontarget screening analysis and data processing

NTS was conducted using GC-quadrupole time-of-flight mass spectrometry (GC-QTOFMS; Agilent 7890B GC with a 7200 QTOFMS; Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1.2 mL min^{-1}. A DB-5MS UI column was employed for compound separation. Detailed instrument conditions are provided in Table S7. The identification of AhR agonist candidates from GC-QTOFMS followed a systematic five-step selection process: 1) deconvolution for nontarget feature extraction (Mok et al., 2023), 2) NIST library matching (Cha et al., 2019; Kim et al., 2019; Zedda and Zwiener, 2012), 3) quality filtering based on matching scores ≥70 and peak shape quality scores ≥70 (Mok et al., 2023), 4) selection of compounds whose retention time index (RI), calculated using an n-alkane mixture, deviated by less than 10% from those in the NIST library (Mok et al., 2024; Moschet et al., 2018), and 5) confirmation of the presence of aromatic ring(s) (Mekenyan et al., 1996). To minimize analytical interference, nontarget features detected in solvent blanks were excluded from the analysis, ensuring that the identified candidates reflect the actual sample composition (Mok et al., 2024). Among the 24 AhR agonist candidates, seven compounds were commercially available for standard materials. These included 2,2′,5,5′-tetramethylbiphenyl, 2,6-di-*iso*-propylnaphthalene, 1,1,3,3,5,5-hexamethyl-1,5-diphenyl-trisiloxane (Sigma-Aldrich), 1,4-dicyclohexylbenzene (1,4DCHB), 2,4-dimethylbenzaldehyde, diethyl phthalate, and α-methylstyrene dimer (Tokyo Chemical Ind., Tokyo, Japan). This study used single-run measurements for both targeted and nontargeted analyses. Although this may limit reproducibility assessment, analytical quality was supported by method validation and rigorous blank controls. In addition, triplicate NTS analyses were previously performed on sediment samples to evaluate reproducibility. The total numbers of deconvoluted components (3040, 3029, and 3059), library-matched compounds (770, 765, and 745), and RI-filtered compounds within 10% (306, 313, and 309) were all comparable across replicates. Furthermore, when 18 target compounds were spiked into the sediment matrix and analyzed in triplicate, all compounds were detected in all three replicates, and their detected areas were highly consistent, with RSDs ranging from 2.1 to 18% (mean = 8.7%), demonstrating the reproducibility of the workflow (Table S8).

2.5. Toxicological and chemical confirmation

To confirm the toxicological properties of the AhR agonist candidates, standard solutions were prepared in DMSO at a concentration of 1000 μg mL^{-1}. Six different concentrations were prepared via threefold serial dilution, starting from 1000 μg mL^{-1}. The ReP for AhR agonist was calculated based on the observed EC_{20} value compared to BaP. Chemical confirmation of a novel mid-polar AhR agonist was performed using GC-MSD, with quantification ions, confirmation ions, and LODs detailed in Table S9.

2.6. Potency balance analysis

The contribution of AhR agonists ($BaP\text{-}EQ_{chem}$) to the overall

bioassay results (BaP-EQ_{bio}) in the egg extracts was evaluated using potency balance analysis. BaP-EQ_{bio} values, representing AhR-mediated potency in the egg extracts over 4 h, were derived from dose-response relationships across six dilution levels. BaP-EQ_{chem} concentrations were calculated by multiplying the concentrations of AhR agonists in the samples by their assay-specific RePs (Table S6).

2.7. Prediction of additional toxic effects of AhR agonist candidates

The binding affinities of 24 AhR agonist candidates with various cellular receptors, including the AhR, androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), and thyroid receptor (TR), were predicted using VirtualToxLab in silico modeling. VirtualToxLab evaluates binding affinities by analyzing thermodynamic interactions between chemical structures and receptors within cells (Vedani et al., 2015). Additionally, the toxic effects of AhR agonist candidates on receptors such as AhR, AR, ER, GR, and TR were further predicted using the open-source EPA ToxCast database (https://comptox.epa.gov/dashboard) (Neale et al., 2015). This database provides bioactivity information for a wide range of chemicals, categorizing results as either active or inactive based on experimental data. VEGA quantitative structure–activity relationships (QSARs) were utilized to predict further potential toxicities of the AhR agonist candidates (Marzo et al., 2016). VEGA QSARs encompass a range of toxicity prediction models, providing insights into various toxicity endpoints, including carcinogenicity, developmental toxicity, and mutagenicity.

3. Results and discussion

3.1. AhR-mediated potencies in egg extracts of black-tailed gulls

The H4IIE-*luc* bioassay results demonstrated significant AhR-mediated potencies in all REs (~11.1 BEq mL^{-1}) of black-tailed gull eggs, except for EG2 (Fig. 1a). This suggests that substances transferred from black-tailed gull mothers to their eggs can bind to AhR and trigger associated activities. Among the silica gel fractions, AhR-mediated potencies were notably higher in mid-polar fraction (F2, mean = 90 % BaP_{max}) compared to non-polar (F1, mean = 11 %BaP_{max}) and polar (F3, mean = 2.8 %BaP_{max}) fractions (Fig. 1b). Notably, significant AhR-mediated potency was also observed in F2 of EG2, despite the absence of clear activity in its REs. Various xenobiotics in black-tailed gulls are known to be transferred to eggs primarily via lipids or proteins (Verreault et al., 2006). This maternal transfer is believed to affect the chemical processing, metabolism, and physiological characteristics of the mother birds (Jouanneau et al., 2022). Interestingly, distinct patterns of AhR-mediated potencies were observed in the liver of black-tailed gulls from the East Sea Coast of Korea, with F3 exhibiting significant activity (Cha et al., 2022). This contrast can be explained by differences in the physicochemical properties of the compounds and tissue-specific conditions. The liver is a major site of biotransformation, and relatively polar compounds that are more readily metabolized are less likely to be transferred to the eggs, whereas lipophilic compounds are preferentially transferred via lipids (Cha et al., 2022; Verreault et al., 2006). Seabird eggs contain high levels of lipids, which promote the accumulation of lipophilic contaminants such as PAHs (Grace et al., 2024), and these compounds are primarily eluted in the F2. Moreover, the metabolic activity within eggs is limited, allowing lipophilic

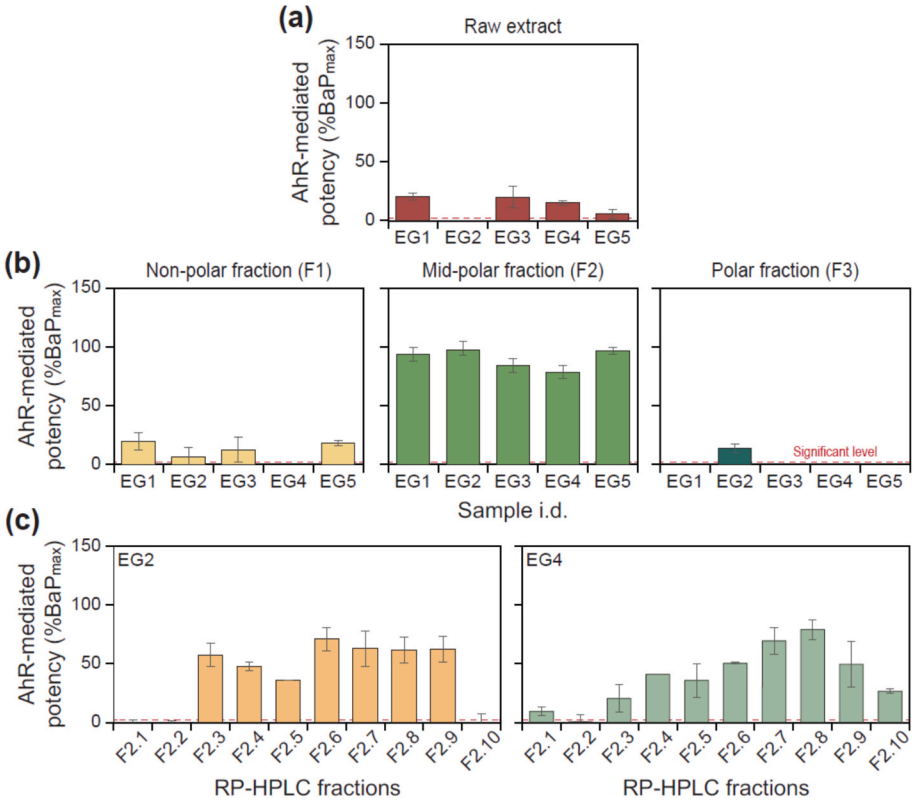

Fig. 1. (a) AhR-mediated potencies of raw organic extracts from black-tailed gull egg samples. (b) AhR-mediated potencies in silica gel fractions, including non-polar (F1), mid-polar (F2), and polar (F3) fractions. (c) AhR-mediated potencies of RP-HPLC subfractions (F2.1 to F2.10) from F2 in egg extracts of EG2 and EG4 (error bar: mean ± SD; n = 3). Error bars are not visible in some cases because the variability was minimal. The red dotted line indicates the significance threshold (3.7%) for AhR-mediated potency, calculated by dividing the standard deviation of RLU values from the solvent control group by the RLU value at the highest concentration in the positive control group.

compounds to persist for extended periods (Grace et al., 2024), which likely accounts for the higher AhR-mediated activity observed in egg extracts. The AhR-mediated potencies in the recombined fractions (F1, F2, and F3) were higher than those in the REs for all samples, with the exception of EG4 (Fig. S3). The enhanced AhR-mediated potencies observed in the recombined fractions are likely attributable to the removal of interfering compounds during the fractionation process (Hong et al., 2023). In contrast, EG4 did not show a similar increase in AhR-mediated potency, which may be influenced by sample-specific factors such as the presence of antagonists (Zhang et al., 2003) and matrix effects (Hashmi et al., 2020).

RP-HPLC fractionation was conducted on F2 from EG2 and EG4, collected from Nando and Gyeongyeolbi-yeoldo, respectively, as these samples were considered representative of the distinct characteristics of each island. In the RP-HPLC fractions, AhR-mediated potencies in F2.3 (log K_{OW} 2–3) and F2.6–F2.9 (log K_{OW} 5–9) exceeded 50% of the BaP_{max} values in at least one sample (Fig. 1c). These results highlight the significant potencies of mid-polar compounds with a wide range of log K_{OW} values in these samples. However, isolating highly potent fractions from RP-HPLC proved challenging, likely due to the complex mixture in biological samples. The patterns of AhR-mediated potencies in fraction samples did not exhibit significant differences between Nando and Gyeongyeolbi-yeoldo, suggesting that regional differences may not be substantial. The AhR-mediated potencies of silica gel and RP-HPLC fractions were significantly greater than those observed in the REs, possibly due to the removal of interfering substances during the fractionation process (Brack et al., 2016). The H4IIE-*luc* bioassay results presented in this study may not fully capture ecotoxicological responses observed *in vivo*, particularly in species such as black-tailed gulls. Future studies should incorporate *in vivo* bioassays to provide a more comprehensive understanding of the ecological implications.

$BaP\text{-}EQ_{bio}$ values were determined based on EC_{50} values obtained from dose-response curves of F2 from EG1–EG5 (Fig. S4), with calculated values of 23, 25, 16, 31, and 47 ng BaP-EQ g^{-1} wet mass (wm) (mean = 28 ng BaP-EQ g^{-1} wm), respectively. These values were utilized in the potency balance analysis. F2 was specifically chosen for this analysis, as it was assumed that the REs might be influenced by masking effects on AhR activity. In contrast, the RP-HPLC fractions posed challenges for direct comparison due to the insufficient separation of AhR-mediated potencies. The previous study reported $BaP\text{-}EQ_{bio}$ concentrations in F2 from liver extracts of black-tailed gulls along the southeastern coast of Korea, ranging from 9.2 to 10 ng BaP-EQ g^{-1} wm (Cha et al., 2022), suggesting that the concentrations in the egg extracts are approximately threefold higher. This disparity is likely influenced by factors such as reproductive activities and the chemical handling processes in black-tailed gull mothers (Ucan-Marin, 2010). Additionally, during the stages of hatching and chick growth, dilution of AhR agonists is expected to occur (Ucan-Marin, 2010). Another possible explanation is that pollution levels along the west coast of Korea may be higher than those on the southeast coast, warranting further investigation.

