Determinations of dioxinlike activity in selected mollusks from the coast of the Bohai Sea, China, using the H4IIE-luc bioassay

Maoyong Song\textsuperscript{a,b}, Yan Xu\textsuperscript{b}, Qinting Jiang\textsuperscript{b}, Paul K.S. Lam\textsuperscript{b}, Desmond K. O’Toole\textsuperscript{b}, John P. Giesy\textsuperscript{b,c}, Guibin Jiang\textsuperscript{a,*}

\textsuperscript{a}State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China
\textsuperscript{b}Research Centre for Coastal Pollution and Conservation, Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China
\textsuperscript{c}Department of Zoology, National Food Safety and Toxicology Center, Institute of Environmental Toxicology, Michigan State University, East Lansing, MI 48824, USA

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Abstract

Extracts of mollusks collected from eight cities along the coast of the Bohai Sea, China, were used to determine dioxinlike activity using an in vitro bioassay approach. Dioxinlike activity of total extracts was detected by measuring luciferase activity in a stable transfected rat hepatoma cell line, H4IIE, containing an aryl hydrocarbon receptor (AhR)-responsive element linked to a luciferase reporter gene. Luciferase activity was expressed as a percentage of the maximum response observed for 2,3,7,8-TCDD (% TCDD\textsubscript{max}). All mollusk samples elicited significant dioxinlike activity except those from Dalian (N1). The greatest magnitudes (\pm SD, data ex N1) of activity observed for these extracts ranged from 4.88 \pm 0.48\% to 11.38 \pm 1.43\% TCDD\textsubscript{max}. The mean activity was 7.37 \pm 1.85\% TCDD\textsubscript{max}. Concentrations of TCDD-EQs in mollusk extracts were from 227.4 to 547.5 pg g\textsuperscript{-1} lipid. The cytotoxic effect of the mollusk extracts on cells was assessed at the same time. Six of the mollusk extracts (N2, N3, N4, S1, S2, and S4) caused noticeable growth inhibition over the exposure period at concentrations higher than 0.33\% of the extracts concentrations (0.83 mL extract well\textsuperscript{-1}). All the dilutions of sample N1 were cytotoxic to the H4IIE cells.

Keywords: Bioassay; H4IIE-luc; Dioxinlike activity; Bohai Sea coast; Mollusk

1. Introduction

Many xenobiotic pollutants, which are released into the environment through industrial and municipal wastewaters, represent a potential threat to aquatic ecosystems. Some substances are persistent while others are (bio)transformed into additional compounds, further increasing the apparent number of chemicals released into the environment (Gagné and Blaise, 1998). A group of toxicants of current interest is the dioxinlike compounds that elicit toxic effects similar to those of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). These compounds are persistent and accumulate in the food chain (Ahlborg et al., 1994). Such dioxinlike compounds include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) (Van den Berg et al., 1998), and other compounds that may elicit the aryl hydrocarbon receptor (AhR) mediated biochemical and toxic response (Behnisch et al., 2001). They are of great concern due to their hepatotoxicity, embryotoxicity, teratogenicity, immunotoxicity, dermal toxicity, lethality, carcinogenicity, association with a wasting syndrome, and tumorogenicity, found expressed in many different species at low concentrations (Hilscherova et al., 2000; Peterson et al., 1993).

