Technology Benchmarking Workshop for Sediment and Floodplain Remediation

Ann Arbor, Michigan
March 25-26, 2004

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Zoology Department, National Food Safety and Toxicology Center &
Center for Integrative Toxicology, Michigan State University

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

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Key References:

Hilscherova, K., M. Miroslav, K. Kannan, A.L. Blankenship and J.P. Giesy. 2000. Cell Bioassays for Detection of Aryl Hydrocarbon (AhR) and Estrogen Receptor (ER) Mediated Activity in Environmental Samples. *Environ. Sci. Pollut. Res.* 7 (3): 159-171

Villeneuve, D. L., J. S. Khim, K. Kannan, J. Falandysz, A. L. Blankenship and J. P. Giesy. 2000. Relative Potencies of Individual Polychlorinated Napthalenes to Induce Dioxin-like Responses in Fish and Mammalian *In Vitro* Bioassays. *Arch. Environ. Contam. Toxicol.* 39:273-281.

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

isadvantages of *in vitro* bioassays relativ to instrumental analysis

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

n vitro bioassay-based TIE: Key Concept

oxicity Identification and Evaluation

Bioassay Directed Fractionation

Mass (Potency) Balance Analysis

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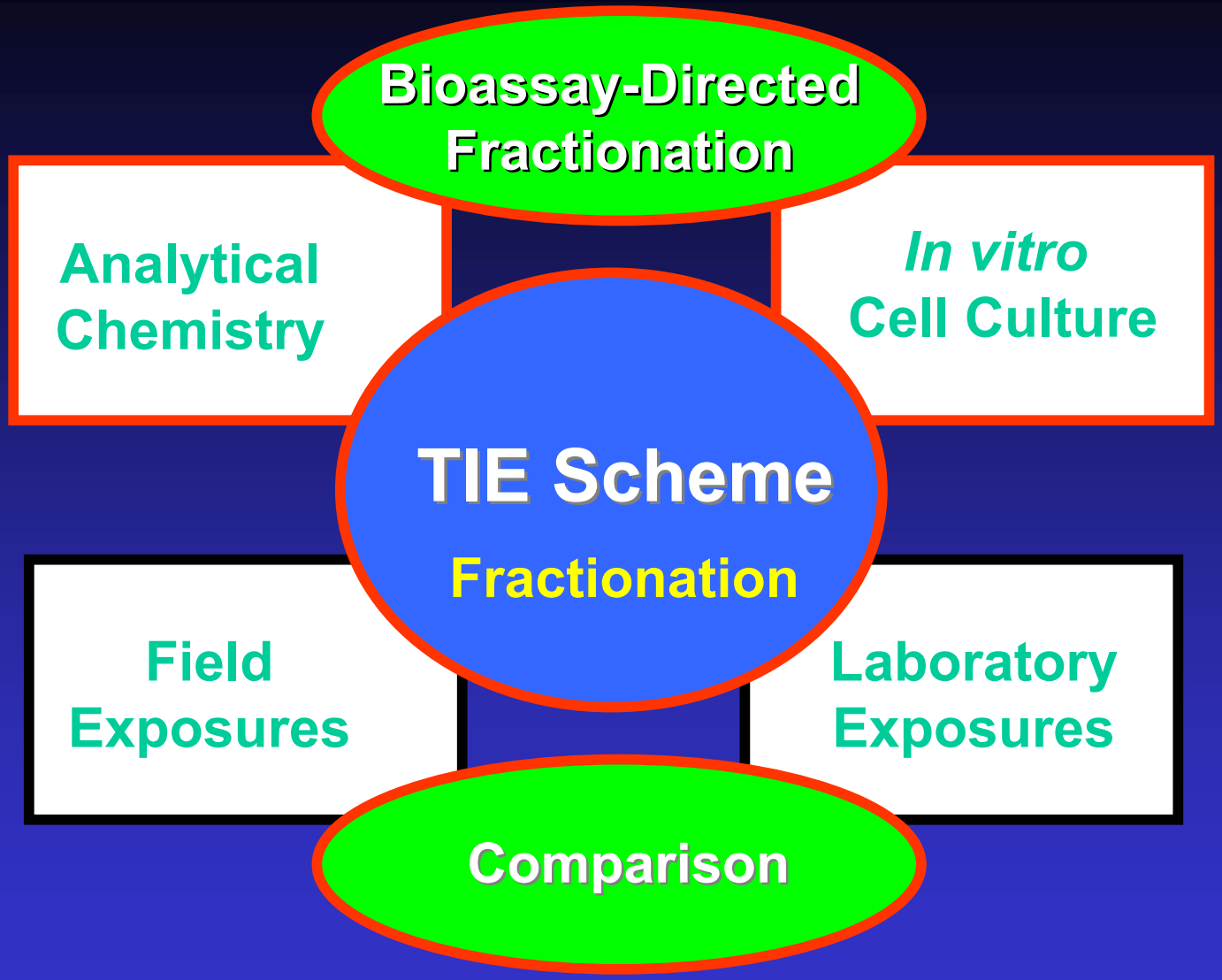
Bioassay-Directed Fractionation
Analytical Chemistry
In vitro Cell Culture
TIE Scheme
Fractionation
Field Exposures
Laboratory Exposures
Comparison