3.2. Concentrations of t-PAHs, e-PAHs, and SOs in egg extracts

The concentrations and compositions of targeted t-PAHs, e-PAHs, and SOs in egg samples collected from Nando and Gyeongyeolbi-yeoldo were found to be similar, likely reflecting the comparable feeding habits, home ranges, and life histories of black-tailed gulls (Fig. 2a and Fig. S5). According to GPS-based tracking conducted by the National Institute of Biological Resources, black-tailed gulls inhabiting the West Sea exhibit a wide foraging range that encompasses the entire coastal region (NIBR, 2021). Since Nando and Gyeongyeolbi-yeoldo are located only about 20 km apart, the eggs collected from these sites are likely to reflect similar exposure to diffuse pollution sources across the West Sea. Thirteen of the 15 t-PAHs were detected in at least one egg extract, with concentrations ranging from 3.3 to 11 ng g^{-1} wm, averaging 6.3 ng g^{-1} wm (Table S10). Among the detected t-PAHs, phenanthrene (Phe) was found at relatively high concentrations. Notably, elevated levels of Phe have been reported in the eggs of black-tailed gulls, as well as in other avian species (Pereira et al., 2009). Seven of the 14 e-PAHs were detected in at least one egg sample, with concentrations ranging from 3.3 to 6.0 ng g^{-1} wm. Despite receiving relatively recent attention compared to t-PAHs, e-PAHs were found at levels comparable to those of t-PAHs in the egg extracts. Particularly notable were the elevated concentrations of benzo[*b*]naphtho[*2,3-d*]furan (BBNF; mean = 1.4 ng g^{-1} wm) detected in the eggs. BBNF primarily originates from the incineration of municipal solid waste and has been previously detected in sediments in several industrialized areas of Korea (Gwak et al., 2022; Kim et al., 2019; Li and Ellis, 2015). Adult gulls may accumulate these byproducts by ingesting prey contaminated with incineration emissions and residues (Weber et al., 2011), with part of the burden subsequently transferred to their eggs.

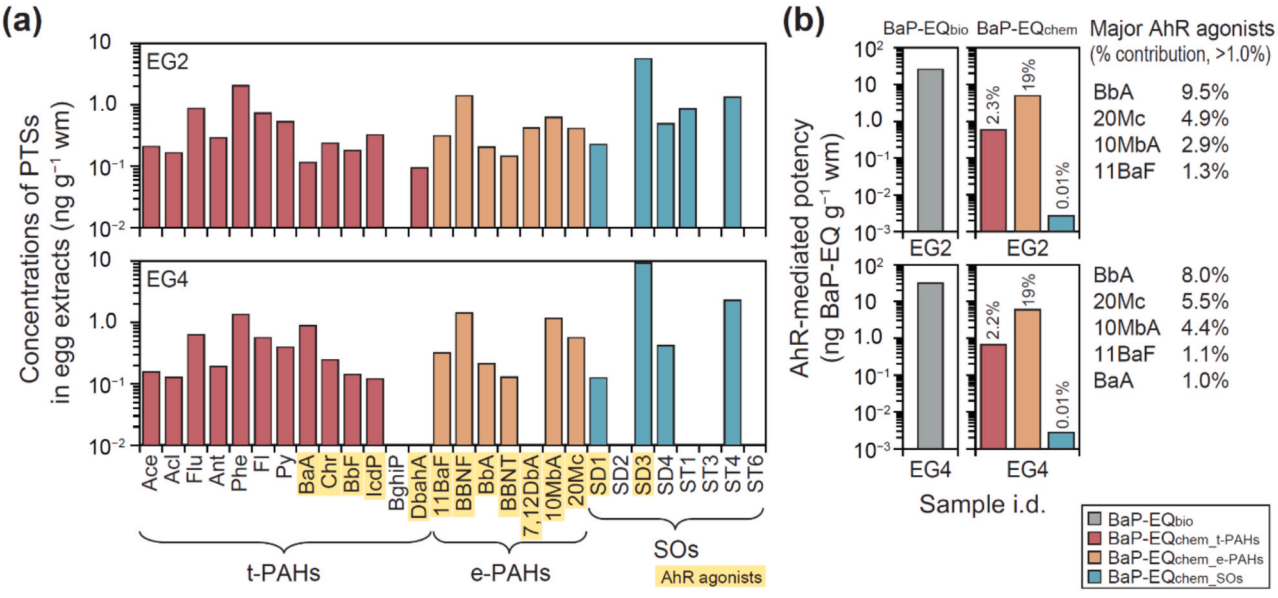

Fig. 2. (a) Concentrations of persistent toxic substances, including t-PAHs, e-PAHs, and SOs, in egg extracts (EG2 and EG4) of black-tailed gulls. (b) Comparison of instrument-derived $BaP\text{-}EQ_{chem}$ concentrations and bioassay-derived $BaP\text{-}EQ_{bio}$ concentrations in F2 of EG2 and EG4. Results for EG1, EG3, and EG5 samples are presented in Fig. S5. Error bars are not shown because the analyses were based on single measurements.

The composition of PAHs in the egg extracts was characterized by a predominance of low molecular weight (LMW)-PAHs (2–3 rings) over high molecular weight (HMW)-PAHs (4–6 rings). Seabirds are primarily exposed to PAHs through trophic transfer from contaminated prey, atmospheric deposition, and contact with petroleum-derived pollutants (Curtosi et al., 2009). LMW-PAHs, which are more abundant in marine environments, tend to accumulate in lower trophic level organisms, such as plankton and small fish, the primary diet of black-tailed gulls (Curtosi et al., 2009). In contrast, HMW-PAHs exhibit limited bioavailability and preferentially partition into lipid-rich tissues of marine organisms, reducing their transfer to eggs (Ball and Truskewycz, 2013). Seabirds metabolize HMW-PAHs via cytochrome P450 enzymes, which are particularly efficient at detoxifying these compounds (Pacyna-Kuchta et al., 2024). Physiological factors, including metabolic detoxification efficiency, bioavailability, and cytochrome P450 enzyme activity, significantly influence the distribution of PAHs in seabirds and vary among species. Additionally, the wide-ranging movements and diverse diets of black-tailed gulls may further affect the accumulation and metabolism of PAHs (Kim et al., 2024a). Future research should investigate the metabolic fate and trophic transfer of PAHs in marine food chains and explore the specific physiological and ecological traits influencing their distribution in black-tailed gulls.

Comparatively, PAH concentrations in these egg extracts from the West Sea Coast of Korea were higher than those reported in seabird eggs from other regions (Table S11). These regional differences likely reflect variations in PAH accumulations among seabird populations and disparities in local PAH sources and emissions. Additionally, the West Sea Coast of Korea is a highly industrialized area, home to petrochemical and oil refinery complexes, chemical plants, steel mills, and power generation facilities (Thang et al., 2019). These findings underscore the substantial industrial impact on the West Sea coast, which differs markedly from other coastal regions of Korea. For example, legacy POPs are predominant in black-tailed gull eggs collected from the East Sea, whereas emerging POPs are more prevalent in eggs from the West Sea, reflecting regional variations in pollution sources and accumulation patterns (Jang et al., 2022). Moreover, a previous study has reported significantly higher concentrations of pollutants, such as SOs and alkylphenols, in sediments along the West Sea coast compared to the East and South Sea coasts (Kim et al., 2021).

Of the 10 SOs, eight were detected in the egg extracts, with concentrations comparable to those of PAHs, ranging from 4.6 to 15 ng g^{-1} wm (mean = 11 ng g^{-1} wm). Notably, 2,4-diphenyl-1-butene (SD3; mean = 7.5 ng g^{-1} wm) was detected at the highest concentrations. SD3 has been reported at significant levels in offshore sediments around Korea (Kim et al., 2021). SOs, primarily derived from polystyrene plastics, are frequently discharged into the marine environment (Hong et al., 2016b; Kitamura et al., 2003; Kwon et al., 2015). The presence of SOs in the egg extracts suggests that black-tailed gulls may have ingested these compounds, possibly through foraging in plastic-contaminated marine environments. Given the documented genotoxic and reproductive toxicities of SOs to aquatic organisms (Kwon et al., 2015), continued monitoring and research on these contaminants are warranted. These findings show that previously less recognized contaminants, such as e-PAHs and SOs, can enter marine ecosystems, bioaccumulate in seabirds, and be maternally transferred to eggs, highlighting their ecological significance. Their detection in eggs collected from the highly industrialized West Sea of Korea further highlights the regional importance of combustion- and plastic-derived pollutants. This study was limited by a relatively small sample size (n = 10) from two breeding sites on the West Sea Coast and by the absence of control samples, including eggs from unpolluted areas or from other bird species. Further studies should expand sampling efforts to include control samples and additional nesting sites, providing a more comprehensive assessment of ecological impacts.

3.3. Contribution of known AhR agonists to total AhR-mediated potencies

Among the targeted PAHs and SOs, 24 compounds were identified as AhR agonists, with 14 detected in at least one egg extract (Fig. 2a and Fig. S5). A potency balance analysis was performed to evaluate the contribution of targeted AhR agonists to the overall AhR-mediated potencies in F2 of the egg extracts (Fig. 2b and Table S12). The results indicated that 20-methylcholanthrene (20Mc), benzo[*b*]anthracene (BbA), and 10-methylbenzo[*a*]pyrene (10MbA) contributed over 1.0% to the total AhR-mediated potencies in F2 of all egg extracts. Among these, 20Mc, a compound derived from the combustion of organic matter (Kumar et al., 2011), accounted for 3.5–39% (mean = 14%). BbA, commonly used in film layers for transistors (Takahashi et al., 2007), contributed 6.1–12% to the overall AhR-mediated potencies. Despite its lower concentration relative to other substances, BbA exhibited a high contribution due to its strong AhR binding affinity. Due to its specific structural properties, such as a linear arrangement of benzene rings, BbA is less prone to degradation and transformation and is metabolized less efficiently than other AhR agonists (Flesher and Lehner, 2016). Additionally, BbA was previously identified as a major contributor to AhR activity in freshwater fish in South Korea (Cha et al., 2025), highlighting its broader ecological relevance across different taxa and exposure pathways. Similarly, 10MbA, which originates from coal combustion and vehicle emissions (Venkataraman et al., 1994), accounted for approximately 4.5% of the observed bioassay results. These AhR agonists are generated intentionally or unintentionally, released into the environment, accumulate in eggs via environmental pathways, and may expose individuals to AhR-related toxicological effects before hatching.

Typical AhR ligands are characterized by aromaticity, planarity, and hydrophobicity, which play a pivotal role in determining their binding affinity to AhR (Stejskalova et al., 2011). Hydrophobic compounds containing three or more aromatic rings generally exhibit higher intrinsic ReP values (Casado et al., 2006), as the increased number of rings enhances binding strength, contributing to greater stability and resistance to degradation (Eduardo, 2004). Compounds such as 20Mc, BbA, and 10MbA demonstrate these properties and display higher RePs than the reference compound, BaP. However, the relationship between structural traits and AhR binding is complex, with additional factors such as ligand flexibility and receptor binding site dynamics significantly influencing binding affinity (Vidal et al., 2011). The 14 targeted AhR agonists explained 15–61% of the total bioassay response. Among them, e-PAHs contributed about 30%, compared with 2.6% from t-PAHs and 0.02% from SOs, showing the much greater role of e-PAHs. Although the accumulation of SD3 in the eggs was significant, its contribution was small due to its low RePs. Overall, unexplained gaps remain in AhR-mediated potencies in F2, suggesting the presence of unmonitored AhR agonists in the egg extracts.

3.4. Identification of novel AhR agonists

NTS using GC-QTOFMS was performed on F2.3 and F2.6–F2.9 of EG2 and EG4, which underwent RP-HPLC fractionation, where at least one 50 %BaP_{max} value was observed. The RP-HPLC fraction was selected for NTS because it is more effective in identifying unknown AhR agonists in samples with reduced complexity by separating compounds based on their log K_{OW} values (Hashmi et al., 2020). AhR agonist candidates were meticulously selected using a five-step selection criterion, a methodology proven effective in previous environmental studies (Fig. 3a and Table S13) (Cha et al., 2019; Kim et al., 2019). The identification process began with deconvolution to extract nontarget features from raw data, yielding approximately 2000 chromatographic peaks per fraction (Mok et al., 2023). In the second step, these compounds were matched with the NIST library, reducing the number of potential compounds to approximately 300 per fraction (Zedda and Zwiener, 2012). Target compounds detected in each fraction were excluded. The third step

