Ecotoxicology
Biology 5868

Biomarkers:
Molecular Effects

2009
Levels of Biological Organization

- Biosphere
- Landscape
- Ecosystem
- Community
- Population
- Individual
- Organ
- Tissue
- Cell
- Molecular

Contaminant

Assessment:
- Exposure
- Effects
- Screening
- Mechanistic
  - in vitro
  - in vivo
  - in silico
Contaminant Analysis

Key Question: what is the predictive ability of any given test?
Biomarkers

**Biomarker** - “...a biochemical, physiological, morphological, or histological quality used to imply exposure to or effect of a toxicant.”

**Biomarker Types:**
- **Exposure biomarkers** - mechanism necessary or implied
- **Effects biomarkers** - no mechanism necessary

**Biomarker Tests:**
- **Tier I** - screening
- **Tier II** - mechanistic

**Biomarker efficiency:**

\[ E = \frac{U_i}{B_i} \]

- \( E \) = efficiency of the biomonitoring methodology
- \( i \) = population of interest
- \( U_i \) = concentration at which the undesirable effect occurs
- \( B_i \) = concentration at which the method can detect or predict
Ideal Biomarker Characteristics

Newman & Unger criteria for biochemical/molecular biomarkers:

1. Measurable before an adverse effect occurs at a higher level of biological organization.

2. Rapid, inexpensive, easy. (*Pragmatic importance.*)


4. Specific to a single toxicant or class of toxicants. (*Unlikely*)

5. Concentration-effect relationship should exist. (*Linear over exposure range if possible.*)

6. Applicable to a broad range of sentinel species. (*If these even exist.*)

7. Linkage of biomarker changes with some toxicant-related decrease in individual fitness.

8. System should be familiar; incorporate qualities of organism that influence biomarker. (*Asking a lot, especially if using native species.*)
Ideal Biomarker Characteristics

(Melancon, 2002 criteria)

1. Know specificity of response to contaminant presence of exposure.

2. Some correlation of presence or magnitude of response to amount of contaminant.


4. Response should precede harm, but ideally predict future harm.

5. Samples should be readily obtainable (in quantities needed for measurement).

6. Sampling should be nonlethal (ideally).

7. Assay method should be accurate and reproducible.

8. Overall costs should be reasonable.
Biomarker Signal:Noise Ratio

Signal:noise ratio, e.g.
- immune system
- stress-response system

response to "normal" stressors

response to xenobiotic stressors

Stressor B = higher signal:noise

Stressor A = low signal:noise

normal stress response = noise
### Molecular Biomarkers

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Biomarker</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>lead</td>
<td>Inhibition of ALAD</td>
<td>alternative to Pb analysis</td>
</tr>
<tr>
<td>Me, PCBs</td>
<td>poryphyrin profiles</td>
<td></td>
</tr>
<tr>
<td>OPs, carbamates</td>
<td>AChE inhibition</td>
<td>signal:noise issues?</td>
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<tr>
<td>rodenticides</td>
<td>anticoagulant proteins</td>
<td></td>
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<tr>
<td>OCs, PAHs</td>
<td>MFO (P450)</td>
<td>gene/enzyme induction multiple forms</td>
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<tr>
<td>cadmium</td>
<td>metallothionein</td>
<td>signal:noise issues?</td>
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<tr>
<td>OCs</td>
<td>retinol profiles</td>
<td>considerable natural variation</td>
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<tr>
<td>EDCs</td>
<td>vitellogenin (♂)</td>
<td>sensitive indicator</td>
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<tr>
<td>Me, OCs, PAHs</td>
<td>other serum enzymes</td>
<td>wide variety, many tests</td>
</tr>
<tr>
<td>metals, OCs</td>
<td>stress proteins</td>
<td>wide variety, many tests</td>
</tr>
</tbody>
</table>
Heme Synthesis

\[ \text{Succinyl-CoA} + \text{Glycine} \rightarrow \delta\text{-aminolevulinic acid synthase (ALA synthase)} \]

\[ \alpha\text{-amino-\(\beta\)-keto-}
\text{adipate} \rightarrow \delta\text{-aminolevulinate} \]

\[ \delta\text{-aminolevulinate} + \delta\text{-aminolevulinate} \rightarrow \text{porphobilinogen} \]

\[ \text{4 Porphobilinogen} \rightarrow \text{Linear tetapyrrole (Polypyrrol methane)} \]

\[ \text{Heme} \]
Lead, PCB effects on porphyrin synthesis

Glycine + Succinyl CoA

$\delta$-Aminolevulinic Acid

$\delta$-aminolevulinic acid synthetase

Porphobilinogen

$\delta$-aminolevulinic acid dehydratase

Uroporphyrinogen

Uroporphyrinogen I synthetase

Heptacarboxyporphyrinogen

Uroporphyrinogen III cosynthetase

Hexacarboxyporphyrinogen

Uroporphyrinogen decarboxylase

Pentacarboxyporphyrinogen

Uroporphyrinogen decarboxylase

Coprophyrinogen

Coprophyrinogen oxidase

Protoporphyrinogen IX

Protoporphyrinogen oxidase

Protoporpyrin IX

Ferrochelatase + Fe(II)

