Metabolomics in Small Fish Toxicology: Assessing the Impacts of Model EDCs

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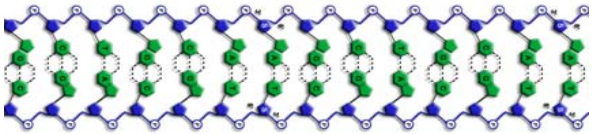
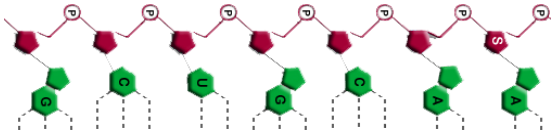
SETAC 2009
OMICS in toxicology: Using the molecular toolbox to study aquatic contaminants

‘omic Tools in the Molecular Toolbox

DNA → **mRNA** → **Protein** → **Metabolites** → **Function**

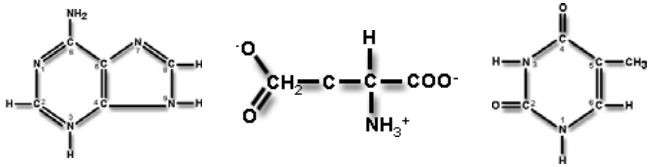


Genotype

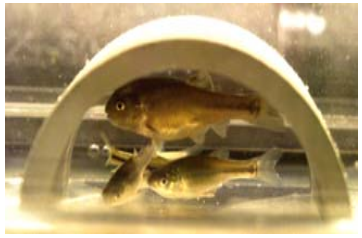


Transcriptomics
~ 100,000 Transcripts

Proteomics
~ 1,000,000 Proteins



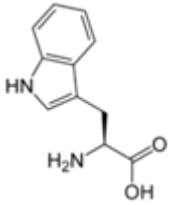
Metabolomics
~ 2,400 Compounds



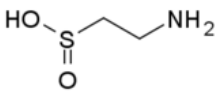
Phenotype

adapted from
Metabolon, Inc., 2004

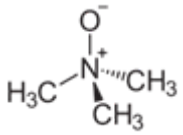
Endogenous Metabolites are Physically, Chemically, and Functionally Diverse



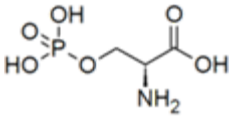
tryptophan



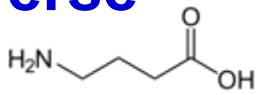
hypotaurine



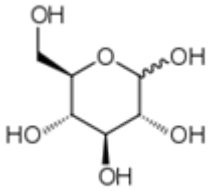
trimethylamine N-oxide



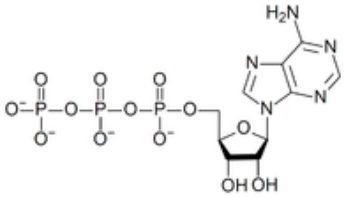
phosphoserine



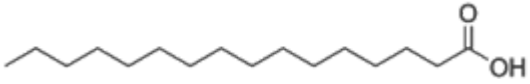
gamma-aminobutyric acid (GABA)



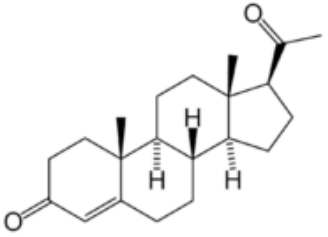
glucose



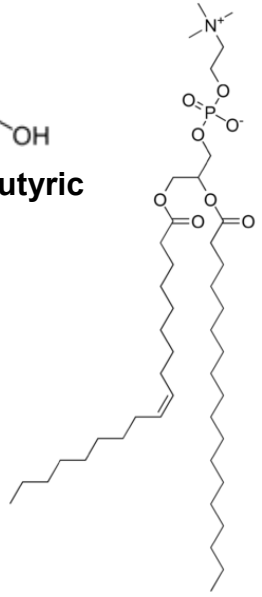
adenosine triphosphate (ATP)