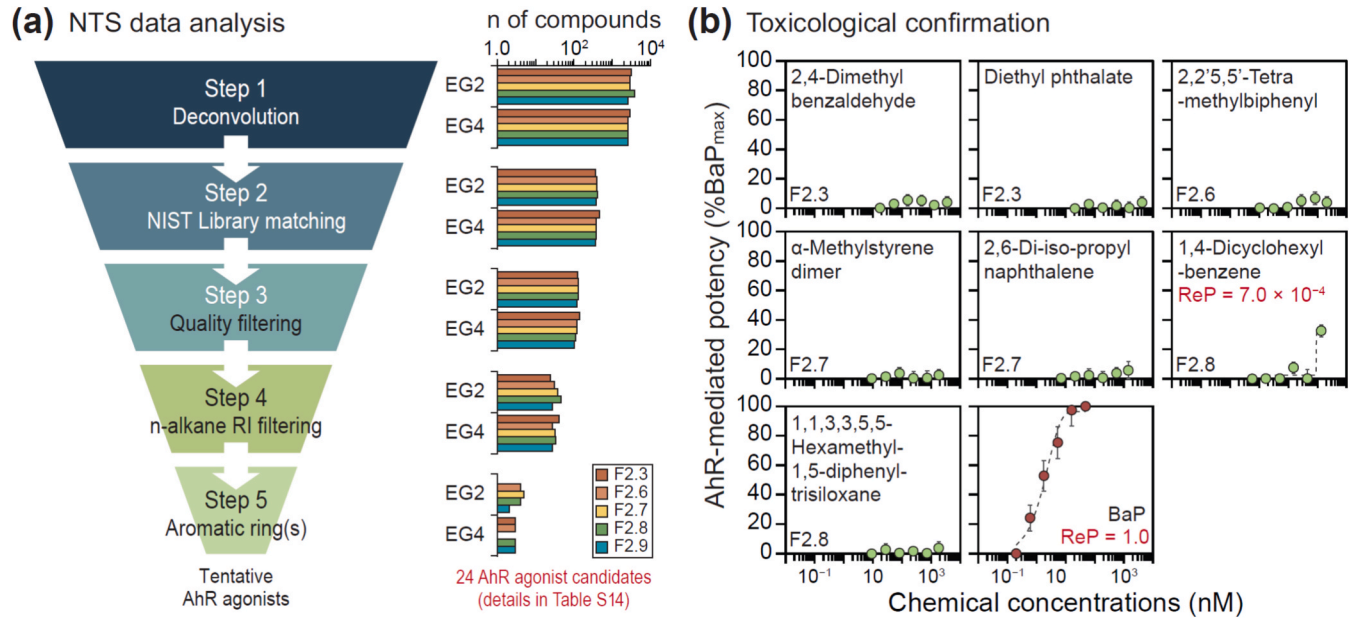

Fig. 3. (a) Step-by-step procedure for GC-QTOFMS data analysis used to identify AhR agonist candidates in F2.3 and F2.6–F2.9 fractions of EG2 and EG4 extracts. The list of 24 AhR agonist candidates is shown in Table S14. (b) Dose-response curves showing AhR-mediated efficacies of 7 AhR agonist candidates at six concentrations compared to BaP in the H4IIE-*luc* bioassays. Error bars represent the mean ± standard deviation (n = 3), but are not visible in some cases because the variability was minimal.

involved quality filtering, selecting compounds with both matching scores and peak shape quality scores of 70 or higher (Mok et al., 2023), which narrowed the selection to approximately 100 compounds per fraction. Given the complexity of environmental matrices, where chromatographic peaks may be flat or indistinct, careful evaluation of peak shape quality is critical to ensure the reliability and accuracy of NTS results (Renner and Reuschenbach, 2023). In the fourth step, filtering based on RI calculated from n-alkane mixture further reduced the number of compounds to 23–43 per fraction (Mok et al., 2024; Moschet et al., 2018) (Table S14). Finally, only compounds containing aromatic ring(s) were selected (Mekenyan et al., 1996). This systematic workflow ultimately identified 24 AhR agonist candidates, three of which were common to fractions from both samples (Fig. S6 and Table S14). These findings highlight that nontarget compounds can exhibit sample-specific variability, even among eggs with broadly similar characteristics.

Meanwhile, aromatic ring-containing compounds were not selected in F2.3 of EG2 and in F2.7 of EG4, despite the presence of significant AhR-mediated potencies. This occurred because candidate compounds were excluded during selection due to scores falling below the 70-point threshold for library matching and shape quality. While these score criteria are essential for ensuring accuracy and reliability in the compound selection, they may also result in the elimination of potentially significant candidates. An empirical and practical approach is essential when selecting causative agents, balancing whether to lower the score threshold to expand the range of potential candidates or to raise the criteria to improve the accuracy and reliability of compound identification. This strategy addresses the inherent limitations of spectral matching and enhances the screening process. In this study, VirtualToxLab in silico modeling was employed to predict the binding affinities of 24 AhR agonists to AhR. *p,p'*-DDE were not available for analysis in VirtualToxLab (Table 1). The results indicated that all compounds, except for 1,1,3,3,5,5-hexamethyl-1,5-diphenyl-trisiloxane, exhibited the potential to bind to AhR (Fig. S7). Compounds identified in F2.3 and F2.6 showed relatively low AhR binding affinities, whereas those detected in F2.7–F2.9 demonstrated moderate toxic potency.

Toxicological confirmation of seven candidates revealed significant AhR activity only for 1,4DCHB in F2.8, with RePs values calculated accordingly (Fig. 3b). 1,4DCHB was detected at quantifiable concentrations in all egg extracts (Table S15). As a known constituent of pyrolysis oil (Zhang et al., 2017), 1,4DCHB was found at concentrations ranging from 0.25 to 5.1 ng g^{-1} wm. With a log K_{OW} value of 7.6, 1,4DCHB exhibits a high potential for bioaccumulation in black-tailed gull mothers (Table S16) (Kelly et al., 2007). However, data on the environmental concentrations and toxicological effects of 1,4DCHB remain very limited. Given its bioaccumulation potential and maternal transfer characteristics, continued monitoring and ecological risk assessment of combustion-derived AhR agonists are warranted. Nevertheless, this study is the first to identify 1,4DCHB as a novel AhR agonist in a relatively understudied species, providing valuable insights into the occurrence of combustion-derived compounds in seabirds. Meanwhile, compounds such as 2,4-dimethylbenzaldehyde, diethyl phthalate, 2,2′,5,5′-tetramethylbiphenyl, 2,6-di-*iso*-propylnaphthalene, and α-methylstyrene dimer were predicted to have AhR binding potential. However, no significant AhR activity was observed in the H4IIE-*luc* bioassay. Discrepancies between predicted and experimental results are not uncommon in toxicity prediction models, such as VirtualToxLab. This can be attributed to several limitations of VirtualToxLab, including reduced accuracy for chemicals with uncommon or reactive functional groups, large molecular sizes, or its reliance solely on thermodynamic considerations (Vedani et al., 2015; Xie et al., 2022).

3.5. *Contribution of novel AhR agonists to total AhR-mediated potencies*

The relative contributions of the newly identified AhR agonist to the observed BaP-EQ_{bio} were evaluated by comparing their BaP-EQ_{chem} concentrations in the eggs. The identified AhR agonists (i.e., targeted and newly identified) accounted for an average of 32% of the total AhR-mediated potencies, with no significant increase (Fig. 4a). 1,4DCHB contributed between 0.001% and 0.02% (mean = 0.01%) (Table S12), indicating a minimal contribution to the overall AhR-mediated activity of the egg extracts (Fig. 4b). Despite analyzing 15 AhR agonists, about 70% of the observed AhR-mediated potency remained unexplained. This suggests that unidentified AhR agonists may still be present in black-tailed gull eggs. The inability to identify these substances is likely due to 1) the lack of commercially available standards for several selected candidates, which limited toxicological confirmation, and 2) inherent

Table 1
Predicted toxicity of 24 AhR agonist candidates in egg extracts of black-tailed gulls from the West Sea, South Korea.

Fractions and compounds	VirtualToxLab					EPA ToxCast					VEGA QSAR		
	AhR	AR	ER	GR	TR	AhR	AR	ER	GR	TR	Carcino-genicity[a]	Developmen-tal toxicity[b]	Muta-genicity[c]
F2.3													
2,4-Dimethylbenzaldehyde	+[d]	−[e]	−	−	+	NA	NA	NA	NA	NA	− + + −	− +	− − + − −
Diethyl phthalate	+	+	−	+	+	NA	NA	NA	NA	NA	− + − +	+ +	− − − − −
1-(2′,4′-Dichlorophenacyl)pyrazole	+	−	+	+	+	NA	NA	NA	NA	NA	+ − − −	− +	+ + − + +
F2.6													
2,2′5,5′-Tetramethylbiphenyl	+	−	−	−	+	NA	NA	NA	NA	NA	− − + −	− +	− + − + +
1,1-Bis(p-toly)ethane	+	−	+	+	+	NA	NA	NA	NA	NA	− − + −	− −	− − − − −
1-(3-Methyl-cyclopent-2-enyl)-cyclohexene	+	−	−	−	−	NA	NA	NA	NA	NA	+ − − −	− −	− − − − −
3,4-Dimethyl-7-*exo*-methylene-bicyclo [4.3.0]non-3-ene	+	−	−	−	−	NA	NA	NA	NA	NA	+ − − +	− −	− − − − −
2-[(Butoxycarbonyl)oxy]phenyl 4-butylbenzoate	+	−	−	+	−	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
Bi-2-cyclohexen-1-yl	+	−	−	−	−	NA	NA	NA	NA	NA	+ − − −	− −	− − − − −
F2.7													
Calamenene	+	−	−	−	−	NA	NA	NA	NA	NA	− − + +	+ +	− − − − −
2,6-Di-*iso*-propylnaphthalene	+	−	+	+	+	+	NA	+	NA	NA	+ − + −	− +	− − − − −
α-Methylstyrene dimer	+	−	+	+	+	NA	NA	NA	NA	NA	− − + −	− −	− + − − −
p,p′-DDE	NA[f]	NA	NA	NA	NA	+	+	+	+	NA	+ + − −	+ +	− − − − −
1,1,3-Triphenylpropane	+	+	+	+	+	NA	NA	NA	NA	NA	− − + −	− −	− − − − −
F2.8													
1,4-Dicyclohexylbenzene	+	−	+	+	+	NA	NA	NA	NA	NA	− − + −	− +	− − − − −
4-Phenylbicyclohexyl	+	+	+	+	+	NA	NA	NA	NA	NA	− − + −	− +	− − − − −
2-Phenyl-1,1′-bi(cyclohexan)-1-ene	+	+	+	+	+	NA	NA	NA	NA	NA	+ − + −	− +	− + − − −
2-Phenyldecane	+	−	+	+	+	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
3-Phenylundecane	+	−	+	+	+	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
1,1,3,3,5,5-Hexamethyl-1,5-diphenyl-trisiloxane	−	−	−	−	−	NA	NA	NA	NA	NA	− − − −	+ +	− − − − −
F2.9													
5-Phenyltridecane	+	+	+	+	+	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
6-Phenyltridecane	+	+	+	+	+	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
2-Phenyldodecane	+	+	+	+	−	NA	NA	NA	NA	NA	− − − −	− −	− − − − −
3-Phenyldodecane	+	−	+	+	+	NA	NA	NA	NA	NA	− − − −	− −	− − − − −

[a] Carcinogenic potential of AhR agonist candidates predicted with four models (IRFMN/ISSCAN-CGX, ISS, CAESAR, and IRFMN/Antares). [b] Developmental toxicity of AhR agonist candidates predicted with two models (PG and CAESAR). [c] Mutagenicity of AhR agonist candidates predicted with five models (SarPy/IRFMN, KNN/Read-Across, ISS, CONSENSUS, and CAESAR). [d] +: Active. [e] −: Not active. [f] −: Not available.

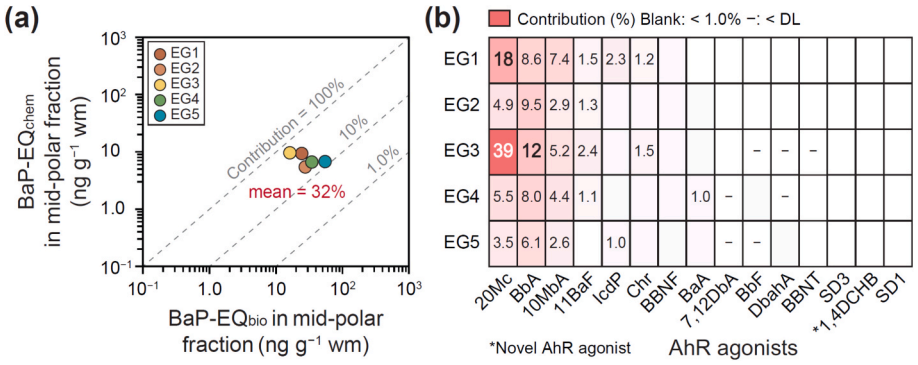

Fig. 4. (a) Potency balance between BaP-EQ$_{chem}$ and BaP-EQ$_{bio}$ concentrations in F2 from egg extracts of black-tailed gulls after inclusion of a novel AhR agonist. Targeted AhR agonists explained on average 32% of the total AhR-mediated potencies. (b) Contribution of each AhR agonist to the total induced AhR-mediated potencies. Numbers in the cells indicate the contribution of individual compounds in each sample; blank cells indicate contributions < 1.0%, and "–" denotes values below the detection limit (DL).

structural limitations of the NTS approach used to discover novel AhR agonists. A notable limitation of the NTS approach is the considerable reduction in candidate compounds after matching with the NIST library. This suggests that many compounds in biological samples may have undergone metabolism, rendering their mass signals difficult to interpret and match with existing library entries (Broeckling et al., 2014). This highlights the need to improve matching capabilities by incorporating additional data such as molecular formulas, MS/MS spectra, and retention times for both known and unknown candidate compounds (Bletsou et al., 2015). More sophisticated selection criteria,

such as molecular formula identification of compounds with potential AhR binding affinity, may also be necessary to improve the identification of AhR agonist candidates (Arturi and Hollender, 2023). Alternatively, refining the selection criteria, such as adjusting the NIST library matching score or incorporating additional criteria to detect compounds with structures resembling aromatic rings, may enhance the identification process. Although this study could not directly identify all potent AhR ligands through NTS, future improvements in these approaches are expected to allow identification of stronger AhR ligands in environmental samples.