Complex PCDD/DF Mixtures

75 PCDD Congeners

135 PCDF Congeners

Dioxin-Like Mechanism of Action

Toxic Effects Mediated by AhR

Aromatic Hydrocarbon Receptor

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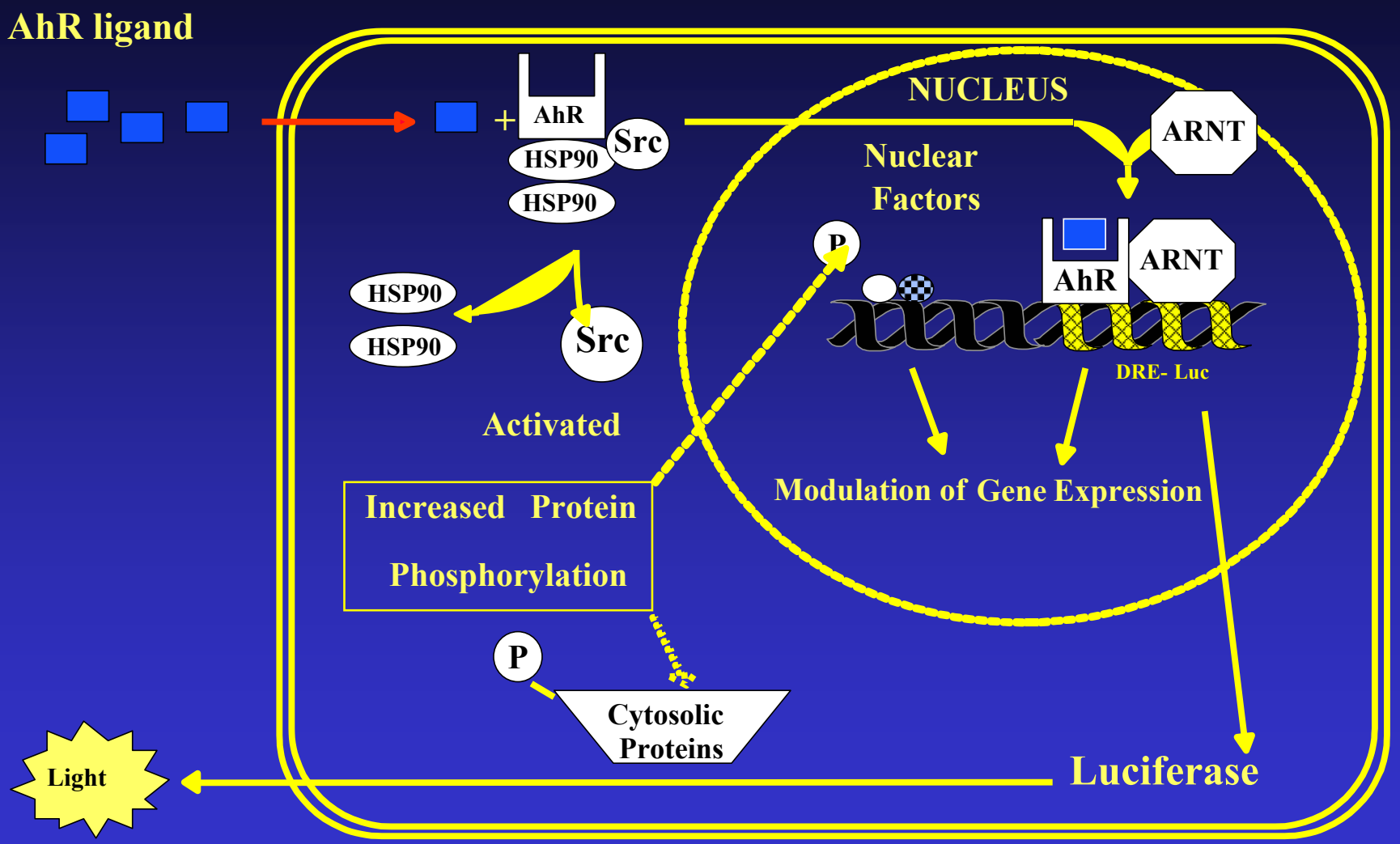
Mechanism of Action for AhR-Activation



METHODOLOGY

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

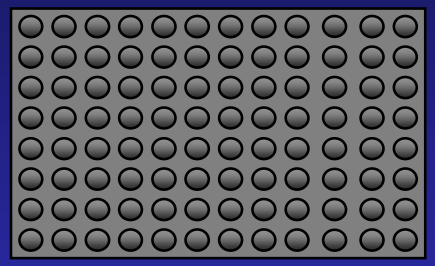
Bioassay directed fractionation

- 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

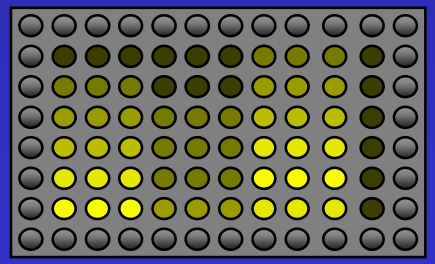
Luciferase Bioassay Methods Using H4IIE-Luc or other Ah-R-responsive Cells

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



ter exposure, media is
pirated, cells rinsed with PBS,
dpoints measured, luciferase
tivity measured after addition
reagent Luclite as
miniscence in plate-reading
minometer

Time course for exposure:
72 hours

Relative Potency Estimation

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.

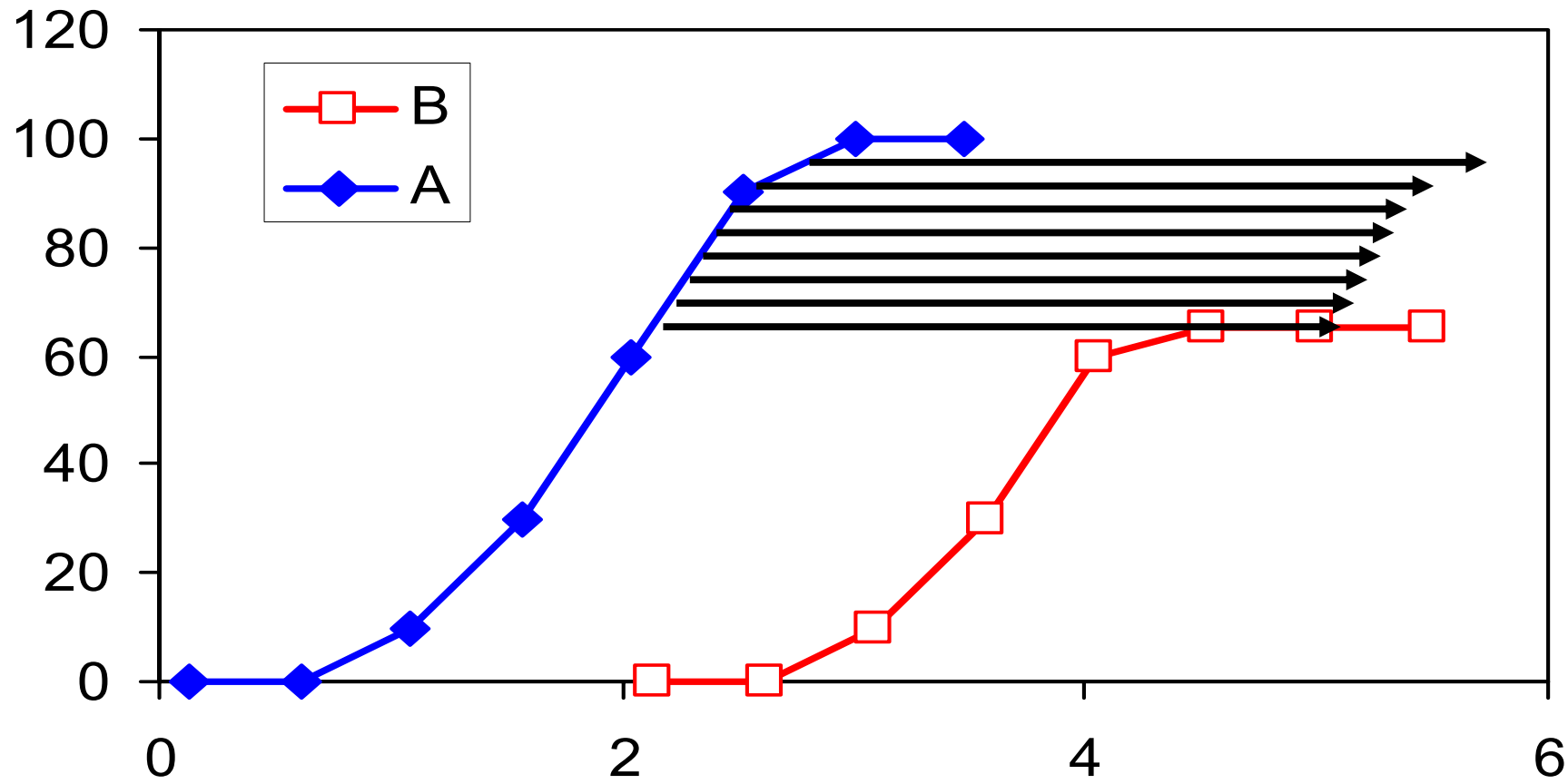
The dose-response relationships being compared have equal (or parallel) slopes