High-resolution gas chromatography and mass spectrometry (HRGC-MS) are used for the detection and
quantification of dioxinlike compounds and are considered the “Gold Standard” technique (Vanderperren et al., 2004), but they are quite expensive and time-consuming. In the environment, chemicals exist and occur as complex mixtures including different congeners and isomers. Quantitative instrumental analysis of complex mixtures of these chemicals is complicated by the fact that techniques and standards for identifying many of the congeners and isomers do not exist (Garrison et al., 1996). In addition, instrumental analysis provides little information about the additive, synergistic, or antagonistic interactions produced by the chemical mixtures. In recent years, a variety of in vitro assays using cultured cells have been developed as an alternative to the use of animals in assessing the risk to fish and wildlife posed by these dioxinlike compounds, which may also reflect the risk to humans. In this study the stable transfected rat hepatoma cell line, H4IIE, has been applied in the H4IIE-luc assay, which is based on the AhR-dependent mechanism for the detection of dioxinlike compounds. Recombinant H4IIE cells (H4IIE-luc) exhibit AhR-mediated luciferase expression (Aarts et al., 1995). It has proved to be a rapid, sensitive, and relatively inexpensive technique compared with some instrumental analytical methods (Sakai and Takigami, 2003). The Bohai Sea is a semienclosed sea of China and has a coastline length of nearly 3800 km. There are many heavy industries and a variety of fisheries located along the coast. To investigate distribution of these xenobiotic substances four kinds of mollusks distributed along the Bohai Coast that are harvested for human consumption were chosen as our test organisms. Most of the mollusk samples were collected from the important seaports. The dioxinlike activity in the mollusk extracts was studied using the recombinant H4IIE-luc cell line.

2. Materials and methods

2.1. Reagents

The following standards and reagents were used in this study: 2,3,7,8-TCDD (Sigma, Germany), n-hexane (99%) (Merck, Germany), dichloromethane (DCM, 99%) (TEDIA company, USA), silica gel 60, column chromatography (Merck, Germany), acid-activated copper powder, 99% (Fluka), and anhydrous sodium sulphate (Na2SO4) (Sigma, Germany). All the solvents in this study were redistilled.

The following were used for cell culturing and testing: Dulbecco’s Modified Eagle’s Medium (Sigma), the LIVE/DEAD® Viability/Cytotoxicity Kit (L-3224) (Molecular Probes, Invitrogen, Eugene, OR), the luminescence receptor gene assay system (LucLite™ kit) (Packard BioScience, Netherlands). Milli-Q purified water was used throughout. The bioassay was conducted with the rat hepatoma cell line, H4IIE, that has been stably transfected with a receptor gene to allow expression of the luciferase enzyme. The cell line was provided by Department of Biology and Chemistry, City University of Hong Kong.

2.2. Sampling

Fig. 1 shows the eight sampling sites, four of them are spread out along the northern Bohai coast and the other four are along the southern coast. The sampling was carried out in August 2003. Four species were selected for this study: Rapana venosa collected from N1, S1, S3 and S4; Neverita didyma collected from N2 and N4; a species of Amusium collected from N3 and Neptunia cumingii collected from S2. The species of mollusks (Table 1) were identified according to the catalog of marine mollusks (Zhao et al., 1982). The collected mollusks were stored in an ice-cooled box during the transportation and then kept at −20 °C in the laboratory. Before analysis, stainless-steel scalpels blades were used to cut open the mollusks and remove the soft tissues, which were then thoroughly rinsed with Milli-Q water to remove extraneous impurities. Samples were homogenized in a stainless-steel blender, and subsamples were freeze-dried for analysis.

2.3. Sample extraction and clean-up

Sample extraction was performed as follows. Five grams of the freeze-dried mollusk samples was mixed with 15 g anhydrous Na2SO4 to remove residual moisture, 10 g of copper powder to remove any sulfur, and 10 g silica gel. Then the mixture was extracted with 300 mL of a mixture (1:1) of DCM and hexane for 16 h in a Soxhlet apparatus. The procedural blank consisted of the same amount of Na2SO4, silica, and copper powder. After the initial extraction steps the sample was rinsed three times with 15 mL hexane to ensure total recovery of the extract. The extract was evaporated (~40 °C) and eventually transferred to 1 mL n-hexane. The freeze-dried sample (1 g) was put into a glass column; the lipids were eluted with a mixture of hexane and DCM. The lipids extract was evaporated under a gentle N2 stream at 37 °C. Lipids were then gravimetrically determined. The concentrated extract was further cleaned up through a column that contained 10 g of activated florisor (Hilscherova et al., 2000). The extraction aliquot was added to the column. A quantity of 100 mL hexane was eluted to obtain Fraction 1, which contained the PCBs and a portion of the PCDD/PCDFs. A mixture of hexane containing 20% DCM was next used to elute organochlorine (OC) compounds, PAHs, alkylphenol-ethoxylates and the remaining PCDD/PCDFs to obtain Fraction 2. The H4IIE-luc bioassay was performed on each fraction to calculate the contribution that each of these fractions made to the overall AhR-mediated activity.