Despite these challenges, it is noteworthy that 20Mc, BbA, and 10MBA, previously identified from sediments of industrial areas in Korea (Cha et al., 2019; Gwak et al., 2022), were detected at high concentrations in black-tailed gull eggs and contributed significantly to the overall toxicity. These e-PAHs have been only limitedly considered as causal compounds in the analysis of AhR activity in environmental samples. The inclusion of these compounds in future monitoring programs is expected to provide a clearer understanding of their overall contribution to AhR activity. As marine environments likely contain additional unidentified AhR agonists, ongoing efforts to identify unmonitored toxicants are crucial for effective marine environmental protection. These substances may contribute to ecological impacts in black-tailed gull populations, particularly through their effects on eggs. For example, activation of the AhR pathway has been shown to increase the production of reactive oxygen species, thereby inducing oxidative stress in organisms (Grishanova and Perepechaeva, 2022). Toxicity studies using avian embryos have reported that AhR-mediated upregulation of CYP1A4 is associated with the onset of uroporphyria and cardiovascular dysfunction, leading to increased mortality during early development (Doering et al., 2023; Farhat and Kennedy, 2021; Farhat et al., 2021; King et al., 2023). In addition, previous studies on herring gulls and related species have demonstrated that AhR activity in eggs can affect reproductive outcomes, including altered sex ratios and reduced hatchling body weights (Erikstad et al., 2011; Muusse et al., 2014). Similarly, exposure to AhR agonists such as BaP, chrysene, and 7,12-dimethylbenz[*a*]anthracene, in mallard eggs has been associated with significant reductions in embryo growth and increased incidences of abnormal chick development (Hoffman and Gay, 1981; Pereira et al., 2009). These findings suggest that the accumulation of AhR agonists can affect black-tailed gulls across multiple life stages, highlighting the need to study their toxic effects in eggs and the associated ecological risks. Therefore, it is important to consider that not only well-known AhR-active substances but also unidentified compounds may contribute to actual ecological toxicity.

3.6. *Potential transfer of toxic substances from black-tailed gulls to eggs*

In avian embryos, two principal exposure routes have been recognized: (1) endogenous transfer from the mother during oogenesis and (2) exogenous contamination of the eggshell through contact with polluted feathers or abdominal skin (Albers, 2006). In this study, the detection of AhR agonists in black-tailed gull eggs provides indirect evidence that maternal transfer of toxic substances may occur, even though direct measurements from adult tissues were not available. The behavior of compounds in biological samples, such as bioaccumulation, biomagnification, and maternal transfer, is known to be influenced by partition coefficients (Baert, 2012; Kelly et al., 2007; Zheng et al., 2018). Specifically, compounds with log K_{OW} (log octanol–water partition

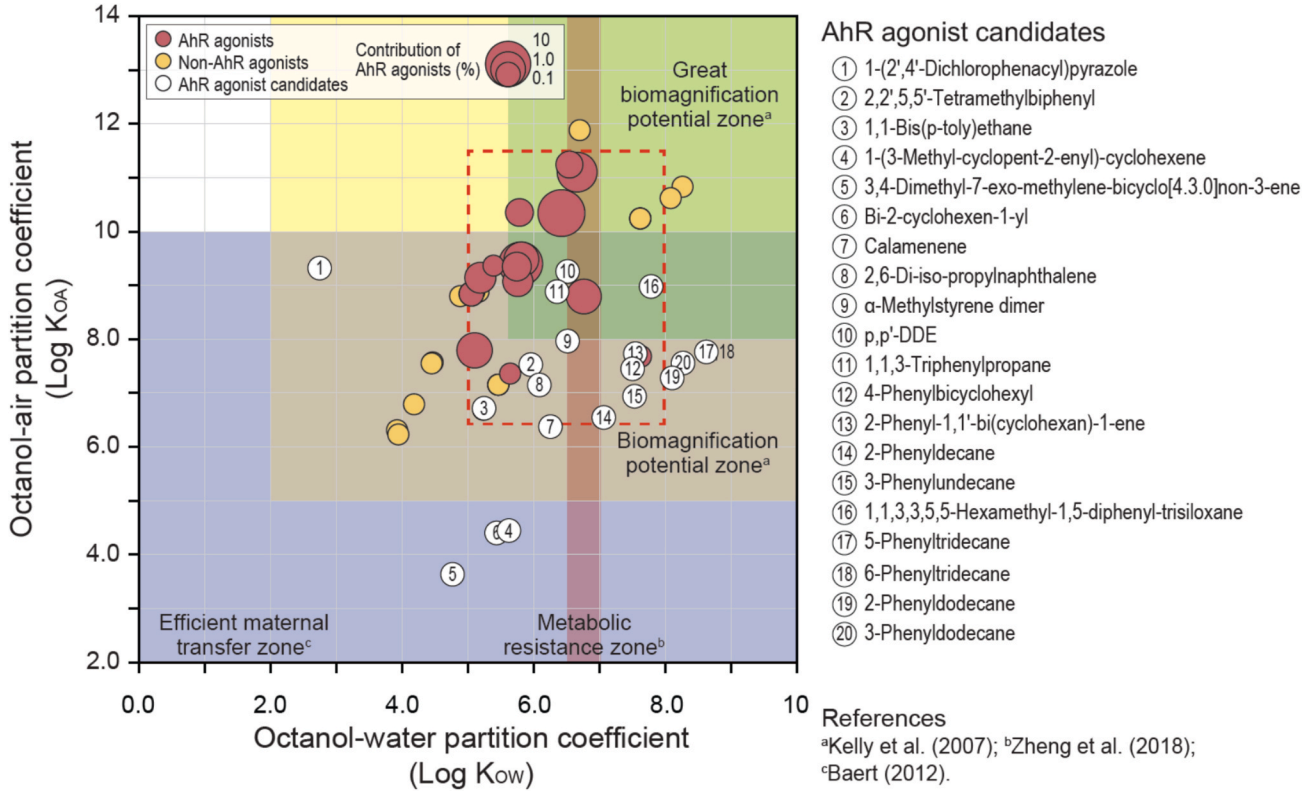

Fig. 5. Chemical space map of AhR agonists, non-AhR agonists, and AhR agonist candidates identified in this study. Information on the partition coefficients of compounds is provided in Table S16. The biomagnification potential zone is defined by a log K_{OW} of 2.0 or higher and a log K_{OA} of 5.0 or higher (yellow box) (Kelly et al., 2007). The great biomagnification potential zone is characterized by a log K_{OW} of 5.5 or higher and a log K_{OA} of 8.0 or higher (green box) (Kelly et al., 2007). The metabolic resistance zone falls within a log K_{OW} range of 6.5 to 7.0 (red box) (Zheng et al., 2018). The efficient maternal transfer zone is defined by a log K_{OA} of less than 10 (purple box) (Baert, 2012). The red dotted box indicates the transferable range of AhR agonists from black-tailed gulls to eggs derived in this study. Among the AhR agonist candidates, 2,4-dimethylbenzaldehyde, diethyl phthalate, and 2-[(butoxycarbonyl)oxy]phenyl 4-butylbenzoate, for which partition coefficient data were unavailable, were excluded from the results.

coefficient) greater than 2.0 and log K_{OA} (log octanol–air partition coefficient) greater than 5.0 exhibit potential for biomagnification within the marine-mammalian food web, particularly in air-breathing organisms (Kelly et al., 2007). In the present study, all targeted chemicals in the egg extracts were observed to fall within this range of partition coefficients (Fig. 5 and Table S16). Notably, certain compounds were found in the great biomagnification potential zone, defined by log K_{OW} values exceeding 5.5 and log K_{OA} values surpassing 8.0 (Kelly et al., 2007).

Furthermore, compounds with log K_{OW} values between 6.5 and 7.0 resist metabolic degradation, enhancing their preferential transfer and accumulation from the mother to the eggs (Zheng et al., 2018). Compounds within this zone were identified as HMW-PAHs, such as indeno[*1,2,3-cd*]pyrene, dibenz[*a,h*]anthracene (DbahA), benzo[*g,h,i*]perylene, and 10MbA. Although some HMW-PAHs were generally not detected in the eggs, these four specific compounds were present at detectable concentrations, suggesting that these substances were not fully metabolized in the mother and were transferred to the eggs (Table S10). Moreover, compounds with log K_{OA} values of 10 or lower are known to undergo efficient maternal transfer, whereas those with values greater than 10 may experience restricted transfer due to their strong lipid-binding affinity (Baert, 2012). The range of log K_{OW} and log K_{OA} values for the compounds detected in the eggs was broad, spanning from 3.9 to 8.3 and 6.2 to 12, respectively. Our findings suggest that substances with lower log K_{OW} values, which were expected to have low bioaccumulation potential and to be easily metabolized, as well as those with higher log K_{OA} values and strong lipid affinity, could be partially transferred to the eggs via maternal transfer without complete metabolism. These results imply the potential for maternal transfer of some AhR agonist candidates identified in the eggs.

The maternally transferred AhR agonists detected in the eggs exhibited log K_{OW} and log K_{OA} values ranging from 5.0 to 8.0 and 6.5 to 11.5, respectively. These findings suggest that compounds with these partition coefficients were transferred from black-tailed gull mothers to their eggs, contributing significantly to the observed AhR activities. The observed range of partition coefficients may also serve as a useful reference for future screening and identification of compounds associated with AhR activity in seabird eggs. These AhR agonists and candidate compounds possess physicochemical properties similar to those of PAHs, suggesting that they may accumulate in mothers through similar external exposure routes and subsequently be transferred to the eggs. Notably, substances with high log K_{OA} values (>10) were found to contribute to AhR-mediated potencies in F2. Despite their high log K_{OA} values, compounds such as DbahA and 10MbA were likely transferred to the eggs due to their strong resistance to metabolism. Compounds residing within the metabolic resistance zone may evade enzymatic degradation, allowing selective accumulation and maternal transfer (Verreault et al., 2006; Zheng et al., 2015). Although direct comparisons with maternal tissues were not conducted, precluding confirmation of specific transfer pathways, the presence of incompletely metabolized compounds in eggs lends support to the plausibility of maternal transfer in this species. Even compounds anticipated to undergo metabolism may resist complete breakdown, emphasizing the complex interplay between metabolizability and maternal transfer pathways (Yamashita et al., 2018). Further research should focus on the absorption, distribution, metabolism, and excretion (ADME) processes of AhR agonists in black-tailed gulls (Wu et al., 2022). In particular, lipid- and protein-binding affinities play a critical role during the distribution phase of ADME (Li et al., 2019), and metabolically stable compounds with high binding affinity are likely to accumulate in eggs, thereby contributing significantly to the observed AhR activity. Therefore, identifying key physicochemical properties, such as partition coefficients and lipid/protein-binding affinities, that influence the maternal transfer of AhR agonists is essential.

3.7. Prediction of additional toxicities of AhR agonist candidates

VirtualToxLab in silico modeling also predicted binding affinities of the 24 AhR agonist candidates to various receptors, including AR, ER, GR, and TR (Table 1). The analysis indicated that all candidate compounds, except for 1-(3-methyl-cyclopent-2-enyl)-cyclohexene, 3,4-dimethyl-7-*exo*-methylene-bicyclo[4.3.0]non-3-ene, bi-2-cyclohexen-1-yl, calamenene, and 1,1,3,3,5,5-hexamethyl-1,5-diphenyl-trisiloxane, were predicted to bind to at least one hormone receptor. Meanwhile, the toxicity prediction using the EPA ToxCast database revealed that only 2,6-di-*iso*-propylnaphthalene and p,p'-DDE had available data. Both compounds were predicted to bind to at least one receptor. These findings indicate that publicly available or pre-registered data for the candidates identified through NTS remains highly limited. The VEGA QSAR models predicted that several compounds, including 2,4-dimethylbenzaldehyde, 1-(2′,4′-dichlorophenacyl)pyrazole, 2,2′5,5′-tetramethylbiphenyl, and 2-phenyl-1,1′-bi(cyclohexan)-1-ene, exhibited potential activity across multiple toxicological endpoints. Except for seven compounds, the remaining chemicals were predicted to induce at least one additional form of toxicity. Although these results rely on in silico predictions, they extend beyond direct AhR binding to indicate additional toxicological potentials of uncharacterized compounds, providing new insights into their ecological risks. If these chemicals present in the eggs of black-tailed gulls induce toxic effects such as carcinogenicity, developmental toxicity, or mutagenicity, there could be detrimental impacts on hatching success and chick survival (Jouanneau et al., 2022; Pereira et al., 2009). In severe cases, these toxicities may lead to significant population declines, which could have profound and far-reaching ecological consequences.

4. Conclusion

This study demonstrates that AhR agonists from human activities accumulate in black-tailed gull eggs on the West Sea Coast of Korea. Although black-tailed gull eggs are not consumed by humans, they serve as sentinel indicators of pollutant transfer. As air-breathing top predators, seabirds share ecological similarities with humans, and their eggs reflect contaminant transfer across air–marine–biological systems. Furthermore, contaminants in eggs represent a pathway for maternal transfer to the next generation and provide a valuable long-term indicator of regional pollution levels. Given that these compounds originate from various anthropogenic sources, implementing practical strategies for environmental management and policy is crucial to mitigate their release into marine ecosystems. In addition, sustained monitoring is needed to evaluate whether emissions of these AhR agonists are effectively reduced as a result of regulatory and policy measures. It is particularly important to evaluate the long-term persistence, bioaccumulation, and ecological impacts of these compounds, as well as their potential risks not only to black-tailed gull populations but also to broader marine food webs. This study provides essential insights into the severe impacts of chemical contamination on avian ecosystems and elucidates the mechanisms of pollutant transfer, enhancing our understanding of the ecological risks posed by these contaminants.