The maximum achievable response (efficacy) for the standard and sample must be identical

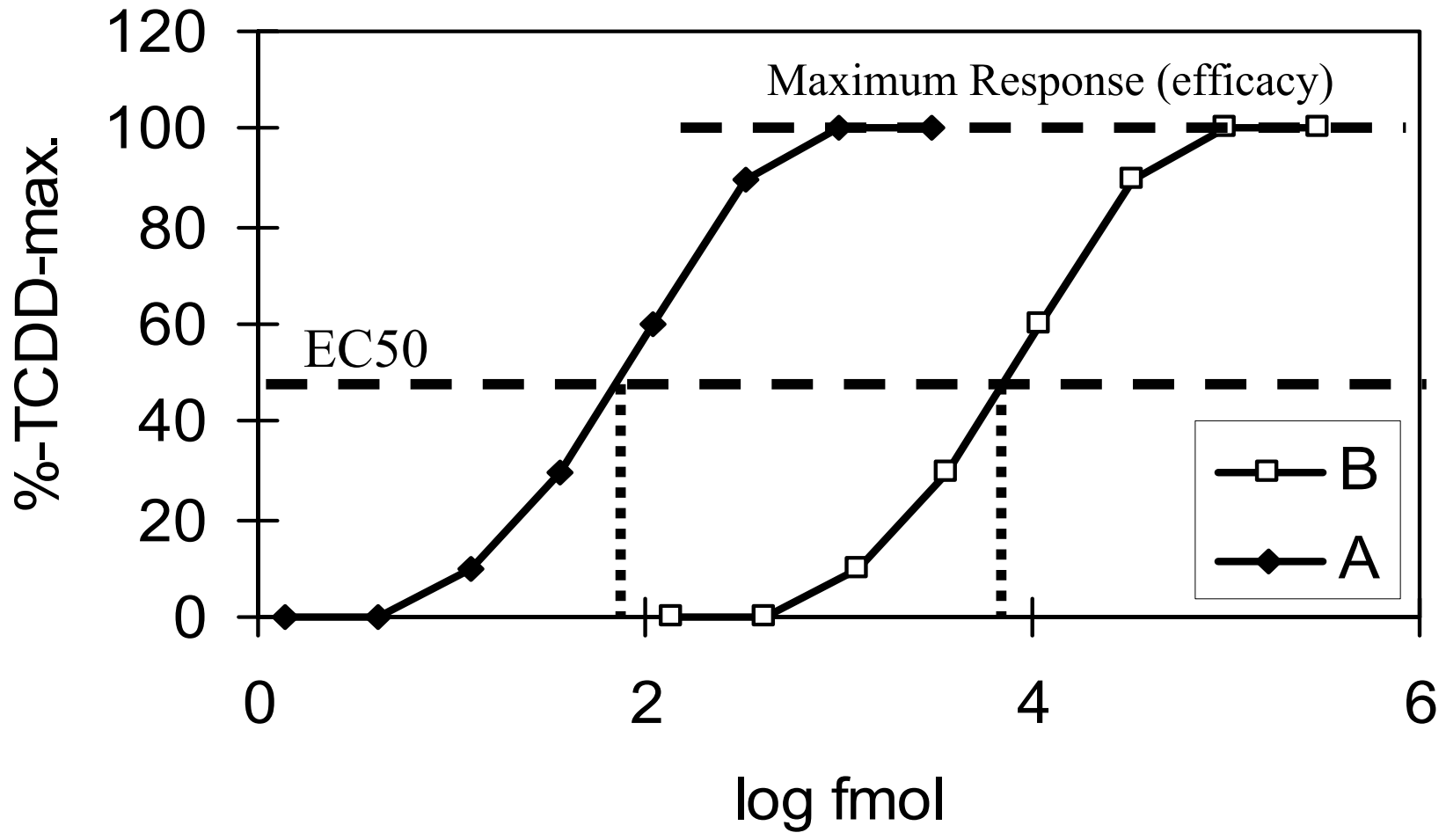
REP Estimation: Limitations

- **Deviation from the assumptions of indirect bioassay are common for *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**