2.4. Assessment of cytotoxicity of mollusk extract

Prior to testing in the H4IIE-luc assay the extracts were tested for cytotoxicity towards the H4IIE cells. Only a dose range without an obvious cytotoxic effect was applied to the subsequent bioassay. Cytotoxicity testing was conducted with H4IIE cells using the procedure in the LIVE/DEADViability/Cytotoxicity Kit. Live cells retain the polyanionic dye AM
calcine which produces an intense uniform green fluorescence in the live cells. Dead cells retain the dye ethD-1 which produces a bright red fluorescence in the dead cells. Graph the average calcine fluorescence and standard deviation for the data visually. If the blank has greater viability decrease than the other treatment, the solvent may be toxic to the cells. If viability decreases with increasing concentration of the test substance, the test substance may be toxic to the cells. In either of these cases, the luciferase data must be regarded with great suspicion.

2.5. Cell culture and bioassay

Culture conditions and the assay procedure for the recombinant H4IIE rat hepatoma cell line, containing a luciferase reporter gene under control of dioxin-responsive enhancers, have been described previously (Khim et al., 1999a, b). In brief, 60 interior wells of a 96-well culture plate were seeded with 250 μL of a cell suspension to give a density of about 15,000 cells well−1, and 250 μL phosphate-buffered saline (PBS) was added to the outer wells. Cells were incubated overnight prior to dosing with 2.5 μL of standard, solvent, and test extract dissolved in hexane. Standard plates were dosed with 2,3,7,8-TCDD at 30, 10, 3, 1, 0.3, and 0.1 pg well−1, prepared by one-in-two dilutions. Test plates were dosed with mollusk extract at 2.5, 0.83, 0.28, 0.09, 0.03, and 0.01 μL well−1, also prepared by one-in-two dilutions. All additions were done in triplicate. Cells were cultured under aseptic conditions in a humidified CO2 incubator at 37 °C, 5% CO2. The luciferase assay was carried out after 72 h exposure. Briefly, H4IIE cells were washed three times with PBS after removal of culture medium. Cells were lysed with 75 μL PBS containing Ca2+ (1 g L−1 CaCl2) and Mg2+ (1 g L−1 MgCl2). Then 75 μL luciferase assay reagent was added to each well. Plates were incubated for 20 min at room temperature in the dark. Luciferase activities were measured with an automate luminometer (Dynatech ML 3000 luminometer; Chantilly, VA). The detailed method for in vitro assays with the H4IIE-luc cell line has been described elsewhere (Sanderson et al., 1996). Responses of extracts, expressed as the mean relative lucinescence units (RLUs), were converted to relative response units, expressed as a percentage of the maximum response observed for 2,3,7,8-TCDD (% TCDDmax). Comparing to the TCDD standard, bioassay-derived dioxin equivalents (TCDD-EQ) were determined directly from the dose–response relationship generated by the testing sample at multiple levels of dilution.