CRediT authorship contribution statement

Jihyun Cha: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation, Conceptualization. **Jiyun Gwak:** Investigation, Formal analysis, Data curation. **Junghyun Lee:** Investigation, Formal analysis, Data curation. **Sori Mok:** Investigation, Formal analysis, Data curation. **Hyo-Bang Moon:** Methodology, Formal analysis, Data curation. **Gi Myung Han:** Investigation, Formal analysis. **Sang Hee Hong:** Writing – review & editing, Investigation, Formal analysis. **Jong Seong Khim:** Writing – review & editing, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Seongjin Hong:** Writing – review & editing, Visualization, Supervision, Project

administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109868.

Data availability

Data will be made available on request.

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Supplementary materials for

Effect-directed analysis and nontarget screening for identifying AhR-active substances in black-tailed gull eggs from South Korea

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References

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Supplementary Methods

Sample Preparation. To prepare the samples, two eggs were combined into a single sample. After weighing 20 g of wet egg, the moisture was completely removed using sodium sulfate, and the samples were extracted with 350 mL of dichloromethane (DCM; J.T. Baker, Phillipsburg, NJ) for 16 h. The raw organic extracts (REs) were then concentrated to 2.0 mL using a rotary evaporator and nitrogen concentrator. To measure the lipid content of the REs, 0.2 mL of the sample was separated and stored in a fume hood for complete evaporation over several days before measurement. Lipid removal from the REs was performed using gel permeation chromatography (GPC) columns packed with Bio-Beads S-X3 (Bio-Rad Laboratories, Hercules, CA). The lipids were removed by eluting the REs with 350 mL of hexane (Honeywell, Charlotte, NC) and DCM mixture (1:1, v/v). The first 100 mL of the eluate was discarded, and the final 250 mL was collected. The REs were then concentrated to approximately 1.8 mL using a rotary evaporator and gentle nitrogen gas, representing a concentration of 20 g of egg sample per 1.8 mL [~11.1 g biota equivalent (BEq) mL^{-1}].

Silica Gel Column and RP-HPLC Fractionations. From the 1.8 mL of REs obtained from black-tailed gull eggs, 1.5 mL was subjected to silica gel column chromatography (70–230 mesh; Sigma-Aldrich, Saint Louis, MO). The remaining 0.3 mL was substituted with dimethyl sulfoxide (DMSO; Sigma-Aldrich) for use in bioassays. The glass column was prepared by packing cotton soaked in hexane and sodium sulfate, followed by 8.0 g of activated silica gel packed with hexane. Sodium sulfate was then added on top of the packed silica gel. The REs were separated into three fractions based on polarity: non-polar (F1), mid-polar (F2), and polar fractions (F3). F1 was collected using 30 mL of hexane, F2 was eluted with 60 mL of 20% DCM

in hexane, and F3 was obtained using 50 mL of 60% DCM in acetone (J.T. Baker). All eluates were concentrated to 1.5 mL using a rotary evaporator and nitrogen gas.

For each 1.5 mL silica gel fraction of the REs, 0.5 mL was allocated for bioassays, 0.5 mL for instrumental analysis, and 0.5 mL for reverse phase (RP)-HPLC fractionation (Agilent 1260; Agilent Technologies, Santa Clara, CA). To identify the major toxic fractions in the egg extracts, F2 was further separated into ten subfractions using RP-HPLC (Hong et al., 2016a). In a previous study, RP-HPLC fractionation conditions were optimized using pure standards, with elution rates of over 85% confirmed (Lee et al., 2020). Compound separation was performed using a C18 column, and the resulting subfractions were concentrated to 0.5 mL using a rotary evaporator and nitrogen gas. Of this, 0.2 mL was allocated for bioassays and 0.3 mL for instrumental analysis.

Supplementary Tables

Table S1. Sample information of eggs of black-tailed gulls in the West Sea of South Korea, including length, width, total mass, mass without shell, and lipid contents.

Samples	Region	Length (mm)	Width (mm)	Total mass (g)	Mass without shell (g)	Lipid contents (%)
EG1	Nando	62	46	66	60	6.8
		62	44	64	57	
EG2	Nando	64	46	70	63	8.5
		66	44	67	60	
EG3	Nando	63	43	61	56	6.8
		63	44	65	58	
EG4	Gyeongyeolbi-yeoldo	65	44	66	60	7.7
		60	45	64	58	
EG5	Gyeongyeolbi-yeoldo	62	43	60	54	7.8
		62	44	65	59	

Table S2. Concentration ranges, slopes, coefficient of determination (R^2), and effective concentrations (EC_{20}, EC_{50}, and EC_{80}) for the positive control (benzo[*a*]pyrene) derived from H4IIE-*luc* bioassays dose-response tests in this study and compared with results from previous studies.

Concentration range (nM)[a]	Slope	Coefficient of determination (R^2)	Effective concentrations (log fmol/well)			References
			EC_{20}	EC_{50}	EC_{80}	
0.2–50	45	0.99	2.1	2.8	3.5	This study
0.2–50	52	0.99	2.6	3.2	3.8	Cha et al. (2021)
0.2–50	57	0.99	2.6	3.1	3.6	Cha et al. (2022)
0.2–50	43	0.99	2.3	3.0	3.7	Cha et al. (2023)

[a] Six concentrations (50, 17, 5.6, 1.9, 0.62, and 0.21 nM) by 3-fold serial dilution.

Table S3. List of target compounds, abbreviations, quantification and confirmation ions, limit of detection, and coefficients of determination (R^2) of dose-response curves for the analysis of traditional polycyclic aromatic hydrocarbons (t-PAHs), emerging PAHs (e-PAHs), and styrene oligomers (SOs).

Compounds	Abb.[a]	Quantification ion (*m/z*)	Confirmation ion (*m/z*)	LOD[b]	R^2 of dose-response[c]
Traditional polycyclic aromatic hydrocarbons (t-PAHs)					
Acenaphthylene	Acl	152	151, 150	0.06	0.9999
Acenaphthene	Ace	153	154, 152	0.12	0.9993
Fluorene	Flu	166	165, 167	0.15	0.9997
Phenanthrene	Phe	178	176, 179	0.06	0.9997
Anthracene	Ant	178	176, 179	0.07	0.9980
Fluoranthene	Fl	202	200, 101	0.09	0.9997
Pyrene	Py	202	200, 101	0.12	0.9996
Benzo[*a*]anthracene	BaA	228	226, 229	0.06	0.9937
Chrysene	Chr	228	226, 229	0.03	0.9977
Benzo[*b*]fluoranthene	BbF	252	253, 250	0.08	0.9923
Benzo[*k*]fluoranthene	BkF	252	253, 251	0.20	0.9958
Benzo[*a*]pyrene	BaP	252	253, 126	0.09	0.9981
Indeno[*1,2,3-cd*]pyrene	IcdP	276	138, 137	0.09	0.9900
Benzo[*g,h,i*]perylene	BghiP	276	138, 137	0.12	0.9938
Dibenz[*a,h*]anthracene	DbahA	278	276, 279	0.07	0.9944
Emerging PAHs (e-PAHs)					
11H-Benzo[*a*]fluorene	11BaF	216	215, 213	0.04	0.9993
11H-Benzo[*b*]fluorene	11BbF	216	215, 213	0.04	0.9995
Benzo[*b*]naphtho[*2,3-d*]furan	BBNF	218	189, 219	0.32	0.9989
Benzo[*b*]anthracene	BbA	228	226, 229	0.12	0.9959
Benzo[*b*]naphtho[*2,1-d*]thiophene	BBNT	234	235, 232	0.12	0.9989
4,5-Methanochrysene	4,5MC	239	240, 241	2.0	0.9967
5-Methylbenzo[*a*]anthracene	5MBA	242	241, 239	0.20	0.9923
1-Methylchrysene	1MC	242	241, 239	0.14	0.9914
3-Methylchrysene	3MC	242	241, 239	0.12	0.9963
7-Methylbenz[*a*]anthracene	7MbA	242	241, 239	0.09	0.9931
Benzo[*j*]fluoranthene	BjF	252	253, 250	0.52	0.9917
7,12-Dimethylbenz[*a*]anthracene	7,12DbA	256	241, 239	0.05	0.9923
10-Methylbenzo[*a*]pyrene	10MbA	266	256, 263	0.32	0.9958
20-Methylcholanthrene	20Mc	268	252, 253	0.12	0.9990
Styrene oligomers (SOs)					
1,3-Diphenylpropane	SD1	105	196, 105	0.19	0.9999
cis-1,2-Diphenylcyclobutane	SD2	78	208, 75	0.19	0.9999
2,4-Diphenyl-1-butene	SD3	104	208, 104	0.89	0.9960
trans-1,2-Diphenylcyclobutane	SD4	78	208, 78	0.11	0.9992
4,6-Triphenyl-1-hexene	ST1	117	194, 117	0.63	0.9945
1e-Phenyl-4e-(1-phenylethyl)-tetralin	ST2	129	207, 129	0.66	0.9948
1a-Phenyl-4e-(1-phenylethyl)-tetralin	ST3	129	207, 129	0.31	0.9960
1a-Phenyl-4a-(1-phenylethyl)-tetralin	ST4	129	207, 129	0.31	0.9291
1e-Phenyl-4a-(1-phenylethyl)-tetralin	ST5	207	105, 129	0.70	0.9970
1,3,5-Triphenylcyclohexane	ST6	117	104, 130	0.41	0.9928
Internal standard					
2-Fluorobiphenyl	IS	172	171, 170		

[a]: Abbreviation.
[b]: Limit of detection.
[c]: Coefficient of determination of the calibration curve used to quantify each compound.

Table S4. Stability of IS abundances in calibration series for PAHs and SOs and in five egg samples.

Sample	IS abundances
PAHs STD 1	46463
PAHs STD 2	49693
PAHs STD 3	47375
PAHs STD 4	44335
PAHs STD 5	48930
PAHs STD 6	55341
PAHs STD 7	51469
SOs STD 1	61692
SOs STD 2	52958
SOs STD 3	54725
SOs STD 4	54382
SOs STD 5	61421
SOs STD 6	63154
SOs STD 7	53440
EG1	46516[a]/55750[b]
EG2	60414/57182
EG3	82624/58850
EG4	58585/57124
EG5	59717/56520
RSD for PAHs (%)	19%
RSD for SOs (%)	5.9%

[a]: IS abundances for PAHs

[b]: IS abundances for SOs

Table S5. Instrumental conditions for the analysis of t-PAHs, e-PAHs, and SOs in egg extracts from black-tailed gulls using GC-MSD.

Instrument	GC: Agilent Technologies 7890B, MSD: Agilent Technologies 5977B	
Samples	Mid-polar fractions from EG1–EG5	
Analytical column	DB-5MS (30 m × 0.25 mm i.d. × 0.25 μm film)	
Carrier gas	Helium	
Injection volume	1.0 μL	
Flow rate	1.0 mL min^{-1}	
Mass range	50–600 *m/z*	
Ion source temperature	230 °C	
Ionization mode	EI mode (70 eV)	
Oven temperature	t-PAHs and e-PAHs	60 °C (hold 2 min) → 6 °C min^{-1} to 300 °C (hold 13 min)
	SOs	60 °C (hold 2 min) → 6 °C min^{-1} to 300 °C (hold 3 min)

Table S6. Relative potency values for mid-polar AhR agonists reported from previous studies. References indicate the source of the potency data for each compound.

Compounds	Effective concentrations[a]	Relative potency values	References
t-PAHs			
BaA	EC_{50}	3.2×10^{-1}	Kim et al. (2019)
Chr	EC_{50}	8.5×10^{-1}	
BbF	EC_{50}	5.0×10^{-1}	
BkF	EC_{50}	4.8×10^{-1}	
BaP	EC_{50}	1.0	
IcdP	EC_{50}	5.8×10^{-1}	
DbahA	EC_{50}	6.6×10^{-1}	
e-PAHs			
11BaF	EC_{50}	1.2	Cha et al. (2019)
4,5MC	EC_{50}	1.0	
BbA	EC_{50}	10.6	
10MbA	EC_{50}	1.2	Gwak et al. (2022)
20Mc	EC_{50}	3.2	
7MbA	EC_{50}	1.4	
7,12DbA	EC_{50}	2.0×10^{-1}	
11BbF	EC_{50}	2.4×10^{-1}	Kim et al. (2019)
BBNT	EC_{50}	3.6×10^{-2}	
BBNF	EC_{50}	8.2×10^{-2}	
BjF	EC_{50}	1.7	
5MBA	EC_{50}	4.2×10^{-1}	
1MC	EC_{50}	6.0	
3MC	EC_{50}	1.5	
SOs			
SD1	EC_{20}	2.3×10^{-3}	Hong et al. (2016b)
SD3	EC_{20}	3.0×10^{-4}	
ST2	EC_{20}	2.7×10^{-3}	

[a]: Relative potency values for AhR agonists were calculated based on effective concentrations at the 20% or 50% level observed in benzo[*a*]pyrene.