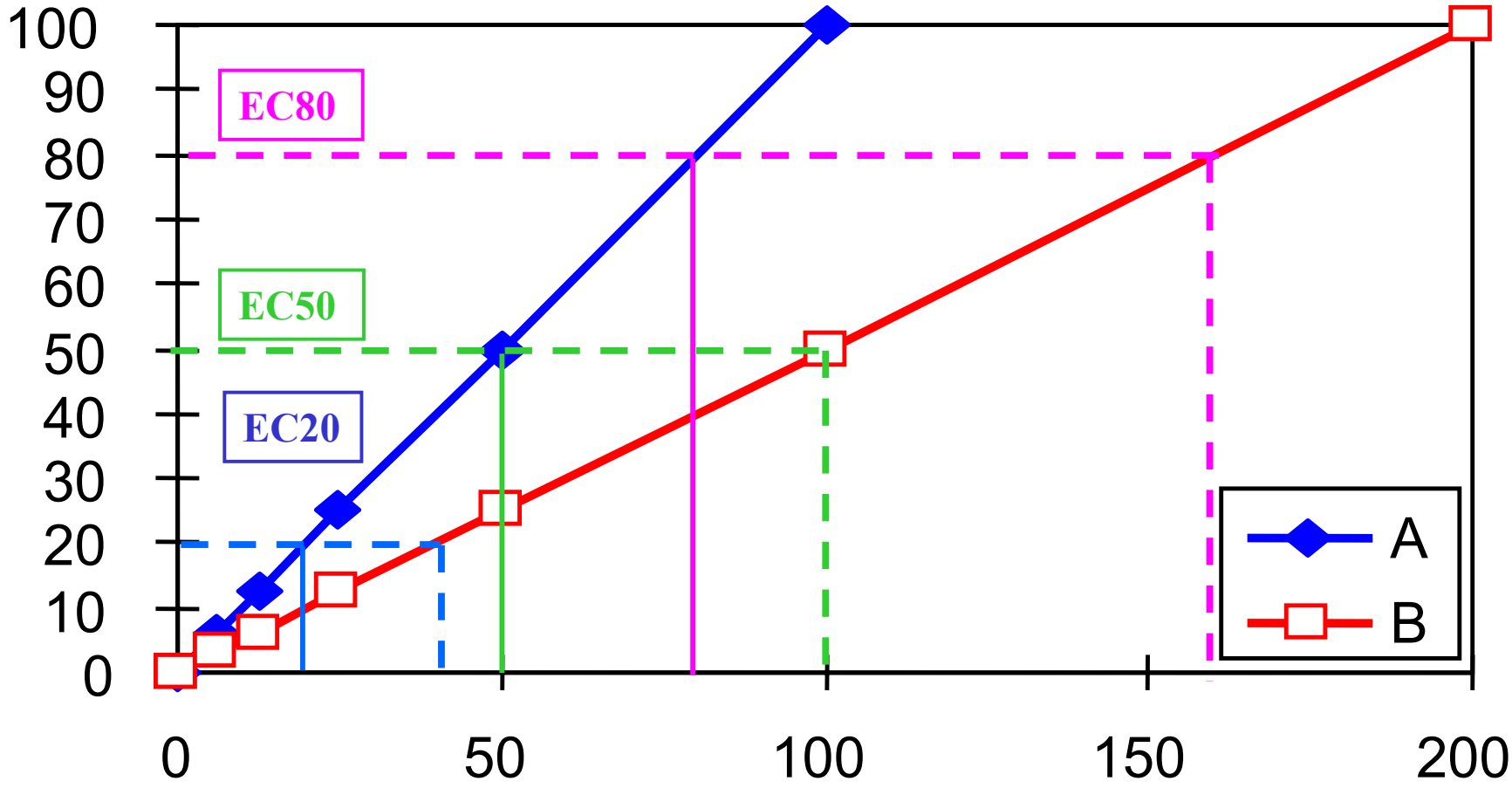
Unequal Efficacy



Equal Efficacy and Parallel Dose-response Curve



Nonparallel Dose-response relationships



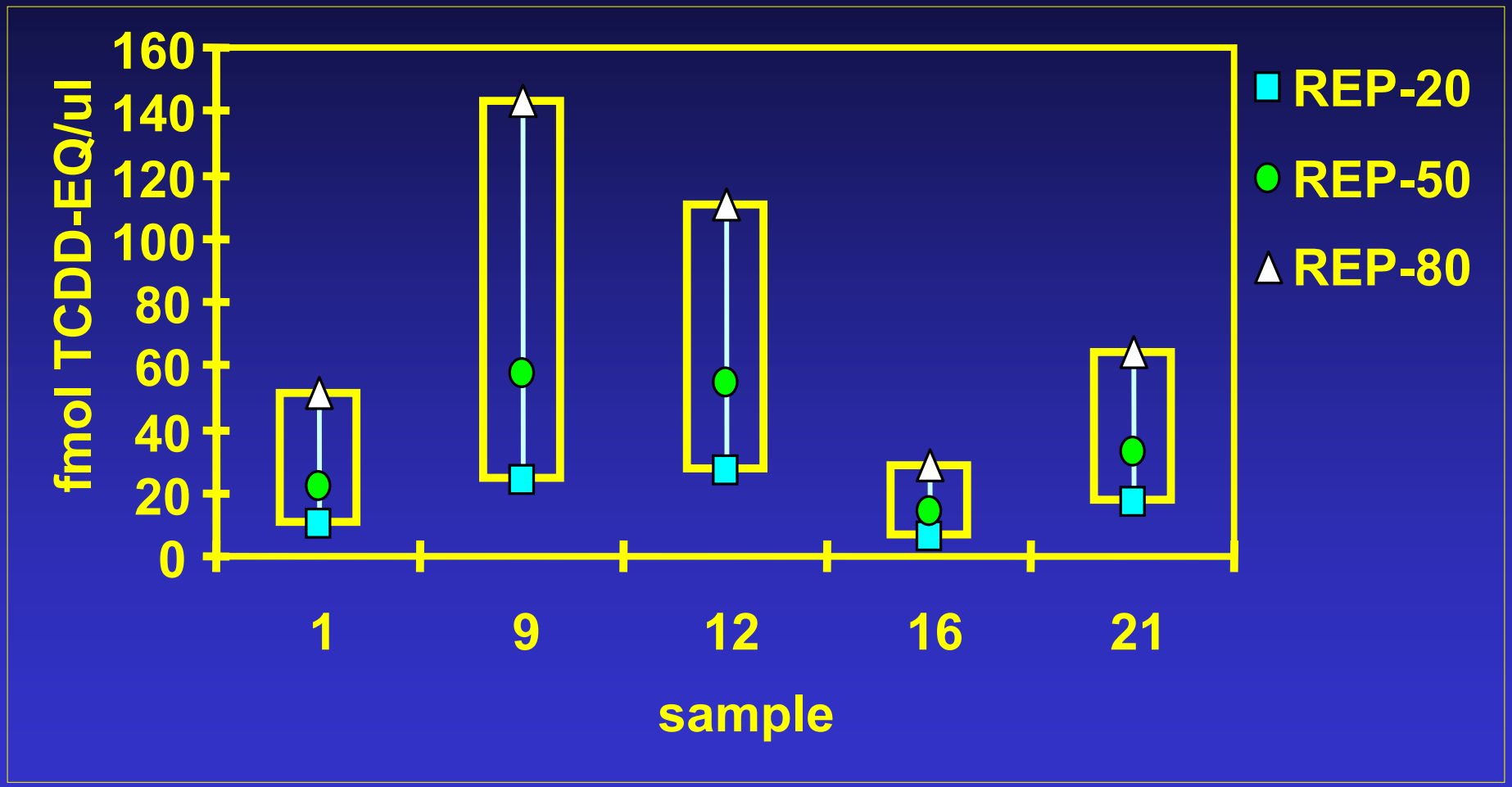
REP_{20-80}-ranges: Standardization

- **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**

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.

$$TEQ = \sum_{I=1}^{N} CONC._i \, OF \; COMPOUND_i \times TEF_i$$

Example TEQ Calculation non-*ortho*-PCBs

PCB	TEF	CON (pg/g)	TEQ (pg/g)
33'44' (77)	0.0001	350	0.035
33'44'5 (126)	0.1	330	33
33'44'55' (169)	0.01	90	0.9
		Total	33.935

Mass (Potency) Balance Analysis: Terms

Instrumentally-derived toxic equivalents

- Calculated by multiplying the analytical concentrations of the compounds identified by their REPs and summing.
  - Σ (concentration$_i$) x (REP$_i$)
  - Assumes an additive model
  - Can only account for known compounds
- TEQ: instrumentally-derived dioxin equivalents