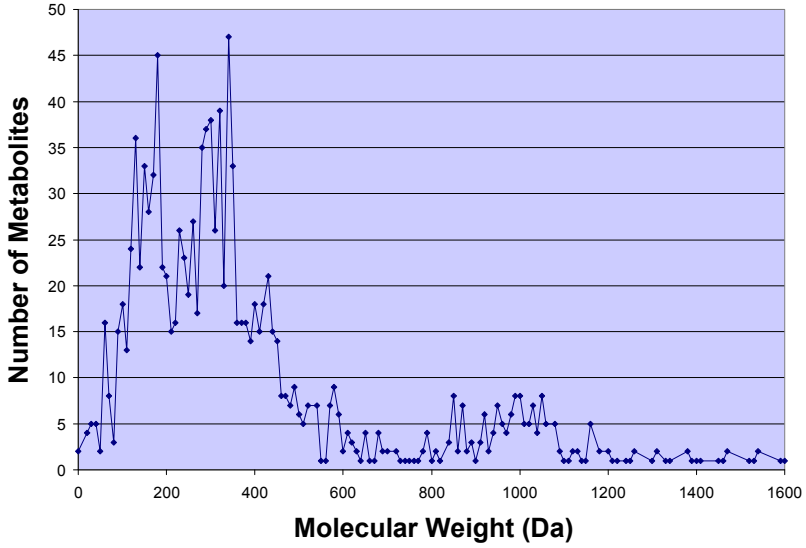
palmitic acid



progesterone



phosphatidylcholine



adapted from
Metabolon, Inc., 2004

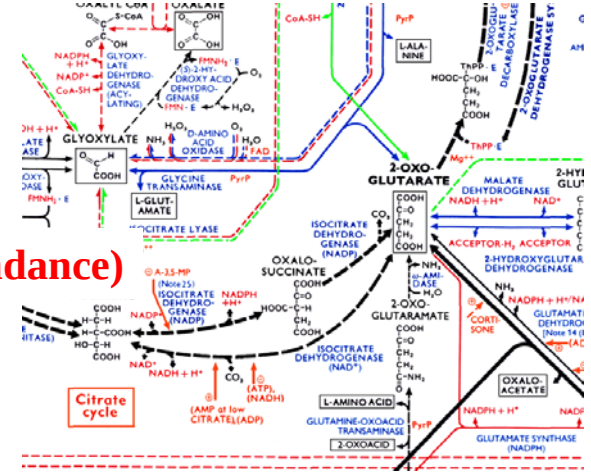
Common Analytical Techniques for Metabolomics

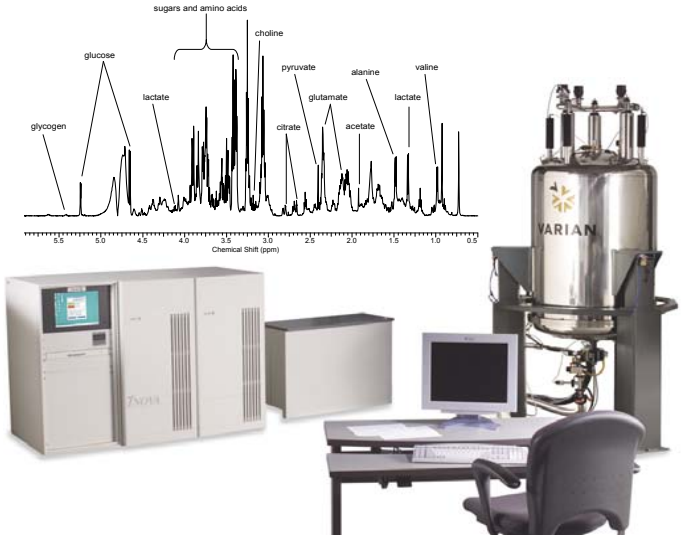
"parallel" analysis	"serial" analysis
^{1}H-NMR	GC-MS
^{13}C-NMR	LC-MS/MS
polar and non-polar extracts	

^{1}H-NMR-based Metabolomics for Toxicology

Advantages of Metabolomics :

- **no need for sequenced genome**
- **open-ended - no user preselection of metabolites** **(observed metabolites "preselected" by abundance)**
- **non-invasive (i.e., urine)**
- **knowledge base from decades of biochemical research**