Table S7. Instrumental conditions for GC-QTOFMS used in nontarget screening of F2.3 and F2.6–F2.9 fractions from egg extracts of black-tailed gulls (EG2 and EG4).

Instrument	GC: Agilent Technologies 7890B; QTOFMS: Agilent Technologies 7200
Samples	F2.3 and F2.6–F2.9 RP-HPLC fractions from EG2 and EG4
Inlet temperature	240 °C
Column	DB-5MS UI (30 m × 0.25 mm i.d. × 0.25 μm film)
Oven temperature	50 ℃ (3 min) – 150 ℃ (10℃/min) – 240 ℃ (20 ℃/min, 10 min)
Carrier gas	Helium
Flow rate	1.2 mL $min.^{-1}$
Injection volume	2 μL
Transfer line temperature	250 ℃
Ionization mode	EI mode (70 eV)
Ion source temperature	230 °C
Mass range	50–1000 *m*/*z*
Scan speed	3 spectra/sec
Monitoring mode	4 Ghz high-resolution
Software	Qualitative analysis B.07.01 MassHunter Quantitative analysis Unknown analysis NIST Library (ver. 2017)

Table S8. Summary of reproducibility test results for 18 spiked compounds in the sediment matrix analyzed by NTS.

Compounds	Molecular formula	M.W.[a]	Library RI[b]	Component RI			Component area			DF[c]	RSD[d] (%)
				Test-1	Test-2	Test-3	Test-1	Test-2	Test-3		
2-Propanol, 1-chloro-, phosphate	$C_9H_{18}Cl_3O_4P$	326	1814	1747	1747	1747	219016	216425	259973	100	11
Alachlor	$C_{14}H_{20}ClNO_2$	269	1894	1852	1852	1852	2051656	1618162	1920673		12
alpha-Endosulfan	$C_9H_6Cl_6O_3S$	404	2114	2092	2092	2092	1584331	1746512	1549581		6.5
Benzyl butyl phthalate	$C_{19}H_{20}O_4$	312	2341	2297	2297	2297	1688217	1771812	1788388		3.1
Butachlor	$C_{17}H_{26}ClNO_2$	311	2128	2080	2080	2080	4189895	4195372	4503948		4.2
Chlorpyrifos	$C_9H_{11}Cl_3NO_3PS$	349	1975	1933	1933	1933	1311223	1425049	1807887		17
Diazinone	$C_{12}H_{21}N_2O_3PS$	304	1791	1745	1745	1745	900624	847505	989410		7.9
Dibutyl phthalate	$C_{16}H_{22}O_4$	278	1965	1914	1914	1914	24992156	24033692	24966025		2.2
Dichlorvos	$C_4H_7Cl_2O_4P$	220	1249	1216	1216	1216	632844	701748	749760		8.5
Dicyclohexyl phthalate	$C_{20}H_{26}O_4$	330	2498	2460	2460	2460	1759726	1842487	2197825		12
Diethyl phthalate	$C_{12}H_{14}O_4$	222	1594	1556	1556	1556	6695713	6555519	6404434		2.2
Endosulfan sulfate	$C_9H_6Cl_6O_4S$	420	2329	2293	2293	2293	2650506	2043035	2686998		15
Etofenprox	$C_{25}H_{28}O_3$	376	2869	2801	2801	2801	2232516	2279133	2327181		2.1
Methoxychlor	$C_{16}H_{15}Cl_3O_2$	344	2454	2429	2429	2429	1368151	1343061	1465472		4.6
Pyrene	$C_{16}H_{10}$	202	2100	2035	2035	2035	563252	539105	735269		18
Tri(2-chloroethyl) phosphate	$C_6H_{12}Cl_3O_4P$	284	1779	1717	1717	1717	409782	404126	362732		6.5
Triethyl phosphate	$C_6H_{15}O_4P$	182	1130	1092	1092	1092	448125	544410	402253		16
Triphenyl phosphate	$C_{18}H_{15}O_4P$	326	2389	2346	2346	2346	2287616	2614895	2715867		8.8

[a] M.W.: Molecular weight.
[b] RI: Retention time index.
[c] DF: Detection frequency.
[d] RSD: Relative standard deviation calculated from the component areas of three replicate analyses.

Table S9. Abbreviations, relative potency values, quantification ion, confirmation ion, and limit of detections for a novel mid-polar AhR agonist identified in egg extracts of black-tailed gulls.

Compound	Abb.[a]	Relative potency value[b]	Quantification ion (*m/z*)	Confirmation ion (*m/z*)	Limit of detection
1,4-Dicyclohexylbenzene	1,4DCHB	7.0×10^{-4}	242	117, 159	0.25

[a]: Abbreviation.

[b]: Relative potency value for a novel AhR agonist was calculated based on effective concentration at the 20% level observed in benzo[*a*]pyrene.

Table S10. Concentrations of t-PAHs, e-PAHs, and SOs in egg extracts of black-tailed gulls in the West Sea of South Korea.

Compounds	EG1	EG2	EG3	EG4	EG5
	(ng g^{-1} wet mass)				
t-PAHs					
Acl	0.17	0.16	0.40	0.13	0.08
Ace	0.18	0.21	2.6	0.16	ND[a]
Flu	0.74	0.88	1.3	0.62	0.30
Phe	1.7	2.0	2.4	1.3	0.59
Ant	0.40	0.29	0.89	0.19	0.07
Fl	0.61	0.73	2.5	0.56	0.26
Py	0.48	0.53	0.61	0.40	0.27
BaA	0.10	0.11	0.11	0.88	0.55
Chr	0.28	0.24	0.25	0.25	0.15
BbF	0.21	0.18	ND	0.14	ND
BkF	ND	ND	ND	ND	ND
BaP	ND	ND	ND	ND	ND
IcdP	0.99	0.32	0.13	0.12	0.91
BghiP	0.17	ND	ND	ND	ND
DbahA	0.15	0.09	ND	ND	0.11
SUM	6.2	5.8	11	4.8	3.3
e-PAHs					
11BaF	0.31	0.31	0.35	0.32	0.21
11BbF	ND	ND	ND	ND	ND
BBNF	1.5	1.4	1.4	1.4	1.1
BbA	0.16	0.20	0.16	0.21	0.24
BBNT	0.26	0.15	ND	0.13	0.13
4,5MC	ND	ND	ND	ND	ND
5MBA	ND	ND	ND	ND	ND
1MC	ND	ND	ND	ND	ND
3MC	ND	ND	ND	ND	ND
7MbA	ND	ND	ND	ND	ND
BjF	ND	ND	ND	ND	ND
7,12DbA	0.94	0.42	0.33	ND	ND
10MbA	1.4	0.62	0.70	1.2	1.1
20Mc	1.4	0.41	2.0	0.56	0.54
SUM	6.0	3.5	5.0	3.8	3.3
SOs					
SD1	0.23	0.23	0.28	0.12	0.27
SD2	ND	ND	ND	ND	0.40
SD3	9.6	5.7	12	9.2	1.5
SD4	0.47	0.49	0.60	0.41	0.56
ST1	2.0	0.86	ND	ND	ND
ST2	ND	ND	ND	ND	ND
ST3	ND	ND	ND	ND	0.76
ST4	2.2	1.3	2.0	2.3	ND
ST5	ND	ND	ND	ND	ND
ST6	ND	ND	ND	ND	1.1
SUM	15	8.5	14	12	4.6

[a] ND: Not detected.

Table S11. Concentrations of PAHs in seabird eggs obtained from this study and previous studies.

Common name	Region	Sampling year	# of targets	Concentrations (ng g^{-1} wm)			References
				Min	Max	Mean	
Black-tailed gull (*Larus crassirostris*)	Korea	2019	29	6.6	16	11	This study
Gannet (*Morus Bassanus*)	Scotland	2002	52			5.0	Pereira et al.
Golden Eagles (*Aquila chrysaetos*)						4.2	(2009)
Guilemot (*Uria aalge*)	Ireland	2017	15	1.8	2.1	2.0	Power et al.
Gannet (*Morus Bassanus*)				1.5	1.7	1.6	(2021)
Arctic Tern (*Sterna paradisaea*)		2018		1.4	1.5	1.4	
Common Tern (*Sterna hirundo*)				0.80	1.4	1.0	
Thick-billed murre (*Uria lomvia*)	Norway	2007	16	ND[a]	0.60	0.40	Miljeteig and
				ND	0.50	0.30	Gabrielsen (2010)
Common eider (*Somateria millisima*)		2012	16	0.63	1.4	1.0	Huber et al.
European shag (*Phalacrocorax aristotelis*)				ND	1.1	0.60	(2015)
Herring gull (*Larus argentatus*)				ND	30	7.6	

[a] ND: Not detected.

Table S12. Potency balance between BaP-EQ$_{chem}$ and BaP-EQ$_{bio}$ concentrations in F2 of the egg extracts of black-tailed gull from Korea.

Compounds	Abbreviation	EG1	EG2	EG3	EG4	EG5
Known mid-polar AhR agonists (BaP-EQ$_{chem}$, ng g^{-1} wm)						
t-PAHs						
Benzo[*a*]anthracene	BaA	4.0×10^{-2}	4.0×10^{-2}	4.0×10^{-2}	3.1×10^{-1}	1.9×10^{-1}
Chrysene	Chr	2.6×10^{-1}	2.2×10^{-1}	2.3×10^{-1}	2.3×10^{-1}	1.5×10^{-1}
Benzo[*b*]fluoranthene	BbF	1.0×10^{-1}	9.0×10^{-2}	ND[a]	7.0×10^{-2}	ND
Benzo[*k*]fluoranthene	BkF	ND	ND	ND	ND	ND
Benzo[*a*]pyrene	BaP	ND	ND	ND	ND	ND
Indeno[*1,2,3-cd*]pyrene	IcdP	5.3×10^{-1}	1.7×10^{-1}	7.0×10^{-2}	6.0×10^{-2}	4.8×10^{-1}
Dibenz[*a,h*]anthracene	DbahA	9.0×10^{-2}	6.0×10^{-2}	ND	ND	7.0×10^{-2}
e-PAHs						
11H-Benzo[*a*]fluorene	11BaF	3.3×10^{-1}	3.3×10^{-1}	3.7×10^{-1}	3.4×10^{-1}	2.2×10^{-1}
4,5-Methanochrysene	4,5MC	ND	ND	ND	ND	ND
Benzo[*b*]anthracene	BbA	1.9	2.4	1.9	2.5	2.9
10-Methylbenzo[*a*]pyrene	10MbA	1.7	7.3×10^{-1}	8.1×10^{-1}	1.4	1.2
20-Methylcholanthrene	20Mc	4.1	1.2	6.0	1.7	1.6
7-Methylbenz[*a*]anthracene	7MbA	ND	ND	ND	ND	ND
7,12-Dimethylbenz[*a*]anthracene	7,12DbA	1.7×10^{-1}	8.0×10^{-2}	6.0×10^{-2}	ND	ND
11H-Benzo[*b*]fluorene	11BbF	ND	ND	ND	ND	ND
Benzo[*b*]naphtho[*2,1-d*]thiophene	BBNT	1.0×10^{-2}	1.0×10^{-2}	ND	5.0×10^{-3}	1.0×10^{-2}
Benzo[*b*]naphtho[*2,3-d*]furan	BBNF	1.4×10^{-1}	1.3×10^{-1}	1.4×10^{-1}	1.4×10^{-1}	1.0×10^{-1}
Benzo[*j*]fluoranthene	BjF	ND	ND	ND	ND	ND
5-Methylbenzo[*a*]anthracene	5MBA	ND	ND	ND	ND	ND
1-Methylchrysene	1MC	ND	ND	ND	ND	ND
3-Methylchrysene	3MC	ND	ND	ND	ND	ND
SOs						
1,3-Diphenylpropane	SD1	6.7×10^{-4}	6.7×10^{-4}	8.3×10^{-4}	3.7×10^{-4}	8.0×10^{-4}
2,4-Diphenyl-1-butene	SD3	3.5×10^{-3}	2.1×10^{-3}	4.2×10^{-3}	3.3×10^{-3}	5.4×10^{-4}
1e-Phenyl-4e-(1-phenylethyl)-tetralin	ST2	ND	ND	ND	ND	ND
Novel mid-polar AhR agonist (BaP-EQ$_{chem}$, ng g^{-1} wm)						
1,4-Dicyclohexylbenzene	1,4DCHB	4.0×10^{-3}	2.3×10^{-3}	2.0×10^{-4}	1.0×10^{-3}	7.0×10^{-4}
BaP-EQ$_{chem}$ (ng g^{-1} wm)[b]		9.4	5.5	9.6	6.7	6.9
BaP-EQ$_{bio}$ (ng g^{-1} wm)[c]		2.3×10	2.5×10	1.6×10	3.1×10	4.7×10
Contribution (%)		41	22	61	22	15

[a] ND: Not detected. [b] Calculated by multiplying the concentrations of known and novel mid-polar AhR agonists by their ReP values. [c] Calculated from dose-response relationships in F2 of egg extracts (based on EC$_{50}$).