Mass (Potency) Balance Analysis: Terms

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**

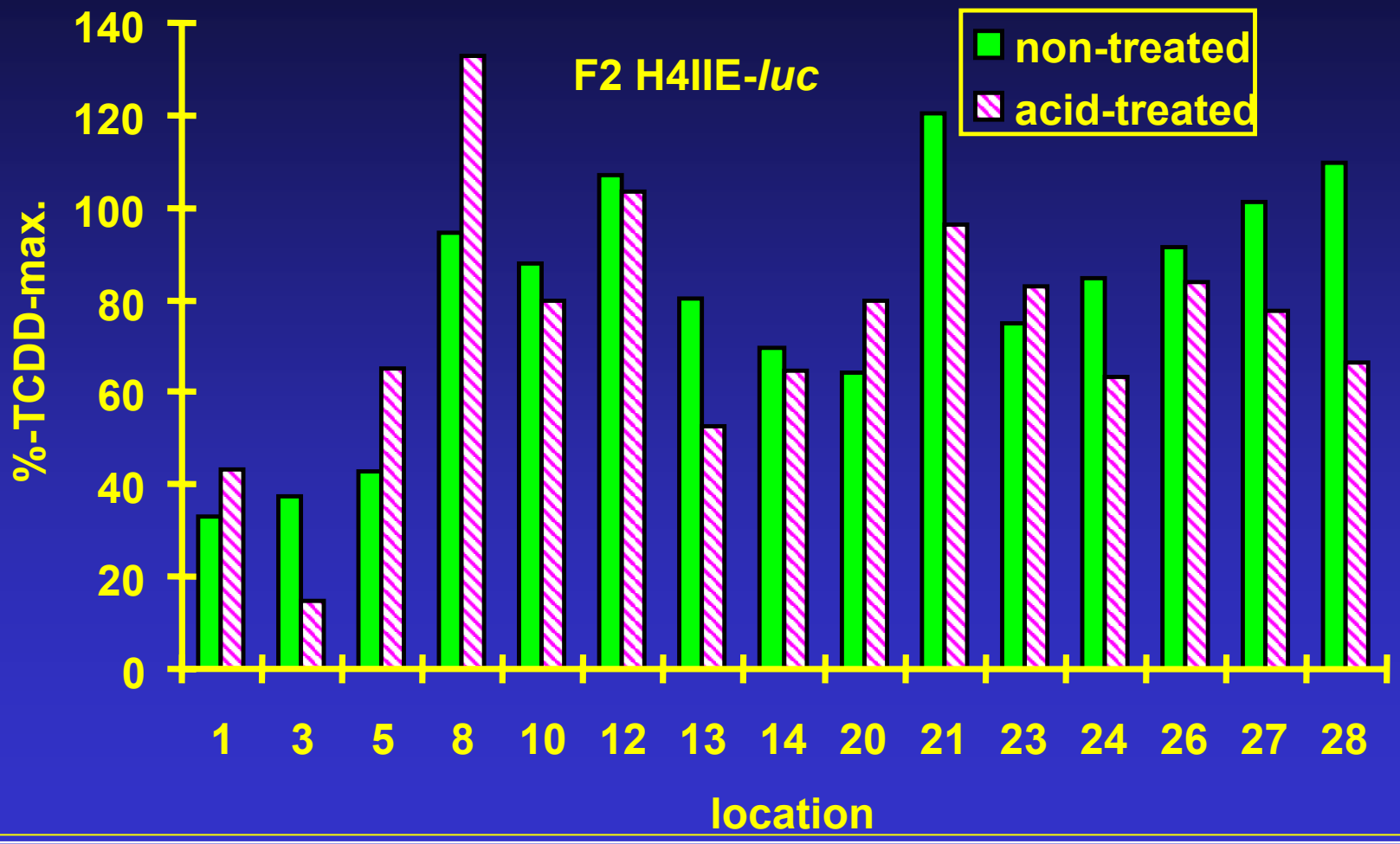
Mass (Potency) Balance Analysis

- 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



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EXAMPLE I

Quensen, J.F., M.A. Mousa, S.A. Boyd, J.T. Sanderson, K.L. Froese and J.P. Giesy. 1998. Reduction in Aryl Hydrocarbon Receptor-Mediated Activity of PCB Mixtures Due to Anaerobic Microbial Dechlorination. *Environ. Toxicol. Chem.* 17:806-813.

TCDD-Equivalents in Sediments Aroclor 1254

Loc/Treat	pmol Teq-assay (umol PCBs)	pmol Teq-calc (umol PCBs)
Non-Dechlorinated	7.5	7.8
SL-Dechlorinated	2.1	1.6
RR-Dechlorinated	<0.6	1.0

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?

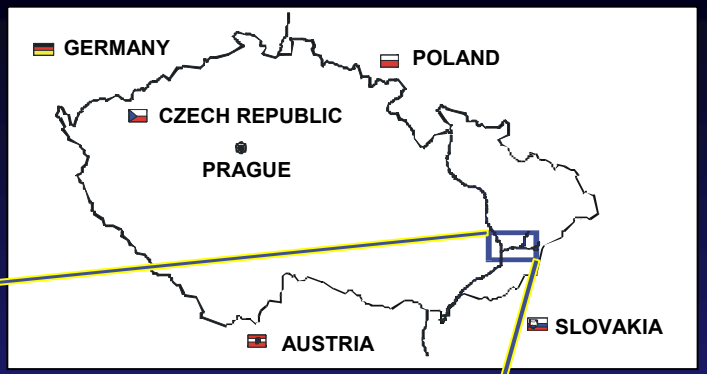
- One of the most difficult aspects of mass balance analysis is determining what magnitude of difference between TEQs and TCDD-EQs, or observed and predicted values is significant.
- Dependent on
  - Variability of the assay
  - Uncertainties in the relative potency estimates
  - Uncertainties or assumptions involved in TEQ or predicted magnitude estimation.