3. Results and discussion

As shown in Fig. 2, the semilogarithmic dose curve of TCDD used as standard in the H4IIE-luc assay has a sigmoidal appearance. Each point of the curve represents the mean of luciferase response to TCDDmax. The detection limit was calculated separately for each multiwell plate and was shown to be very reproducible. The limit of detection (LOD) and EC50 for luciferase induction by 2,3,7,8-TCDD were 0.15 and 2.21 ± 0.13 pg well−1, respectively. Significant response was defined as those outside the range defined by three times the standard deviation (expressed as % TCDDmax) of the mean blank response (0% TCDDmax). For the testing samples, the extract dilutions (μL well−1) were used as a measure of fluorescence induction instead of the concentration of 2,3,7,8-TCDD.

Cytotoxicity is a significant influencing factor when evaluating the dioxinlike activity of complex extracts. In our study, cytotoxicity was assessed as a viability index. If viability decreased with increasing concentration of the test substance, the test substance may be toxic to the cells and any morphological damage to cells can be observed by microscopic examination. All the dilutions of sample N1 were cytotoxic to the H4IIE cells, and only one sample (S3) was not cytotoxic at any test extract concentrations. The other six of mollusk extracts (N2, N3, N4, S1, S2, and S4) caused noticeable growth inhibition over the exposure period at concentrations of 0.33% extracts (0.83 μL extract well−1) and 0.1% extracts (2.5 μL extract well−1). As shown

Table 1
Characteristics and number of mollusk samples collected from sites along the coast of the Chinese Bohai Sea

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sites</th>
<th>Species</th>
<th>Length mm ± SD</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Dalian</td>
<td>Rapana venosa</td>
<td>71 ± 3</td>
<td>5</td>
</tr>
<tr>
<td>N2</td>
<td>Yingkou</td>
<td>Neverita didyma</td>
<td>61 ± 4</td>
<td>10</td>
</tr>
<tr>
<td>N3</td>
<td>Jinzhou</td>
<td>Amusium sp.</td>
<td>85 ± 6</td>
<td>3</td>
</tr>
<tr>
<td>N4</td>
<td>Huладao</td>
<td>Neverita didyma</td>
<td>60 ± 3</td>
<td>4</td>
</tr>
<tr>
<td>S1</td>
<td>Weihai</td>
<td>Rapana venosa</td>
<td>87 ± 6</td>
<td>4</td>
</tr>
<tr>
<td>S2</td>
<td>Yantai</td>
<td>Neptunea caningii</td>
<td>49 ± 6</td>
<td>29</td>
</tr>
<tr>
<td>S3</td>
<td>Laizhou</td>
<td>Rapana venosa</td>
<td>56 ± 3</td>
<td>8</td>
</tr>
<tr>
<td>S4</td>
<td>Yangkou</td>
<td>Rapana venosa</td>
<td>28 ± 5</td>
<td>12</td>
</tr>
</tbody>
</table>

Fig. 2. Dose–response curve of 2,3,7,8-TCDD for the induction of luciferase activity in the H4IIE-luc assay. Luciferase induction is expressed in terms of the percentage of luciferase activity relative to that of 30 pg well−1 2,3,7,8-TCDD (TCDDmax). Values represent the mean ± SD; n = 3.
as Fig. 3, there was still a significant dose-dependent increase of dioxinlike activity in test wells despite the decrease in the viability index.

Results of the in vitro H4IIE-luc assay on the extract and fractions of mollusks collected from different sampling locations are summarized (Fig. 4). Seven of the eight extracts induced significant luciferase activity, except the sample from N1. The greatest magnitudes (±SD) of activity observed for these extracts ranged from 4.88 ± 0.48 to 11.38 ± 1.43% TCDDmax. The mean activity (±SD, data ex N1) was 7.37 ± 1.85% TCDDmax. For F1, no significant induction was observed even for the greatest concentration to this fraction except N2 and N4. Of the two fractions, only F1 caused significant cytotoxicity to H4IIE cells. The range of cytotoxic concentrations was 0.83 μL extract well−1 and 2.5 μL extract well−1 (all concentrations for N1). This means that the cytotoxic compounds were concentrated in F1. The greatest induction of luciferase activity was caused by compounds in F2 for most samples, except N3. This fraction contained PAHs, their derivatives, OC pesticides and a portion of the PCDD/PCDFs. Some PAHs, such as benzo(a)pyrene (BaP), dibenzo(a,h)anthracene (DBA), benzo(a)anthracene (BaA), benzo(k)fluoranthene (BkF), benzo(b)fluoranthene (BbF), chrysene (Chr), and indeno(1,2,3-c,d)pyrene (IdP), are a class of chemicals that can significantly contribute to the induction TEQs. In study these seven PAHs accounted for more than 85% of the calculated total induction equivalents in mollusks (Gardinal and Wade, 1998). Anderson et al. (1999) have described how small mussels deployed in the Naval Station exhibited very high bioaccumulation of PAHs, in addition to very strong induction of CYP1A1 measured by reporter gene system (RGS) response.