Table S13. Number of AhR agonist candidates detected in subfractions of egg extracts from EG2 and EG4 at each step of the five-step selection criteria.

Samples	Fractions	Step 1	Step 2	Step 3	Step 4	Step 5
EG2	F2.3	2642	329	113	23	0
	F2.6	2422	353	119	29	4
	F2.7	2448	355	119	35	5
	F2.8	3216	364	119	43	4
	F2.9	2166	337	109	26	2
EG4	F2.3	2902	463	138	41	3
	F2.6	2594	371	119	27	3
	F2.7	2544	371	119	32	0
	F2.8	2584	370	109	33	3
	F2.9	2528	364	103	27	3

Table S14. List of compounds with aromatic rings(s) detected in egg extracts of black-tailed gulls using GC-QTOFMS.

Samples	Fractions and compounds	Molecular formula	CAS number	M.W.[a]	Component RI[b]	Library RI	Matching factor	Shape quality	AhR activity
EG2	**F2.3**								
	–[c]								
	F2.6								
	2,2'5,5'-Tetramethylbiphenyl	$C_{16}H_{18}$	3075-84-1	210.314	1695	1820	84	86	–[d]
	1,1-Bis(p-toly)ethane	$C_{16}H_{18}$	530-45-0	210.314	1676	1728	76	78	
	1-(3-Methyl-cyclopent-2-enyl)-cyclohexene	$C_{12}H_{18}$	997100-40-8	162.271	1398	1303	86	91	
	3,4-Dimethyl-7-exo-methylene-bicyclo[4.3.0]non-3-ene	$C_{12}H_{18}$	997100-60-9	162.271	1320	1209	72	71	
	F2.7								
	p,p'-DDE	$C_{14}H_8Cl_4$	72-55-9	318.025	2156	2240	94	89	
	1,1,3-Triphenylpropane	$C_{21}H_{20}$	19120-39-9	272.384	2337	2274	78	84	
	α-Methylstyrene dimer	$C_{18}H_{20}$	6362-80-7	236.351	1772	1846	71	74	–
	2,6-Di-iso-propylnaphthalene	$C_{16}H_{20}$	24157-81-1	212.330	1659	1727	81	77	–
	Calamenene	$C_{15}H_{22}$	483-77-2	202.335	1517	1537	85	74	
	F2.8								
	1,4-Dicyclohexylbenzene	$C_{18}H_{26}$	1087-02-1	242.399	1879	2028	85	82	+[e]
	4-Phenylbicyclohexyl	$C_{18}H_{26}$	20273-27-2	242.399	1982	1976	79	90	
	2-Phenyl-1,1'-bi(cyclohexan)-1-ene	$C_{18}H_{24}$	24636-55-3	240.383	2072	2015	71	87	
	2-Phenyldecane	$C_{16}H_{26}$	4537-13-7	218.378	1590	1624	79	73	
	F2.9								
	6-Phenyltridecane	$C_{19}H_{32}$	4534-49-0	260.457	1801	1922	83	78	
	5-Phenyltridecane	$C_{19}H_{32}$	4534-50-3	260.457	1808	1922	86	78	
EG4	**F2.3**								
	1-(2',4'-Dichlorophenacyl)pyrazole	$C_{11}H_8Cl_2N_2O$	108664-58-0	255.100	1908	1983	81	77	
	Diethyl phthalate	$C_{12}H_{14}O_4$	84-66-2	222.237	1577	1639	87	72	–
	2,4-Dimethylbenzaldehyde	$C_9H_{10}O$	15764-16-6	134.175	1217	1208	84	84	–
	F2.6								
	2-[(Butoxycarbonyl)oxy]phenyl 4-butylbenzoate	$C_{22}H_{26}O_5$	997769-33-9	370.439	2643	2799	70	80	
	Bi-2-cyclohexen-1-yl	$C_{12}H_{18}$	1541-20-4	162.271	1329	1305	87	89	
	2,2'5,5'-Tetramethylbiphenyl	$C_{16}H_{18}$	3075-84-1	210.314	1695	1820	79	81	–
	F2.7								
	–								
	F2.8								

1,1,3,3,5,5-Hexamethyl-1,5-diphenyl-trisiloxane	$C_{18}H_{28}O_2Si_3$	17977-72-9	360.670	1720	1845	78	71	–
4-Phenylbicyclohexyl	$C_{18}H_{26}$	20273-27-2	242.399	1983	1976	81	87	
3-Phenylundecane	$C_{17}H_{28}$	4536-87-2	232.404	1652	1724	80	72	
F2.9								
6-Phenyltridecane	$C_{19}H_{32}$	4534-49-0	260.457	1801	1922	80	71	
3-Phenyldodecane	$C_{18}H_{30}$	2400-00-2	246.431	1747	1823	80	76	
2-Phenyldodecane	$C_{18}H_{30}$	2719-61-1	246.431	1785	1823	85	77	

[a] M.W.: Molecular weight.
[b] RI: Retention time index.
[c] –: Not detected.
[d] –: Not significant response in the H4IIE-*luc* bioassay.
[e] +: Significant response in the H4IIE-*luc* bioassay.

Table S15. Concentrations of a novel mid-polar AhR agonist in egg extracts of black-tailed gulls in the West Sea of South Korea.

Compounds	EG1	EG2	EG3	EG4	EG5
	(ng g^{-1} wet mass)				
1,4DCHB	5.1	2.9	0.25	1.3	0.84

Table S16. Partition coefficients, such as log K_{OA}, log K_{AW}, and log K_{OW} values, for target compounds, novel AhR agonist, and AhR agonist candidates identified in this study. The partition coefficients of the chemicals were obtained from ChemSpider (www.chemspider.com) (accessed 2024).

Compounds	Log K_{OA}	Log K_{AW}	Log K_{OW}
t-PAHs			
Acl	6.23	–2.29	3.94
Ace	6.31	–2.39	3.92
Flu	6.79	–2.61	4.18
Phe	7.57	–3.11	4.46
Ant	7.55	–3.10	4.45
Fl	8.88	–3.72	5.16
Py	8.80	–3.92	4.88
BaA	9.07	–3.31	5.76
Chr	9.48	–3.67	5.81
BbF	10.35	–4.57	5.78
BkF	10.73	–4.62	6.11
BaP	7.82	–1.83	5.99
IcdP	8.79	–2.03	6.76
BghiP	11.97	–5.27	6.70
DbahA	11.24	–4.70	6.54
e-PAHs			
11BaF	7.79	–2.69	5.10
11BbF	8.95	–3.18	5.77
BBNF	8.84	–3.79	5.05
BbA	9.45	–3.69	5.76
BBNT	9.14	–3.95	5.19
4,5MC	9.86	–4.08	5.78
5MBA	9.72	–3.65	6.07
1MC	9.72	–3.65	6.07
3MC	9.72	–3.65	6.07
7MbA	9.72	–3.65	6.07
BjF	9.93	–4.15	5.78
7,12DbA	9.40	–3.60	5.80
10MbA	11.10	–4.44	6.66
20Mc	10.34	–3.92	6.42
SOs			
SD1	6.69	–1.46	5.23
SD2	7.15	–1.69	5.46
SD3	7.36	–1.72	5.64
SD4	7.15	–1.69	5.46
ST1	10.83	–2.57	8.26
ST2	10.24	–2.62	7.62
ST3	10.24	–2.62	7.62
ST4	10.24	–2.62	7.62
ST5	10.24	–2.62	7.62
ST6	10.62	–2.54	8.08
Novel AhR agonist			
1,4DCHB	7.68	–0.05	7.63
AhR agonist candidates			
2,4-Dimethylbenzaldehyde	–[a]	–	–
Diethyl phthalate	–	–	–
1-(2',4'-Dichlorophenacyl)pyrazole	9.37	–6.63	2.74
2,2',5,5'-Tetramethylbiphenyl	7.55	–1.60	5.95

1,1-Bis(p-toly)ethane	6.74	–1.63	5.95
1-(3-Methyl-cyclopent-2-enyl)-cyclohexene	4.47	1.15	5.62
3,4-Dimethyl-7-exo-methylene-bicyclo[4.3.0]non-3-ene	3.85	1.15	5.00
2-[(Butoxycarbonyl)oxy]phenyl 4-butylbenzoate	–	–	5.52
Bi-2-cyclohexen-1-yl	4.42	1.01	5.43
Calamenene	6.40	–0.15	6.25
2,6-Di-isopropylnaphthalene	7.17	–1.09	6.08
α-Methylstyrene dimer	7.99	–1.48	6.51
p,p'-DDE	9.28	–2.77	6.51
1,1,3-Triphenylpropane	8.90	–2.55	6.35
4-Phenylbicyclohexyl	7.47	0.03	7.50
2-Phenyl-1,1'-bi(cyclohexan)-1-ene	7.75	–0.21	7.54
2-Phenyldecane	6.57	0.49	7.06
3-Phenylundecane	6.91	0.62	7.53
1,1,3,3,5,5-Hexamethyl-1,5-diphenyl-trisiloxane	8.97	–1.19	7.78
5-Phenyltridecane	7.76	0.86	8.62
6-Phenyltridecane	7.76	0.86	8.62
2-Phenyldodecane	7.45	0.74	8.19
3-Phenyldodecane	7.36	0.74	8.10

[a] –: No information on partition coefficient values.

Supplementary Figures

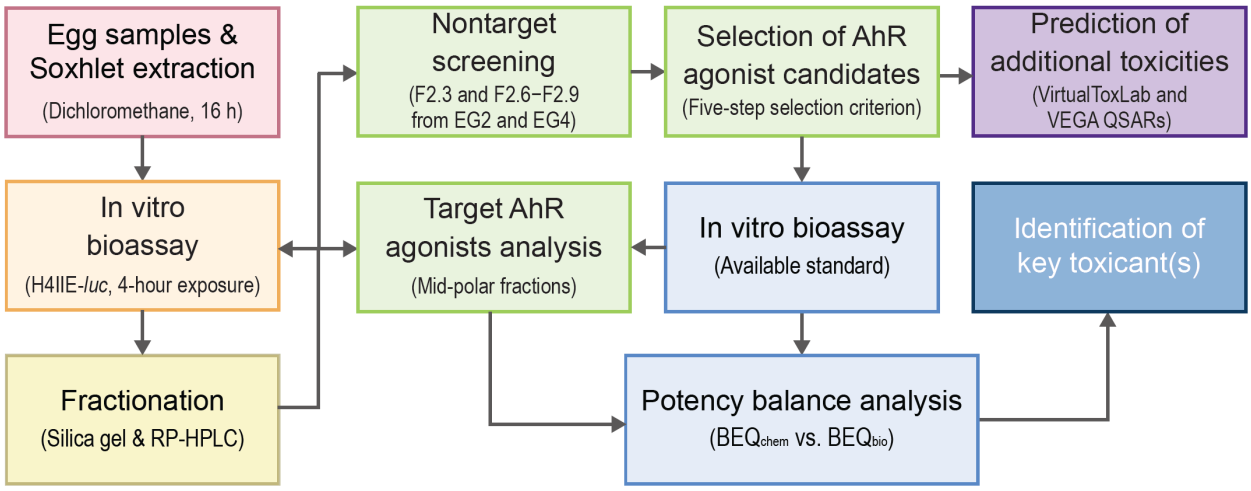

Fig. S1. Flowchart for identifying major AhR agonists in egg extracts of black-tailed gulls in the West Sea of South Korea.

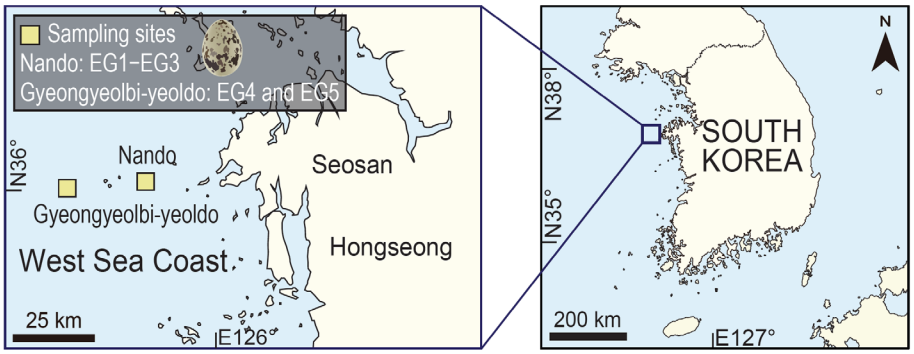

Fig. S2. Map showing the sampling sites of egg samples of black-tailed gulls in the West Sea of South Korea. EG1–EG3 were collected from Nando, and EG4 and EG5 were collected from Gyeongyeolbi-yeoldo in 2019.