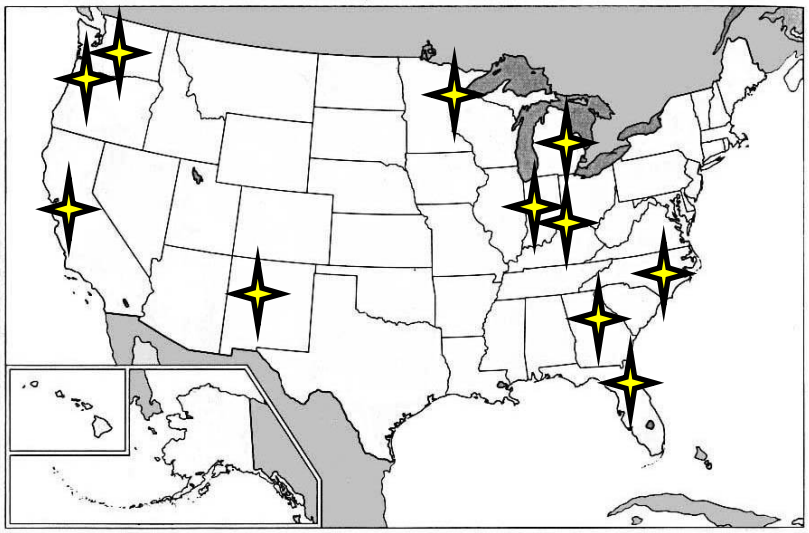




Advantages of ^{1}H-NMR :

- **high throughput**
  - **< 15 min per sample run in most cases**
- **low per sample cost** **(but high initial investment!)**
  - **< $2.00 USD**
- **non-destructive**
- **little or no sample preparation**
- **fully automated (with robotics)**
- **excellent reproducibility (e.g., cross-instrument)**

Determining Effects of Exposure of Small Fish Models to Endocrine Disrupting Chemicals Using Transcriptomics, Proteomics, and Metabolomics



- **EPA – NERL Cincinnati, OH**
- **EPA – NHEERL Duluth, MN, and Grosse Isle, MI**
- **EPA – NERL Athens, GA**
- **EPA – NCCT RTP, NC**
- **EPA STAR Program**
  - **Univ. of Florida**
  - **Florida Atlantic Univ.**
  - **Oregon Health Sciences Univ.**
  - **Purdue Univ.**
- **Other partners**
  - **Joint Genome Institute, DOE (Walnut Creek, CA)**
  - **Sandia, DOE (Albuquerque, NM)**
  - **PNNL (Richland, WA)**
  - **Army Corp of Engineers (Vicksburg, MS)**
  - **Dow Chemical (Midland, MI)**

Application to:

- **reproductive and developmental effects**
- **via hypothalamus - pituitary - gonadal (HPG) axis alterations**
- **within a systems biology modeling context**

Models and 'omic outcomes are anchored with phenotypic data

Metabolite Profiling Serves Two Important Functions in this Project

1) Hypothesis-driven mode:

measuring targeted steroid levels to develop / test systems biology models

Tissue	Compound
Gonad, Plasma, Urine	Testosterone
Testis, Plasma, Urine	11-ketotestosterone
Gonad, Plasma, Urine	Estradiol
Gonad	Pregnenolone
Gonad	17alpha-Hydroxyprogesterone
Gonad	Dehydroepiandrosterone
Gonad, Plasma, Urine	Progesterone
Gonad	17alpha-Hydroxypregnenolone
Gonad	Androstenedione
Gonad	Androstenediol
Gonad	Estrone
Gonad	Estriol
Gonad	17 alpha,20 beta-dihydroxypregn-4-en-3-one
Gonad	17,20,21-trihydroxy-4-pregnen-3-one
Gonad	11-hydroxytestosterone
Gonad	Cholesterol
Gonad	Cyclic AMP
Gonad, Plasma, Urine	17β-hydroxy-5α-androstan-3-one
Gonad, Plasma, Urine	3α-androstanediol
Gonad, Plasma, Urine	3b-androstanediol
Gonad, Plasma, Urine	16β-hydroxytestosterone
Gonad, Plasma, Urine	6β-hydroxytestosterone
Gonad, Plasma, Urine	Estrone-3-glucuronide
Gonad, Plasma, Urine	Estradiol-3-glucuronide