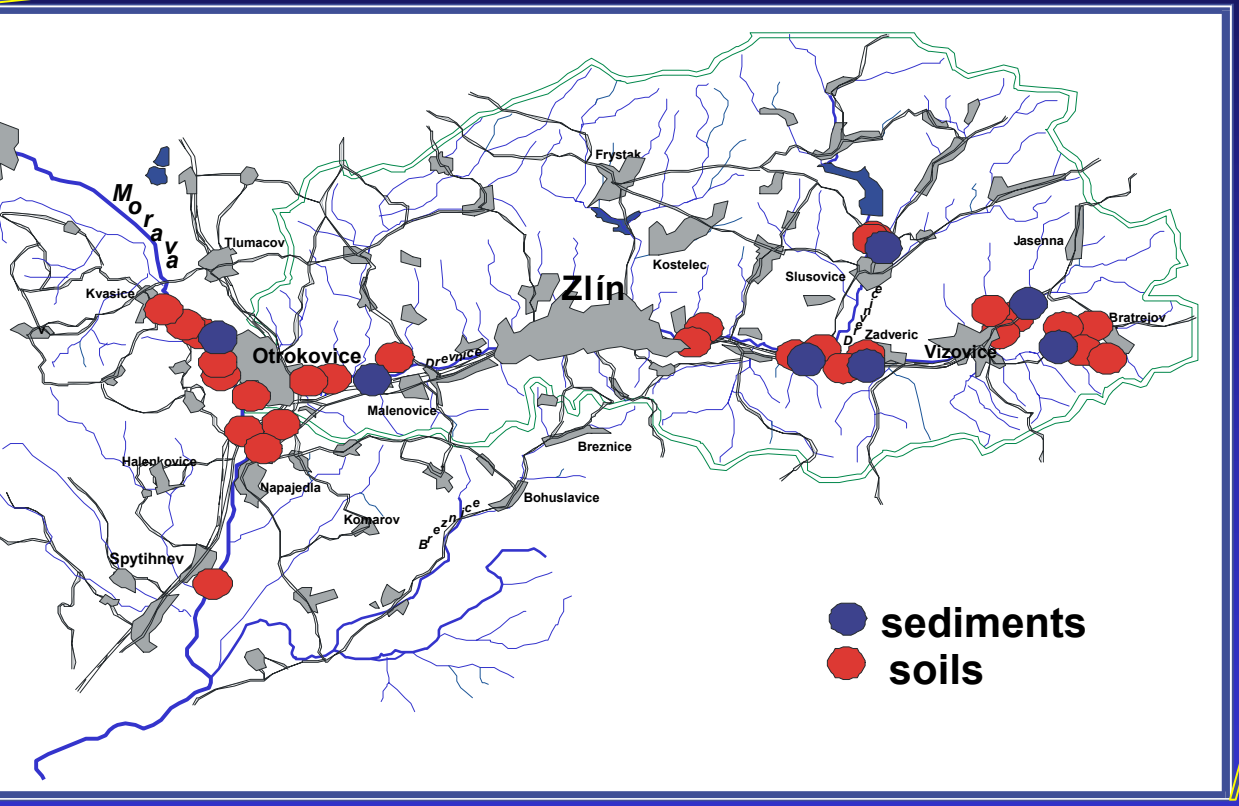
EXAMPLE II

Hilscherova, K., K. Kannan, Y.-S. Kang, I. Holubek, M. Machala, S. Masunaga, J. Nakanishi and J.P. Giesy. 2001. Characterization of Dioxin-like Activity of Riverine Sediments from the Czech Republic. *Environ. Toxicol. Chem.* 20:2768-2777.

Sampling Locations

Morava & Drevnice Rivers





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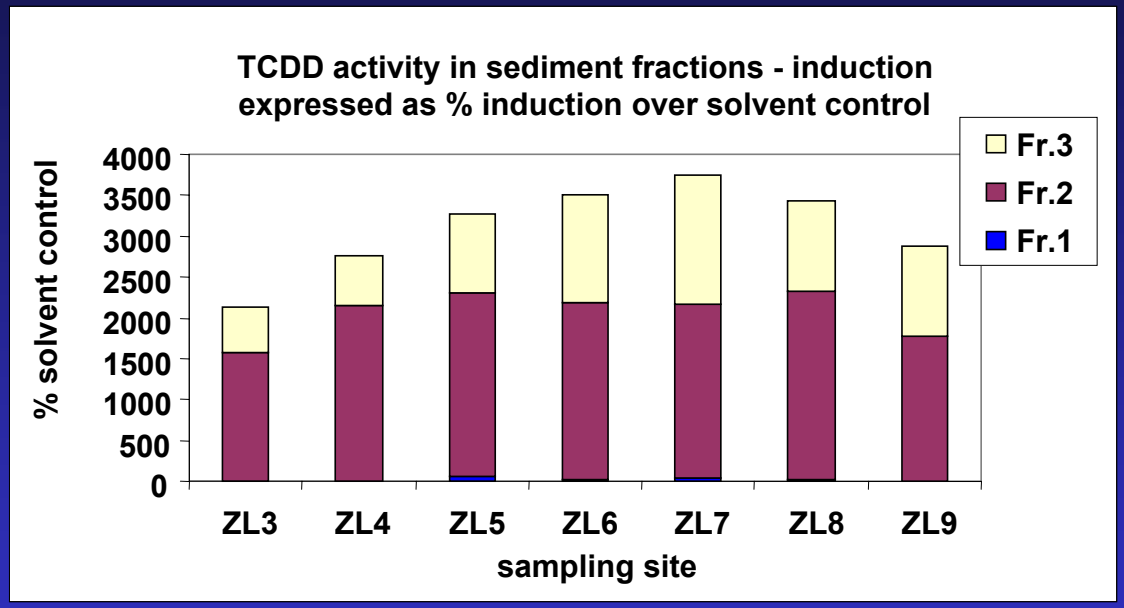
TCDD-EQ in Sediment Extracts from Czech Rivers