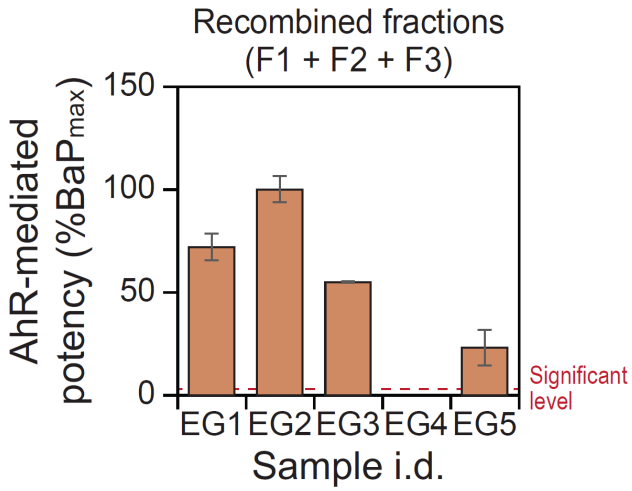

Fig. S3. AhR-mediated potencies of the recombined silica gel fractions (F1, F2, and F3) from black-tailed gull egg samples.

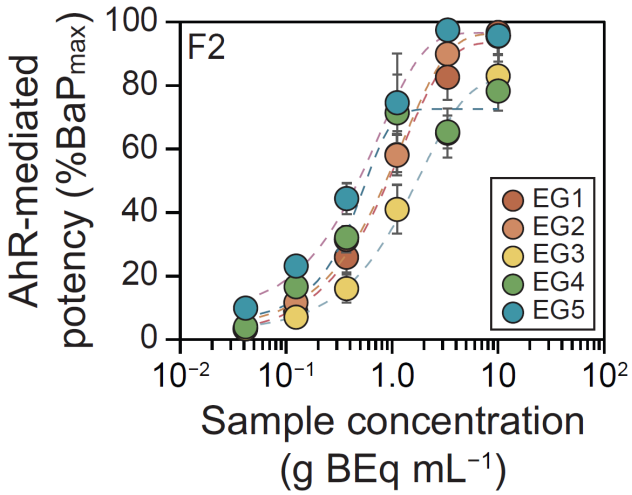

Fig. S4. Dose-response relationships for F2 of egg extracts after 4 hours of exposure in the H4IIE-*luc* bioassay. Error bars represent the mean ± standard deviation (n = 3).

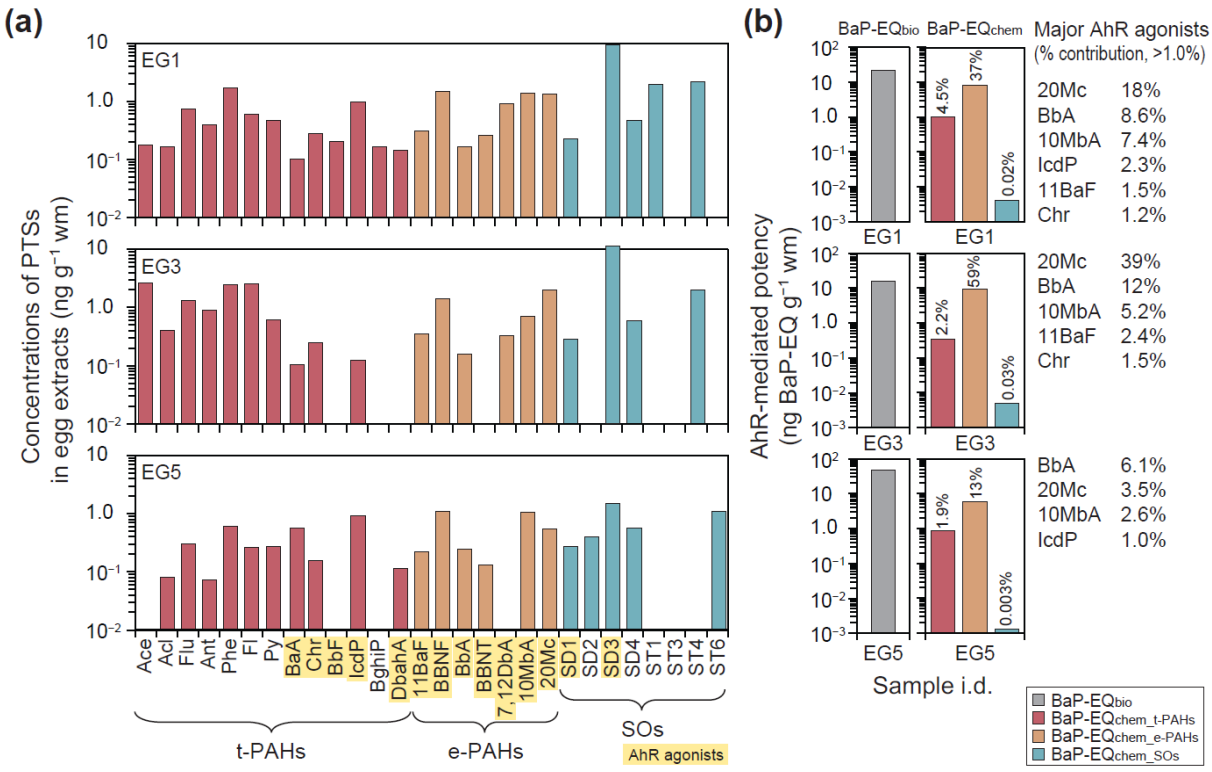

Fig. S5. (a) Concentrations of t-PAHs, e-PAHs, and SOs in egg extracts (EG1, EG3, and EG5) of black-tailed gulls. **(b)** Comparison of instrument-derived BaP-EQ_{chem} and bioassay-derived BaP-EQ_{bio} concentrations in F2 of EG1, EG3, and EG5. Error bars are not shown because the analyses were based on single measurements.

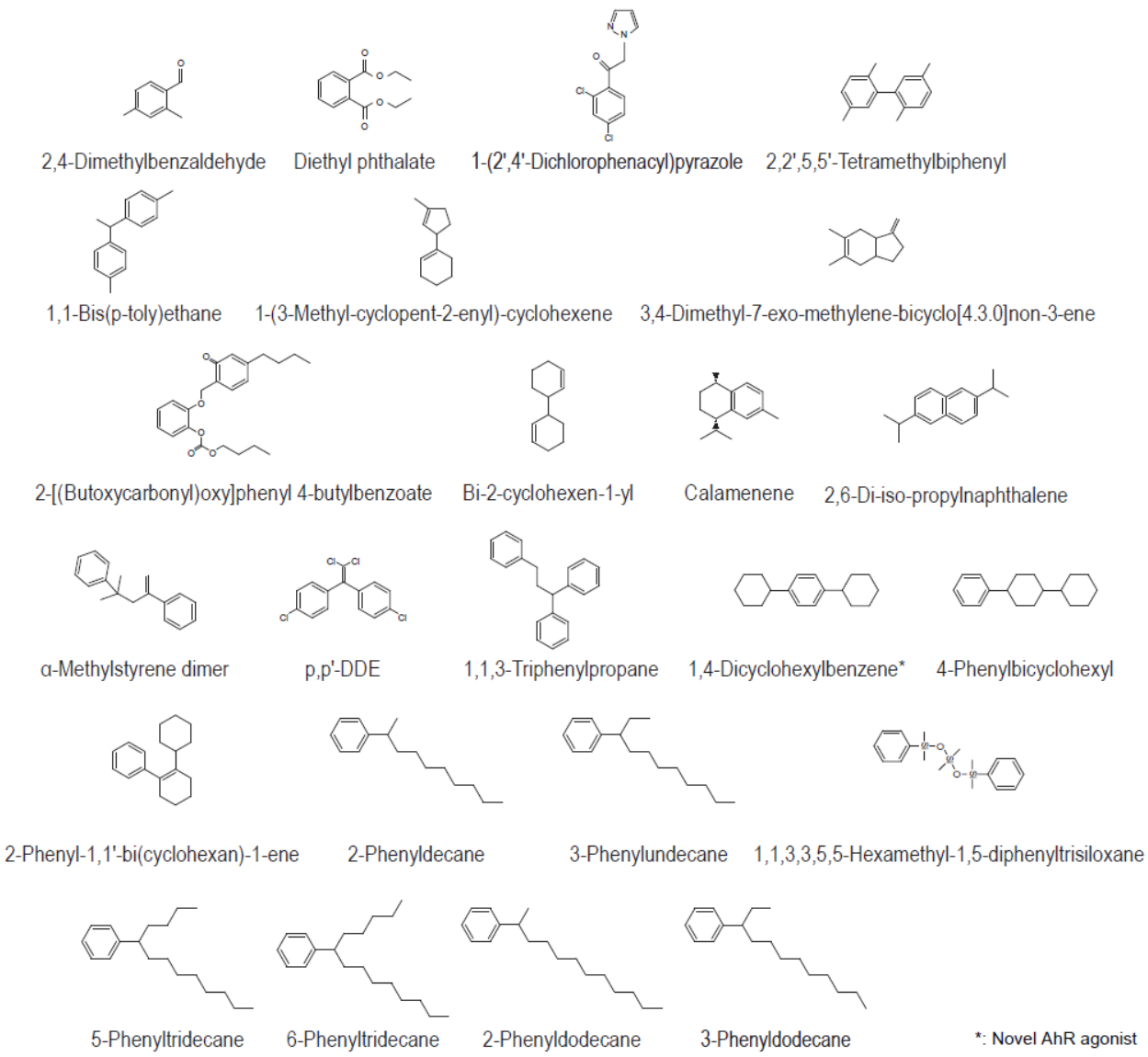

Fig. S6. Chemical structures of 24 AhR agonist candidates identified in F2 subfractions of egg extracts of black-tailed gulls in the West Sea of South Korea.

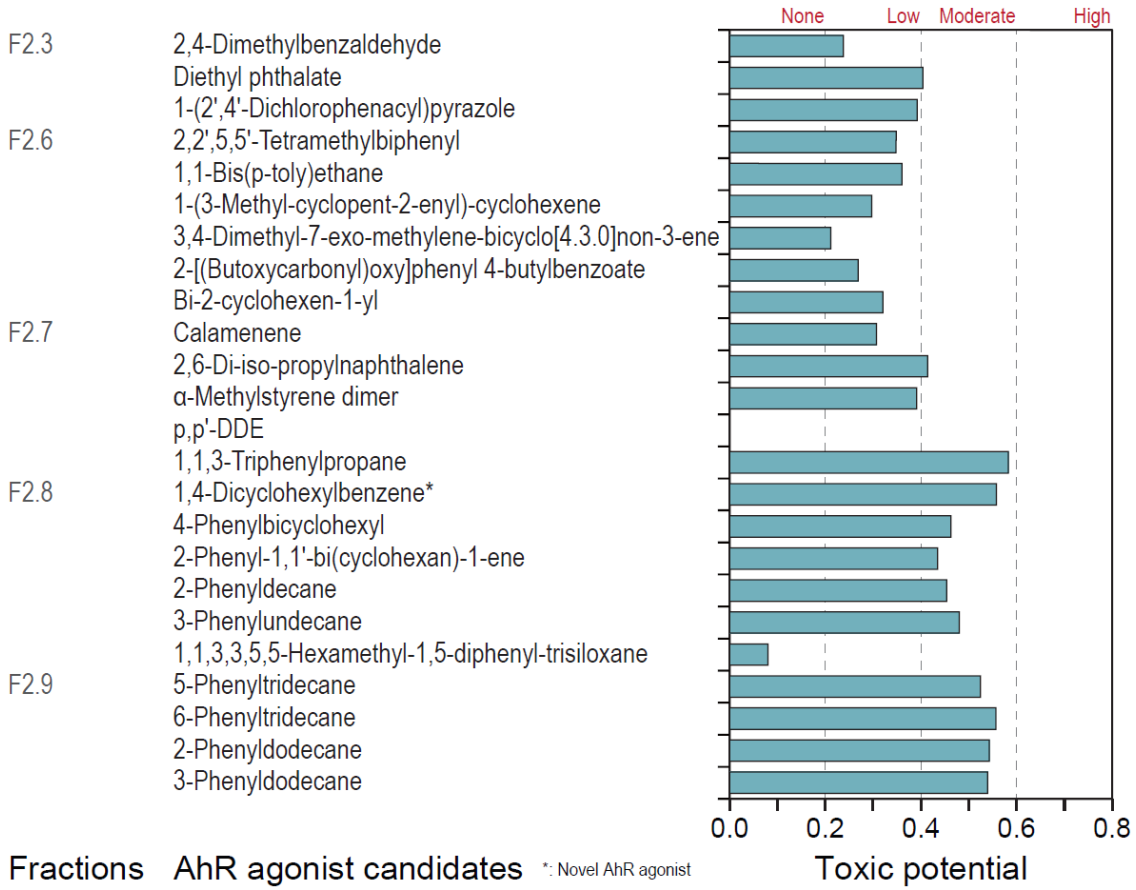

Fig. S7. Predicted toxic potential based on AhR binding affinity for 24 AhR agonist candidates in F2 subfractions of egg extracts using VirtualToxLab in silico modeling. Toxic potential ranges were interpreted as follows: 0.0–0.2 indicates no binding, 0.2–0.4 indicates low binding, 0.4–0.6 indicates moderate binding, and 0.6–0.8 indicates high binding.

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