GC-MS and LC-MS applications

2) Discovery mode:

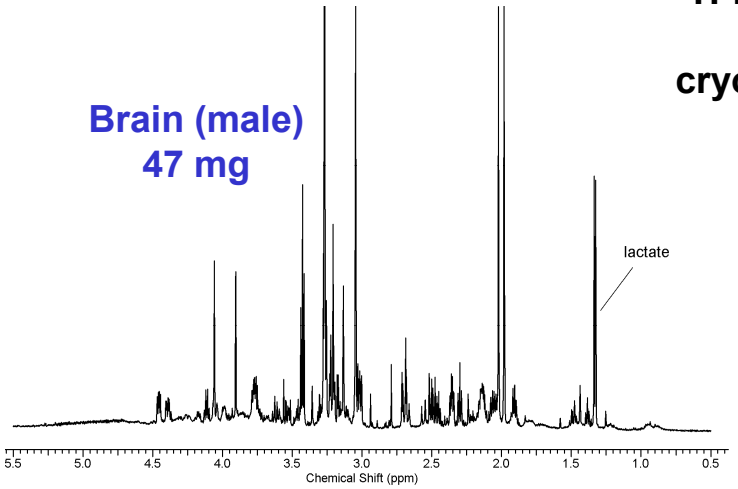
Identifying markers of exposure, mapping temporal effects, etc.

Mostly NMR applications

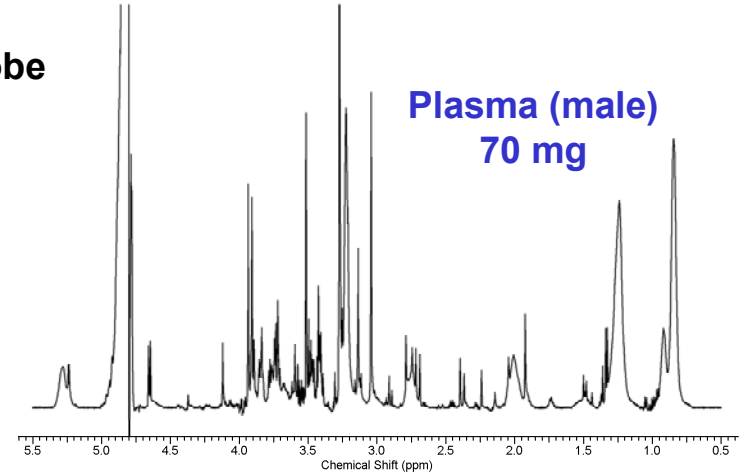
Can We See Metabolites in Individual Fathead Minnow Tissue Extracts ?