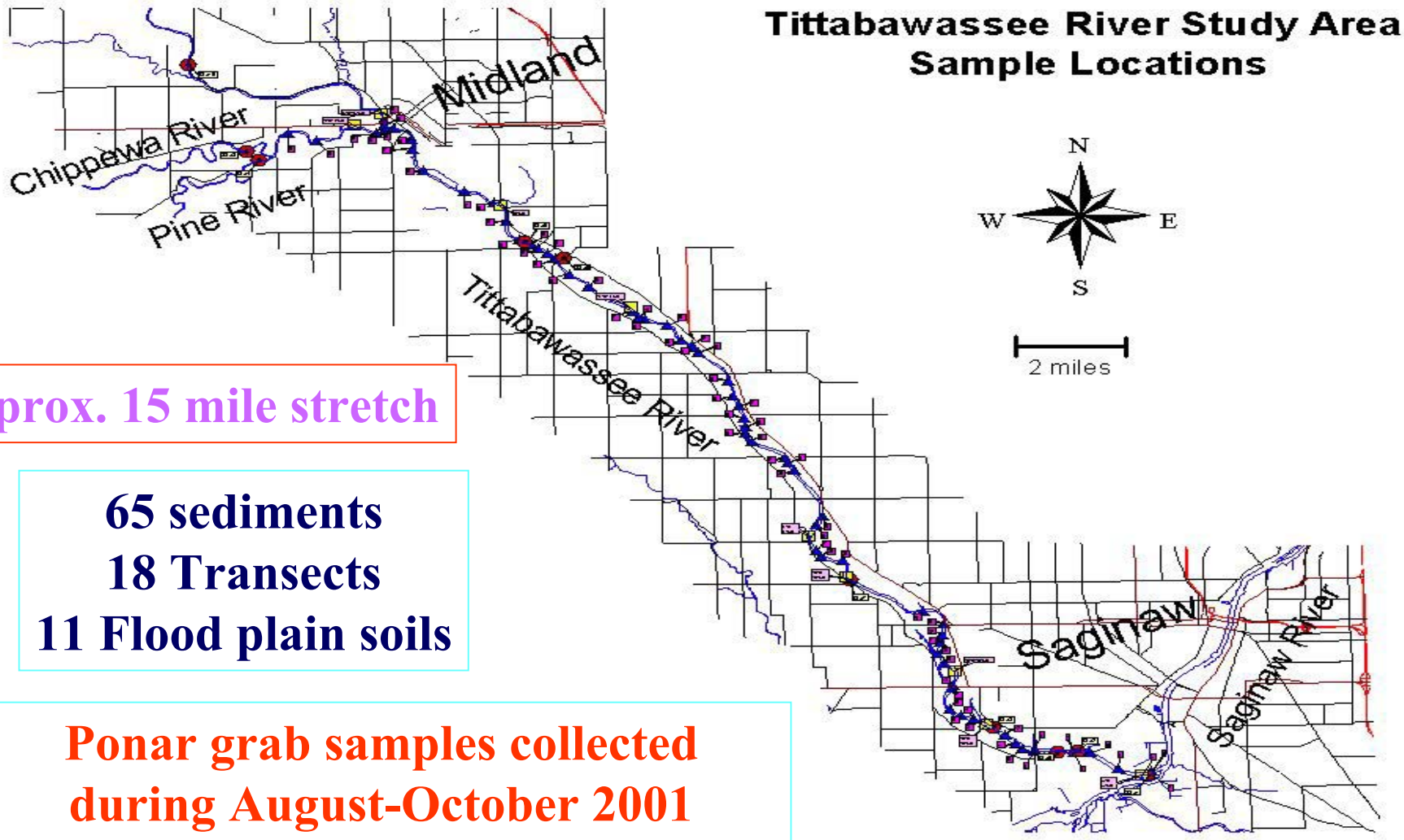
Fraction 1 = PCDD/F PCBs

Fraction 2 = PAHs

Fraction 3 = Polar

EXAMPLE III

Hilscherova, K., K. Kannan, H. Nakata, N. Yamashita, P. L. Bradley, J. M. McCabe, A. B. Taylor, J. P. Giesy. 2003. Polychlorinated Dibenzo-p-dioxin and Dibenzofuran Concentration Profiles in Sediments and Flood Plain Soils of the Tittabawassee River, Michigan *Environ. Sci. Technol.* 37:468-474.