Heme

Globin

Hemoglobin

PCBs cause buildup of multiple porphyrins

(ALAD-deficiency porphyria)

Heme + $\delta$-aminolevulinic acid $\rightarrow$ porphobilinogen

Inhibited by lead

Pb
Hemoglobin Induction

Induction of differential hemoglobin profiles in midges

<table>
<thead>
<tr>
<th>NP</th>
<th>BPA</th>
<th>B[a]P</th>
<th>CP</th>
<th>PQ</th>
<th>Pb</th>
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<tbody>
<tr>
<td>NP(μg L⁻¹)</td>
<td>BPA(μg L⁻¹)</td>
<td>B[a]P(μg L⁻¹)</td>
<td>CP(μg L⁻¹)</td>
<td>PQ(mg L⁻¹)</td>
<td>Pb(mg L⁻¹)</td>
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<tr>
<td>0</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>0</td>
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<tr>
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<td>100</td>
<td>1000</td>
<td>0</td>
<td>100</td>
<td>50</td>
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NP - nonylphenol
BPA - bisphenol A diglycidyl ether
B[a]P - benzo[a]pyrene
CP – chlorpyriphos
PQ - paraquat dichloride
Pb - lead(II)nitrate (Pb)
Hemoglobin Induction

nonylphenol (NP), bisphenol A diglycidyl ether (BPA), benzo[a]pyrene (B[a]P) chlorpyriphos (CP), paraquat dichloride (PQ), and lead(II)nitrate (Pb)

nonylphenol (NP), bisphenol A diglycidyl ether (BPA), benzo[a]pyrene (B[a]P) chlorpyriphos (CP), paraquat dichloride (PQ), and lead(II)nitrate (Pb)

Hemoglobin Induction

<table>
<thead>
<tr>
<th>Methods</th>
<th>Preliminary characterization</th>
<th>NP (µg L⁻¹)</th>
<th>BPA (µg L⁻¹)</th>
<th>B[a]P (µg L⁻¹)</th>
<th>CP (µg L⁻¹)</th>
<th>PQ (mg L⁻¹)</th>
<th>Pb (mg L⁻¹)</th>
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<tbody>
<tr>
<td>PAGE</td>
<td>26 kDa, 17 kDa, 16 kDa, 14 kDa, 10 kDa, 8 kDa, 7 kDa</td>
<td>0 1 10 100</td>
<td>0 5 50 500</td>
<td>0 10 100 1000</td>
<td>0 2 20 200</td>
<td>0 1 10 100</td>
<td>0 5 50 500</td>
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<tr>
<td>IEF</td>
<td>pl 6.2, pl 5.6, pl 4.8, pl 4.3, pl 3.8, pl 3.5</td>
<td>26 kDa↑, 17 kDa↑</td>
<td>14 kDa↑</td>
<td>10 kDa↑</td>
<td>pl 5.6↑</td>
<td>pl 4.3↓</td>
<td></td>
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nonylphenol (NP), bisphenol A diglycidyl ether (BPA), benzo[a]pyrene (B[a]P), chlorpyriphos (CP), paraquat dichloride (PQ), and lead(II)nitrate (Pb)
1. Neurotransmitter **acetylcholine** must be broken down rapidly by the receiving neuron - continued stimulation → uncoordinated muscle movement, seizures, and death
2. **Acetylcholinesterase** (AChE) binds acetylcholine by donating proton from serine - results in the release of the choline group - acetyl group released and the serine reactivated by a proton donated by H$_2$O.
Organophosphate Insecticides

3. Organophosphates inhibit AChE by initially binding to serine at the active site
- allows *irreversible* binding of organophosphate and an active site glutamyl residue
- acetylcholine is prevented from entering the active site = no degradation

![Chemical structures of organophosphates: Methyl Parathion and Diazinon with their mechanisms of action involving serine and glutamyl binding.](image)
Acetylcholinesterase Inhibition

sites at increasing distance from contaminant source

Mixed Function Monooxygenase Induction

MFO; CYP450s, etc.

e.g. RH + O₂ + 2H⁺ + 2e⁻ → ROH + H₂O

(also oxidation, reduction, epoxidation, dealkylation, etc.)