Bioassay-derived dioxin equivalents (TCDD-EQs) were estimated directly from the sample dose–response curve using methods described elsewhere (Sanderson et al., 1996; Villeneuve et al., 2000). Concentrations of TCDD-EQs in mollusk extracts were from 227.4 to 547.5 pg g−1 lipid (Table 2). Bioassay-derived TCDD-EQs were compared to instrumentally derived TEQs in order to assess the contamination level along the Bohai Sea coast. The instrumental analyses were performed previously using the same samples, except N3 and S3 (Zhao et al., 2005). The results are shown in Table 3. The TEQ-values ranged from 0.73 to 51.9 pg g−1 lipid. Currently, the relative toxicological potency of a complex mixture of dioxinlike compounds is assessed by the toxic equivalent factor (TEF) approach. Only seven dioxins (PCDDs), 10 furans (PCDFs) and 12 dioxinlike PCBs have had TEF values attributed to them and it is only these chemicals that are analyzed for as part of the conventional targeted chemical analysis TEQ determination (Hurst et al., 2004). There are a number of other compounds and classes of compounds in the extracts that can interact with AhR and potentially...
cause dioxinlike activity. The H4IIE bioassay will respond to any AhR ligand if it is present in the matrix of interest. It has been observed in other locations that the extracts contained some chemicals that could exhibit the luciferase activity (Koh et al., 2004). These unknown compounds producing dioxinlike response in the H4IIE-luc assay may account for the major differences between the TCDDs with TEQs and TEQs\textsubscript{SHAIE-luc}. One recent study reported that the luciferase induction in the H4IIE-luc cell bioassay elicited by raw extracts of Zhoushan fish was negative (Jiang et al., 2005). While another study reported the concentrations of TEQs in the organisms collected from the Bohai Sea coast ranged from 0.06 to 65 pg g\textsuperscript{-1} wet weight (Wan et al., 2005).

The Bohai Sea is an enclosed inner sea in north China, and the coastal areas are highly developed economically. With the economic development and population growth the environmental impact is likely to be most severe along the Bohai coastline. For example, 1 billion tons of wastewater alone is discharged into the Bohai Sea from Beijing, Tianjin, and Hebei province every year (Wan et al., 2005). People in coastal cities have a greater chance of exposure to toxic contaminants than inland populations due to the consumption of seafood (Jiang et al., 2005). Being highly lipophilic and resistant to metabolic breakdown, dioxin and dioxinlike compounds can accumulate in predators at the top of food chain (Braune and Simon, 2003), resulting in potential risk to wildlife. Because dietary uptake is the major route of dioxin exposure in humans, many studies have used foods such of mollusks as a bioindicator for monitoring and determining the contaminant levels, bioaccumulation, and pollutant bioavailability in marine environments. (Abad et al., 2003; Monod et al., 1995; Wu et al., 2001). Although the use of marine organisms for pollution monitoring has some limitations, evaluation of contaminant profiles in mollusks can provide more information on contaminant levels, bioaccumulation, and pollutant bioavailability to successive consumers in the food chain.

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References