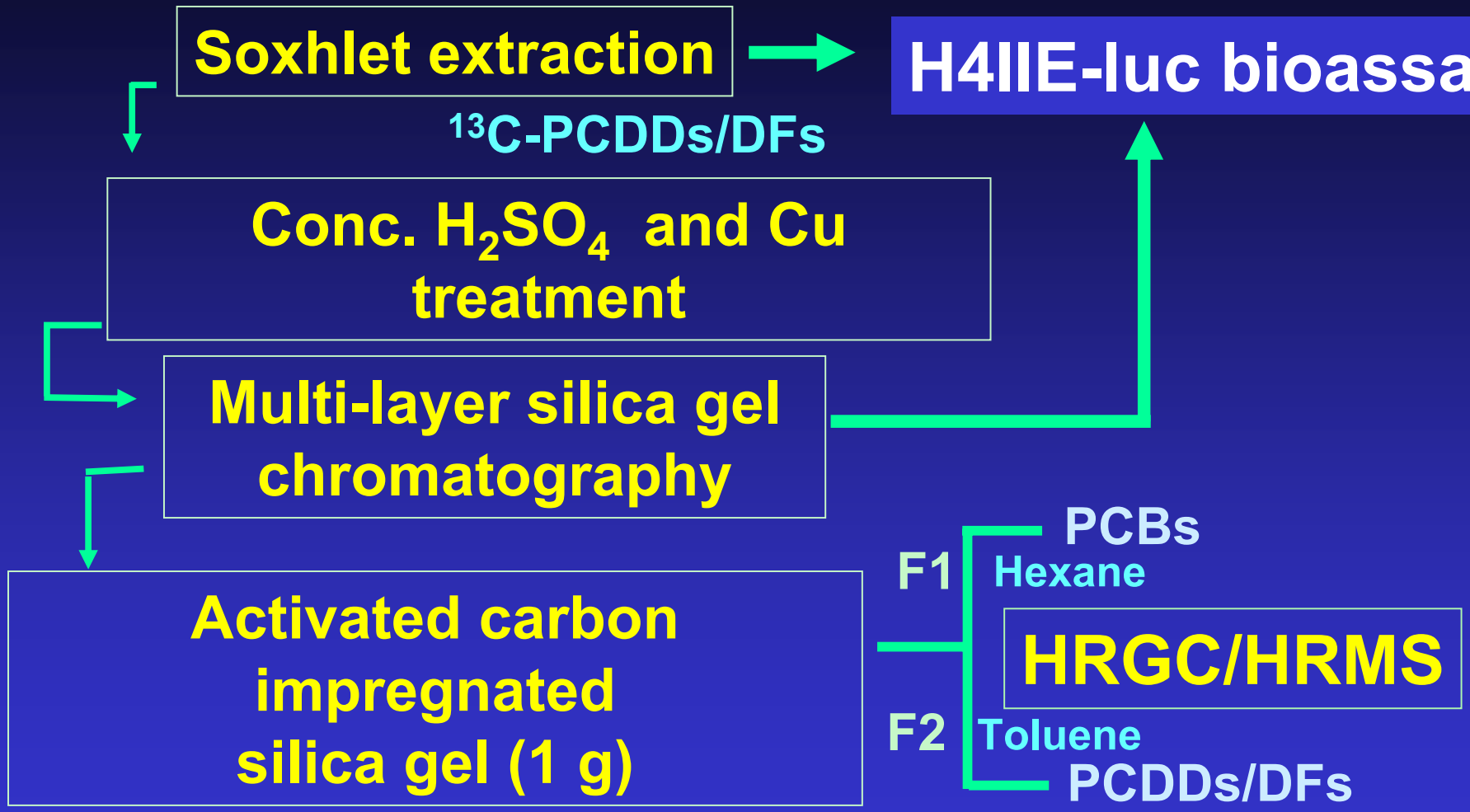


Approx. 15 mile stretch

65 sediments
18 Transects
11 Flood plain soils

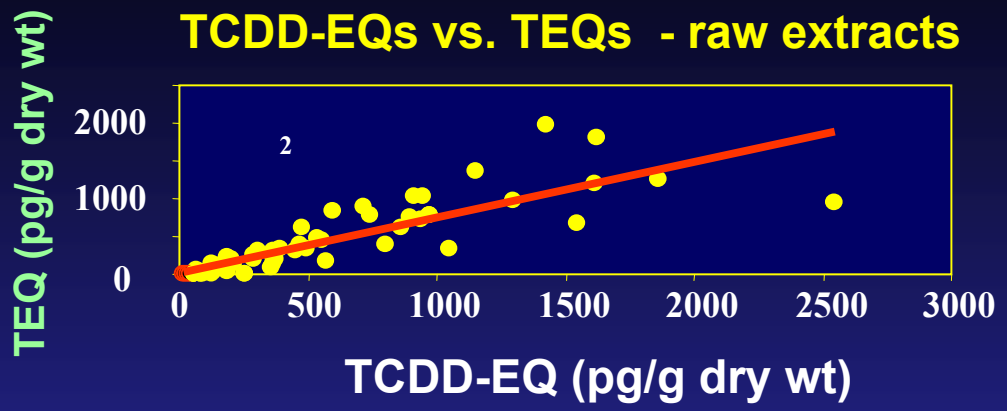
Ponar grab samples collected during August-October 2001

Analysis


Soxhlet extraction
H4IIE-luc bioassay
^{13}C-PCDDs/DFs
Conc. H_2SO_4 and Cu treatment
Multi-layer silica gel chromatography
Activated carbon impregnated silica gel (1 g)
F1
PCBs
Hexane
HRGC/HRMS
F2
Toluene
PCDDs/DFs

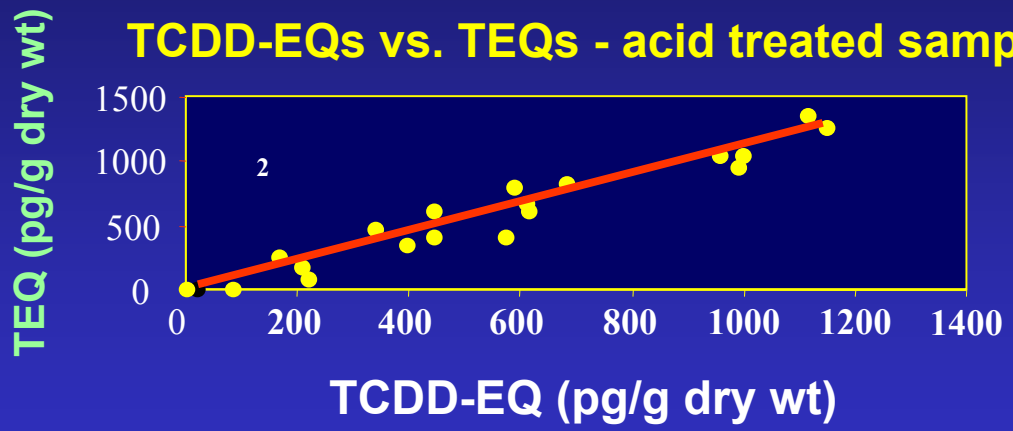
Concentrations (pg/g, dry wt) of TEQs and TCDD-Eqs in sediments/soils from the Tittabawassee River (Mean & Range)

Sample (n)	TEQs	TCDD-Eqs
Composite sediment (16)	550 (41-1,810)	370 (34-2,430)
Transect sediment (18)	440 (6.3-2,770)	300 (8.6-1450)
FP soil (7)	1150 (350-1,890)	1100 (290-2,450)
Ups. Comp sediment (3)	8.2 (2.5-19)	4.3 (0.8-9.8)
Ups. Transect sediment (4)	2.3 (0.56-5.5)	7.6 (0.4-25)
Ups. Soil (3)	6.2 (2.1-10)	165 (20-240)



$y = 0.73x + 11.1$

$R = 0.72$



$y = 1.12x - 36.3$

$R = 0.94$

Relationship between PCDD/DF - TEQs and bioassay derived TCDD-Eqs in soil/sediments from the Tittabawassee River basin

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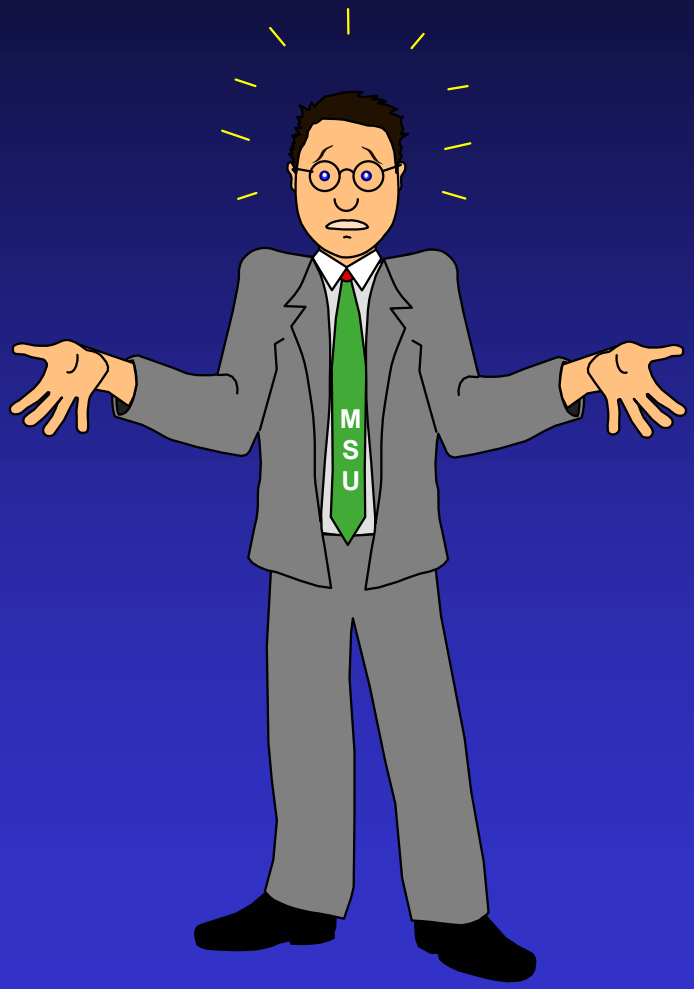
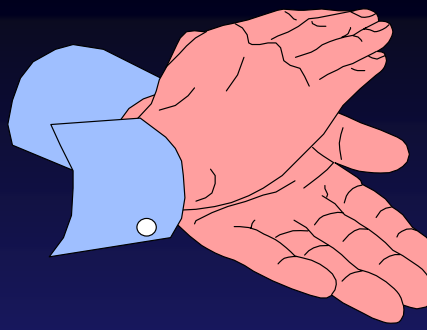
Questions ???????



Thank You



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