Technology Benchmarking Workshop for Sediment and Floodplain Remediation

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In Vitro Cell-based Bioassays for Detection of Aryl Hydrocarbon (AhR)-Mediated Activity in Environmental Samples

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Key References:


Exposure Questions

Are there chemicals in a given environment that can cause a biological response through the Ah-R-Mediated Mechanism of Action?
Exposure Questions

What is the identity of the Ah-R active agent(s) present in the environment?
Advantages of *in vitro* bioassays relative to instrumental analysis

- Biological relevance
- Integrated measure of the combined potency of all chemicals in a complex mixture
- Can account for unknowns
- Can account for compounds for which analytical methods have not been developed
Advantages of *in vitro* bioassays relative to instrumental analysis

- Can account for non-additive interactions between chemicals
- In some cases, more sensitive than instrumental analysis
Disadvantages of *in vitro* bioassays relative to instrumental analysis

- Inability to *quantify* the concentration of active agent(s) present
- Inability to *identify* the active agent(s)

Zoology Department, National Food Safety and Toxicology Center & Center for Integrative Toxicology, Michigan State University
In vitro bioassay-based TIE: Key Concepts

Toxicity Identification and Evaluation

Bioassay Directed Fractionation

Mass (Potency) Balance Analysis
Bioassay-Directed Fractionation

Analytical Chemistry

In vitro Cell Culture

TIE Scheme Fractionation

Field Exposures

Laboratory Exposures

Comparison
Complex PCDD/DF Mixtures

75 PCDD Congeners
135 PCDF Congeners
Dioxin-Like Mechanism of Action

Toxic Effects Mediated by AhR

Aromatic Hydrocarbon Receptor
Mechanism of Action for AhR-Activation

AhR ligand

+ 

Activated

Increased Protein Phosphorylation

NUCLEUS

Nuclear Factors

Modulation of Gene Expression

Cytosolic Proteins

Light

Luciferase

AhR

Src

HSP90

ARNT

DRE- Luc

P

P

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Bioassays - endpoints measured

- **Luciferase activity** is assessed as a measure of binding of ligands present in the samples to Ah-receptor (H4IIE-luc cells - standard TCDD) to evaluate TCDD-like activity or to estrogen-receptor.
- After addition of luciferase assay reagent, the light production, a measure of luciferase activity, is determined with a luminometer.
- **Viability index** measured by fluorimetric method with calcein AM/ethidinium bromide reagents.
- **Protein content** measured by fluorimetric method with reagent fluorescamine.
In vitro bioassay-based TIE: Key Concepts

Mass (Potency) Balance Analysis

• Used to assess whether compounds identified by instrumental analysis can account for the potency of a sample.

• Used to assess whether non-additive interactions are occurring between components of a mixture.
**In vitro bioassay-based TIE: Key Concepts**

**Bioassay directed fractionation**

- Used to narrow the field of potential causative agents
- Involves an iterative process of chemical fractionation or treatment followed by *in vitro* bioassay
  - examples: HPLC, GPC, acid treatment, activated Copper
- Generally [active/inactive] screening-based, but response magnitudes may be considered
Luciferase Bioassay Methods Using H4IIE-Luc or other Ah-R-responsive Cells

H4IIE-Luc Cells

Cells trypsinized and plated at 15,000 cells in 250 ml media/well

After 24 hours cells dosed with standards and sample extracts

Time course for exposure: 72 hours

After exposure, media is aspirated, cells rinsed with PBS, endpoints measured, luciferase activity measured after addition of reagent Luclite as luminescence in plate-reading luminometer
Assumptions of Indirect Bioassay

The sample being analyzed is assumed to respond as if it were simply a dilution of the standard compound.

Dose-response curves should be effectively identical except for their position along the concentration or dose axis.

The dose-response relationships being compared have equal (or parallel) slopes.

The maximum achievable response (efficacy) for the standard and sample must be identical.
REP Estimation: Limitations

- Deviation from the assumptions of indirect bioassay are common for \textit{in vitro} bioassay results
- Parallelism cannot be tested statistically for complex mixtures and unknowns
- Complex or unknown composition limits the ability to assign a meaningful set of dose units which are statistically comparable to those of the standard
Unequal Efficacy
Equal Efficacy and Parallel Dose-response Curves

The graph illustrates parallel dose-response curves for two different substances, labeled A and B. The x-axis represents the log fmol concentration, while the y-axis shows the %-TCDD-max.