^{1}H-NMR spectra
600 MHz
cryogenic probe

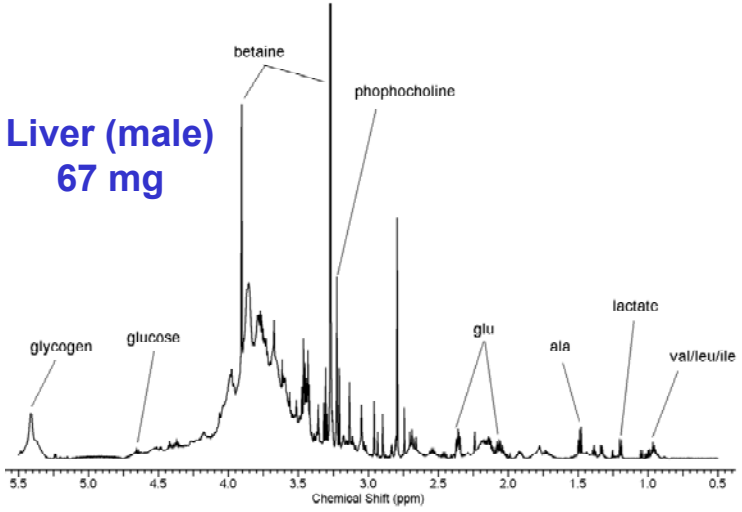
Brain (male)
47 mg



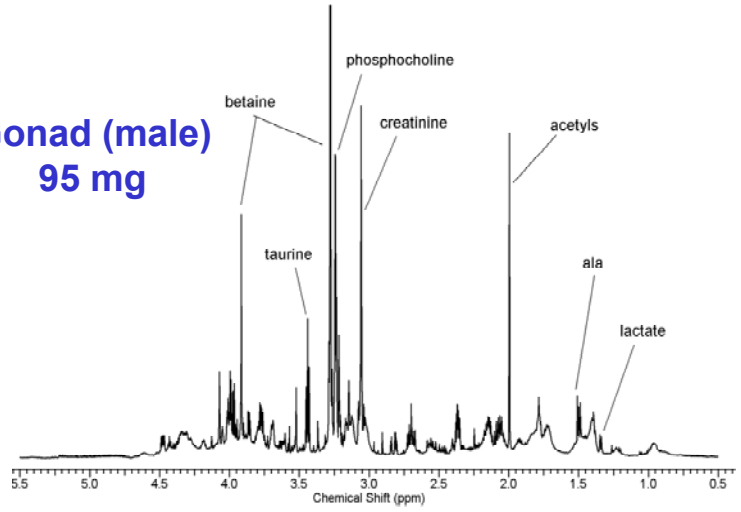
Plasma (male)
70 mg



Liver (male)
67 mg