- 150 - 400 varieties ~ 8800 known sequences
- 28 families; multiple subfamilies

- nomenclature:
  - gene family = cyp
  - family = 1 - 28
  - subfamily = A - Z
  - individuals = 1, …. (#s)

  e.g. CYP1A1, P4501A1

CYP1, 2 – drug, steroid, xenobiotic metabolism
CYP3, 11, 17, 19, 21 - steroid metabolism
CYPx…. arachadonic acid, thromboxine, vitamin D, retinoic acid, etc.
CYP1A Activities

**Benzo[a]pyrene**  
\[ \text{Benzo[a]pyrene} \rightarrow \text{Benzo[a]pyrene diol epoxide} \]

**AHH** - aryl hydrocarbon hydroxylase

**EROD** - ethoxyresorufin O-deethylase

Note – CYP1A1 induced via activated aryl hydrocarbon (Ah) receptor
CYP450 Assays

PAHs

EROD

Carcinogenic PAHs

BPMO

Metabolite Biomarkers

e.g. Pyrethroid metabolites:

Esfenvalerate

Allethrin

Permethrin

Pyrethroid pesticides and their metabolites in vacuum cleaner dust collected from homes and day-care centers.
Metallothionein Induction

**Metallothioneins** (MT) - uptake, internal compartmentalization, sequestration, excretion of essential and non-essential metals
- small, ubiquitous proteins (6-7 kDa); four major isoforms
  - ~ 25% cysteine; no aromatics or histidine
  - metals bind to sulfhydryl (thiol) group of cysteine
- normal homeostatic function
- inducible
  - higher levels (in the presence of metals) = increased fitness
- well conserved; *Drosophila*, fish, mouse, human)
- transcriptional regulation via Zn-dependent transcription factor (MTF-1)
  - MTF-1 binds to Metal Response Element (MRE)
  - induction by metals other than Zn not well understood; via MTF-1??
    - MT gene also has glucocorticoid and antioxidant response element

**MT Detection:**
- antibodies for specific metallothioneins
- cDNA probes
Vitellogenin

**Vitellogenin** (VTG) - protein precursor of several yolk proteins; e.g. phosvitin, lipovitellin in the eggs of various vertebrates and invertebrates
- synthesized in the liver
- estrogen sensitive
  - sensitive assay in males and immature organisms
  - not as sensitive in females (protein is normally present)
- Note - restricted to oviparous organisms

**VTG Detection:**
- RT-QPCR (reverse transcriptase quantitative PCR)
- Western blots
- ELISA

fathead minnow
Stress Proteins

**Cellular stress response** – “orchestrated induction of key proteins that form the basis for the cell’s protein protection and recycling system”

- includes heat shock proteins (hsp) and others
  - hsp 100, hsp90, hsp70, hsp60, low MW, ubiquitin
- inducible by a variety of “stressors”
  - infection, inflammation, ethanol, arsenic, metals, UV, starvation, hypoxia
  - transcriptional regulation by Heat Shock Factor (HSF)
- chaperones – protein folding (re-folding)
  - constitutive expression: stress 90 (high), stress 70 (low)
  - but both are inducible to higher concentrations by stress
  - LMW varies in stress inducibility
- ubiquitin – tags proteins destined for recycling
  - highly conserved; highly inducible

**Stress protein detection:**

- protein fingerprinting
- “suite” of stress response proteins may be functional
Oxidative Stress and Antioxidant Response

**Oxidative stress** – damage to biomolecules from reactive oxygen species (ROS)

- e.g. free oxyradicals
  - superoxide radical (O$_2^-$)
  - hydroxyl radical (·OH)

ROS also produced by:
- aerobic metabolism; i.e. mitochondrial e\textsuperscript{-} transport
- photosynthetic e\textsuperscript{-} transport
- phagocytosis
- catalysis; e.g. prostaglandin synthase, guanyl cyclase, glucose oxidase

**Antioxidant response:**
- e.g. molecular antioxidants:
  - tocopherol (vitamin E)
  - ascorbic acid (vitamin C)
  - retinaldehyde (vitamin A)
  - glutathione (GSH)
  - β-carotene
  - catecholamines
  - uric acid
Antioxidant Response

Enzymatic antioxidants:

Inducible

- **Superoxide dismutase**
  
  \[
  4 \text{O}_2^- \rightarrow 2 \text{O}_2 + 2 \text{H}_2\text{O}_2
  \]

- **Catalase**
  
  \[
  2 \text{H}_2\text{O} + \text{O}_2 \rightarrow 4 \text{H}_2\text{O}
  \]

- **Glutathione reductase**
  
  \[
  \text{NADP}^+ \rightarrow \text{NADPH}
  \]

**Xenobiotics** (e.g. PAH radicals) can produce free radicals after biotransformation

- undergo redox cycling; e.g. xenobiotic is reduced to a radical, produces oxygen radical, recycles to original form
- interfere with antioxidant enzymes
DNA Modification

**Genotoxicity** – damage by a physical or chemical agent to genetic materials; chromosomes, DNA, RNA

- mutagens
- teratogens
- carcinogens
- clastogens (agents that cause chromosome damage)

**Mechanisms:**

- free radicals can cause strand breakage
- oxyradicals oxidize bases
- adducts: xenobiotics bind covalently to nucleotide; alters structure
- metals bind phosphates or bases; e.g.
  - Hg crosslinks DNA strands; Cu displaces Mg on PO$_4$
Genotoxicity Response

DNA repair mechanisms - highly active and efficient, but can be overwhelmed

Assay methods:
- chromosome staining
- flow cytometry (aneuploidy)
- $^{32}$P-labeling (adducts)
- comet assay (single cell DNA assay)