Key points:
- **EC50**: The dose at which 50% of the maximum response is achieved.
- **Maximum Response (efficacy)**: The maximum response achieved for each substance.

The curves are parallel, indicating equal efficacy and parallel dose-response behavior for substances A and B.
Nonparallel Dose-response relationships

EC50
EC20
EC80

A
B
REP$_{20-80}$-ranges: Standardization

• REP-ranges are sensitive to the range of responses over which they are calculated.
• To be directly comparable and give an independent measure of uncertainty due to non-parallel slopes, it is necessary to standardize the range of responses over which REP-ranges are calculated.
• The standard range has arbitrarily been defined as 20-80% of the maximum response achieved for the standard compound.

REP$_{20-80}$-range

• Extrapolation may be necessary for some samples
$\text{REP}_{20-80}$ ranges for Masan Bay sediment extracts
Mass (Potency) Balance Analysis: Terms

- **“Toxic” Equivalents**: An expression of the potency of a sample in terms of the concentration of a well characterized standard compound which elicits the same magnitude of response in a bioassay.
  - Example: 50 pg dioxin-equivalents / g sediment

  - There are two types of “toxic” equivalents estimates
    - Instrumentally derived
    - Bioassay derived
Calculation of Relative Potency

Calculation of TCDD equivalents (TEQs) from analytical results - mass balance calculations

TEQs were calculated for all samples by multiplying the bioassay-specific toxic equivalency factor (TEF) by concentration of specific congener.

\[
TEQ = \sum_{i=1}^{N} \text{CONC}. \text{OF COMPOUND}_i \times \text{TEF}_i
\]
### Example TEQ Calculation
#### non-ortho-PCBs

<table>
<thead>
<tr>
<th>PCB</th>
<th>TEF</th>
<th>CON (pg/g)</th>
<th>TEQ (pg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>33’44’ (77)</td>
<td>0.0001</td>
<td>350</td>
<td>0.035</td>
</tr>
<tr>
<td>33’44’5 (126)</td>
<td>0.1</td>
<td>330</td>
<td>33</td>
</tr>
<tr>
<td>33’44’55’ (169)</td>
<td>0.01</td>
<td>90</td>
<td>0.9</td>
</tr>
</tbody>
</table>

**Total** 33.935
Instrumentally-derived toxic equivalents

- Calculated by multiplying the analytical concentrations of the compounds identified by their REPs and summing.
  - $\sum (\text{concentration}_i) \times (\text{REP}_i)$
  - Assumes an additive model
  - Can only account for known compounds

- TEQ: instrumentally-derived dioxin equivalents
Bioassay-derived toxic equivalents

- Estimated directly from dose-response curves resulting from bioassay analysis of a sample and standard.
  - Does not assume additivity
  - Can account for unknown compounds

- TCDD-EQ: bioassay-derived dioxin equivalents
Mass (Potency) Balance Analysis

- **TCDD-EQ = TEQ**
  - suggests that the compounds identified by instrumental analysis can account for the potency observed
  - suggests additivity

- **TCDD-EQ < TEQ**
  - suggests antagonistic interactions among components of the sample

- **TCDD-EQ > TEQ**
  - suggests the presence of agonists which were not identified by instrumental analysis, or synergistic interactions among components of the sample
Mass (Potency) Balance Analysis

• Ideally the analysis is based on predicted (TEQ) and observed (TCDD-EQ) potency – concentration required to induce a defined magnitude of response.