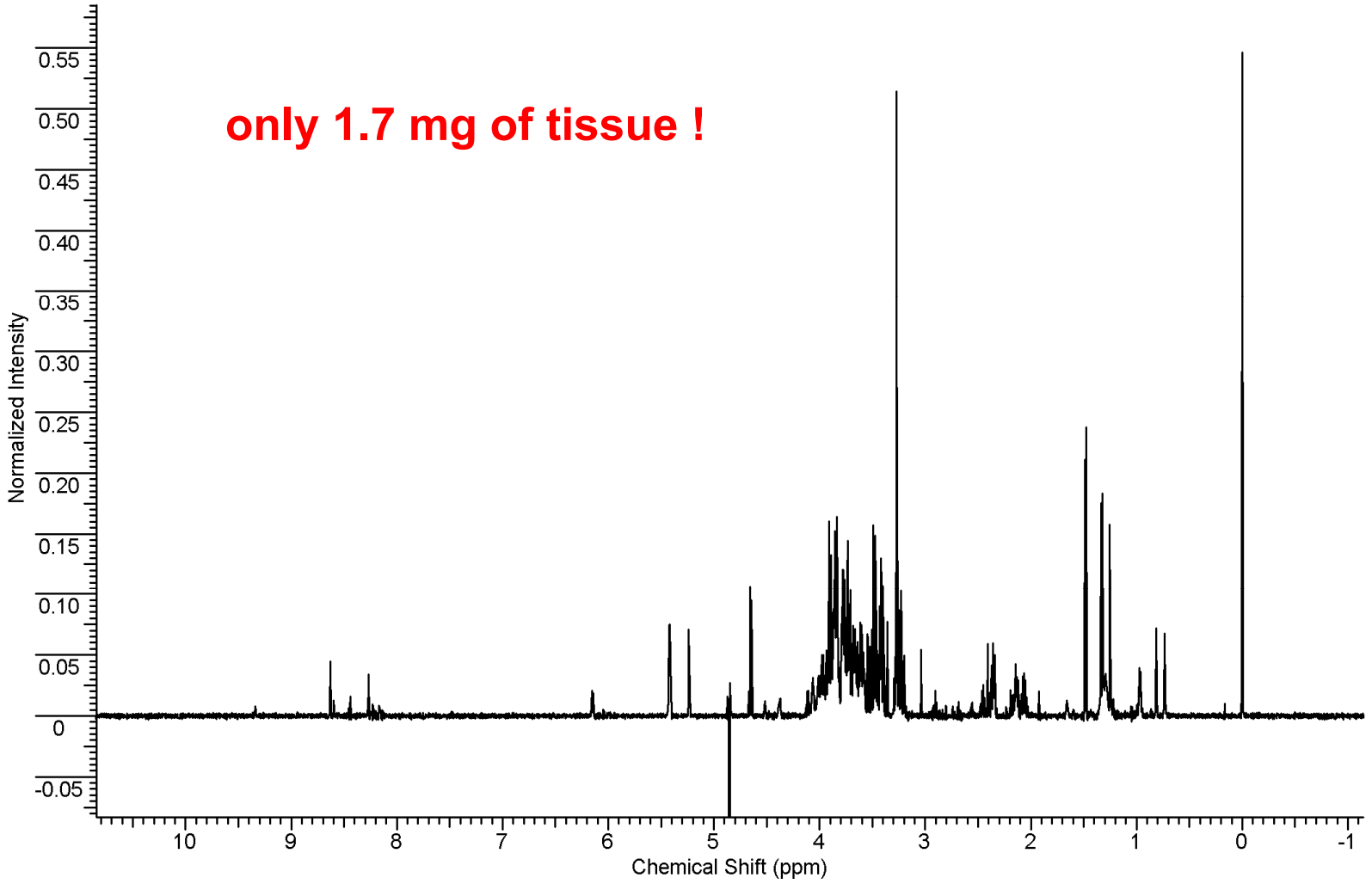
Gonad (male)
95 mg



^{1}H-NMR Spectrum of Zebrafish

Liver Extract

600 MHz (cryogenic probe)



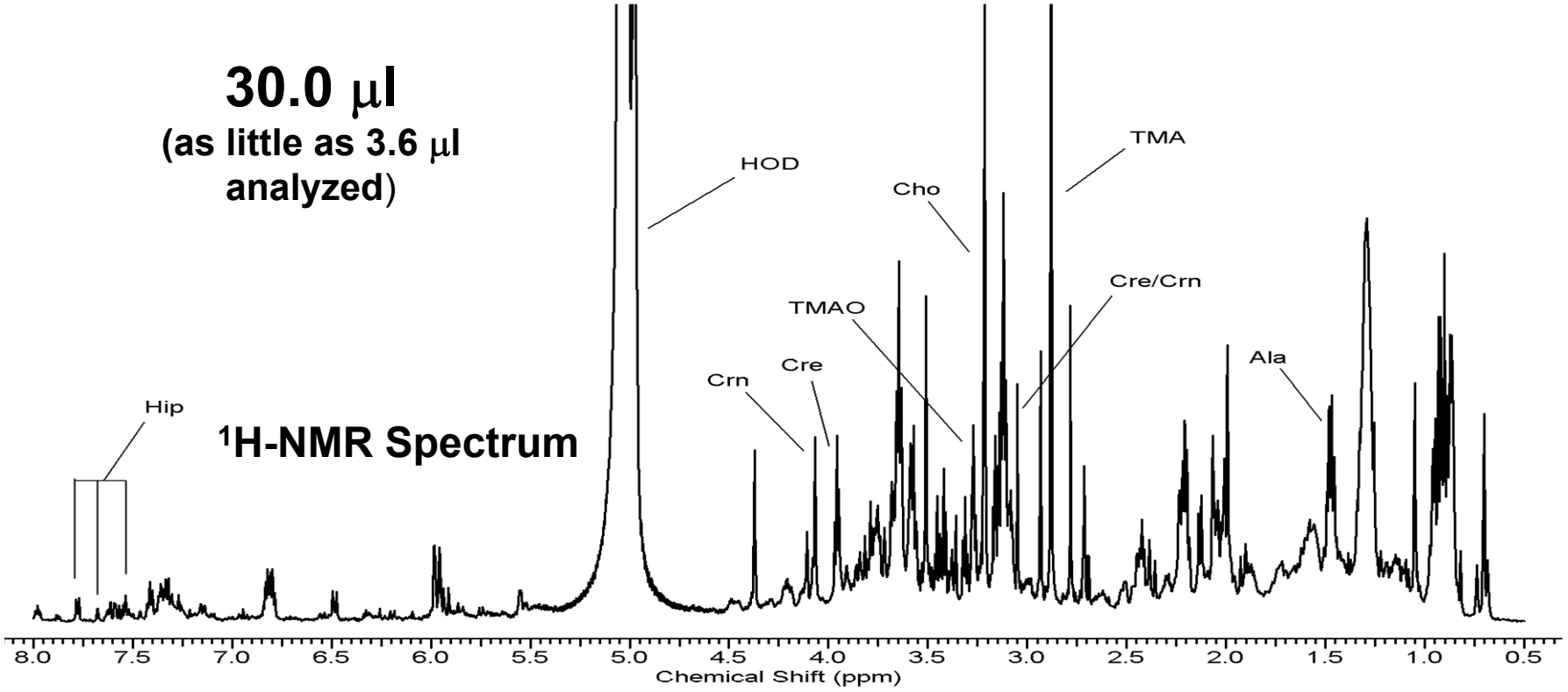
Male Fathead Minnow Urine:

allows repeat, non-lethal sampling

FHM urine-based metabolomic studies –
talk by Drew Ekman Monday 12:50 Belle Chasse

30.0 μl
(as little as 3.6 μl analyzed)

^{1}H-NMR Spectrum



Direct versus Indirect Responses to Chemical Stressors

a critical issue for toxicogenomics and risk assessments!

What is reflective of toxicity ("true harm") versus an adaptive response?

When can organisms compensate for the chemical stress?

When can organisms recover from episodic stress?

A complex function of both concentration and duration of exposure

exposure studies with meaningful concentration- and time-courses are needed

Mapping these response functions requires:

1) low-cost exposure scenarios

2) low-cost analysis, high sample throughput

3) highly reproducible "profiling"

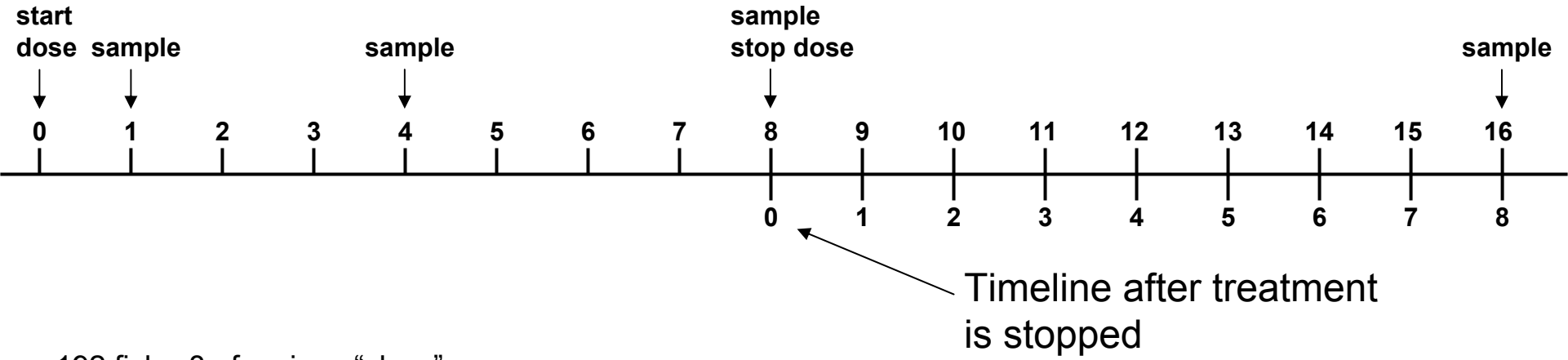
NMR-based Metabolomics with Small Fish Models is Ideal!