• In cases where TEQ estimates are available but TCDD-EQ estimates are not, mass balance analysis may be based on predicted and observed response magnitudes.
Dioxin-like Activity of Sediment from Masan Bay, Korea Before and After Acid Treatment

![Graph showing dioxin-like activity in sediment from different locations before and after acid treatment. The x-axis represents location numbers (1 to 28), and the y-axis represents %-TCDD-max. The graph compares non-treated (green) and acid-treated (pink) samples.]
EXAMPLE I

## TCDD-Equivalents in Sediments
### Aroclor 1254

<table>
<thead>
<tr>
<th>Loc/Treat</th>
<th>pmol Teq-assay (umol PCBs)</th>
<th>pmol Teq-calc (umol PCBs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-Dechlorinated</td>
<td>7.5</td>
<td>7.8</td>
</tr>
<tr>
<td>SL-Dechlorinated</td>
<td>2.1</td>
<td>1.6</td>
</tr>
<tr>
<td>RR-Dechlorinated</td>
<td>&lt;0.6</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Mass (Potency) Balance Analysis: Confirmation

- Interactions between agonists and antagonists could yield an apparent mass balance even when all active compounds have not been identified.

- When possible, mass-balance conclusions should be confirmed empirically.

- Sample fractionation and reconstitution of the sample using analytical standards can be used to help distinguish effects of unidentified compounds from the effects of non-additive interactions between identified compounds.
What Magnitude of Difference is Significant?

• One of the most difficult aspects of mass balance analysis is determining what magnitude of difference between TEQs and TCDD-EQs, or observed and predicted values is significant.

• Dependent on
  – Variability of the assay
  – Uncertainties in the relative potency estimates
  – Uncertainties or assumptions involved in TEQ or predicted magnitude estimation.
EXAMPLE II

Sampling Locations
Morava & Drevnice Rivers

GERMANY
CZECH REPUBLIC
POLAND
PRAGUE
AUSTRIA
SLOVAKIA

Morava & Drevnice Rivers

sediments
soils

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TCDD-EQ in Sediment Extracts from Czech Rivers

TCDD activity in sediment fractions - induction expressed as % induction over solvent control

Fraction 1 = PCDD/F PCBs
Fraction 2 = PAHs
Fraction 3 = Polar
EXAMPLE III

Approx. 15 mile stretch

65 sediments
18 Transects
11 Flood plain soils

Ponar grab samples collected during August-October 2001
Analysis

1. **Soxhlet extraction**
2. **Conc. H$_2$SO$_4$ and Cu treatment**
3. **Multi-layer silica gel chromatography**
4. **Activated carbon impregnated silica gel (1 g)**
5. **$^{13}$C-PCDDs/DFs**
6. **H$_4$IIE-luc bioassay**

**Chromatography Fractions**
- F1: PCBs
  - Hexane
- F2: PCDDs/DFs
  - Toluene

**HRGC/HRMS**
Concentrations (pg/g, dry wt) of TEQs and TCDD-Eqs in sediments/soils from the Tittabawassee River (Mean & Range)
<table>
<thead>
<tr>
<th>Sample (n)</th>
<th>TEQs</th>
<th>TCDD-Eqs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composite sediment (16)</td>
<td>550</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td>(41-1,810)</td>
<td>(34-2,430)</td>
</tr>
<tr>
<td>Transect sediment (18)</td>
<td>440</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>(6.3-2,770)</td>
<td>(8.6-1450)</td>
</tr>
<tr>
<td>FP soil (7)</td>
<td>1150</td>
<td>1100</td>
</tr>
<tr>
<td></td>
<td>(350-1,890)</td>
<td>(290-2,450)</td>
</tr>
<tr>
<td>Ups. Comp sediment (3)</td>
<td>8.2</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>(2.5-19)</td>
<td>(0.8-9.8)</td>
</tr>
<tr>
<td>Ups. Transect sediment (4)</td>
<td>2.3</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>(0.56-5.5)</td>
<td>(0.4-25)</td>
</tr>
<tr>
<td>Ups. Soil (3)</td>
<td>6.2</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>(2.1-10)</td>
<td>(20-240)</td>
</tr>
</tbody>
</table>
TCDD-EQs vs. TEQs - raw extracts

\[ y = 0.73x + 11.1 \]
\[ R = 0.72 \]

TCDD-EQs vs. TEQs - acid treated samples

\[ y = 1.12x - 36.3 \]
\[ R = 0.94 \]

Relationship between PCDD/DF - TEQs and bioassay derived TCDD-Eq in soil/sediments from the Tittabawassee River basin
Questions
Thank You

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