Metabolomics for Investigating Compensation and Recovery in Exposed Fish

male and female fathead minnows exposed to the model estrogen mimic 17α-Ethynylestradiol (EE2)

EE2 is known to feminize male fish

Sampling timeline (in days) for 3 treatment levels (0, 10, 100 ng/L)

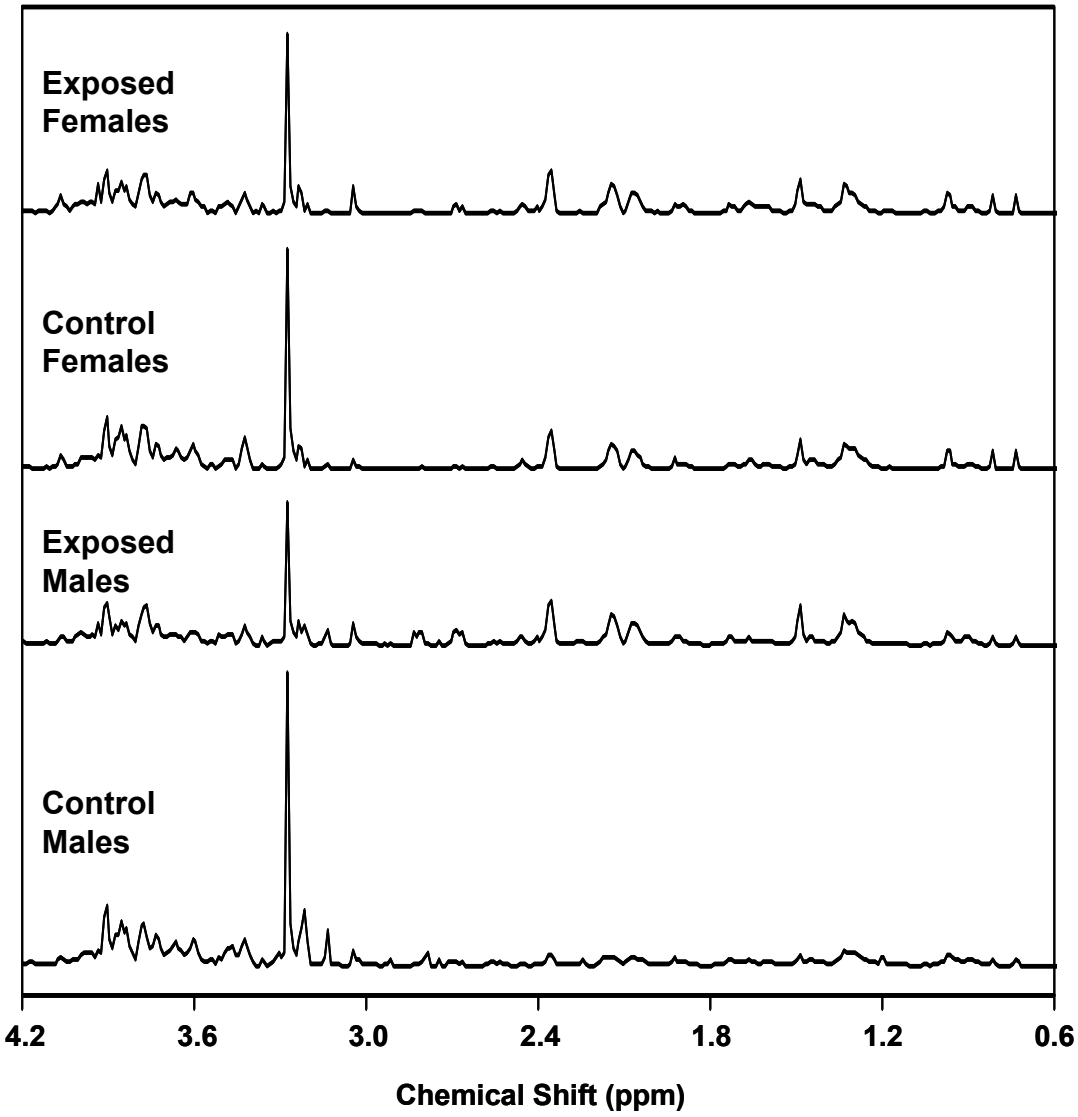


192 fish - 8 of a given “class”, where class is defined by gender, exposure level, and sampling time

Can We See Feminization of Males with Metabolomics ?

One "Snapshot" in Time and Concentration - Day 4, 100ng/L

polar liver extracts averaged ^{1}H-NMR spectra



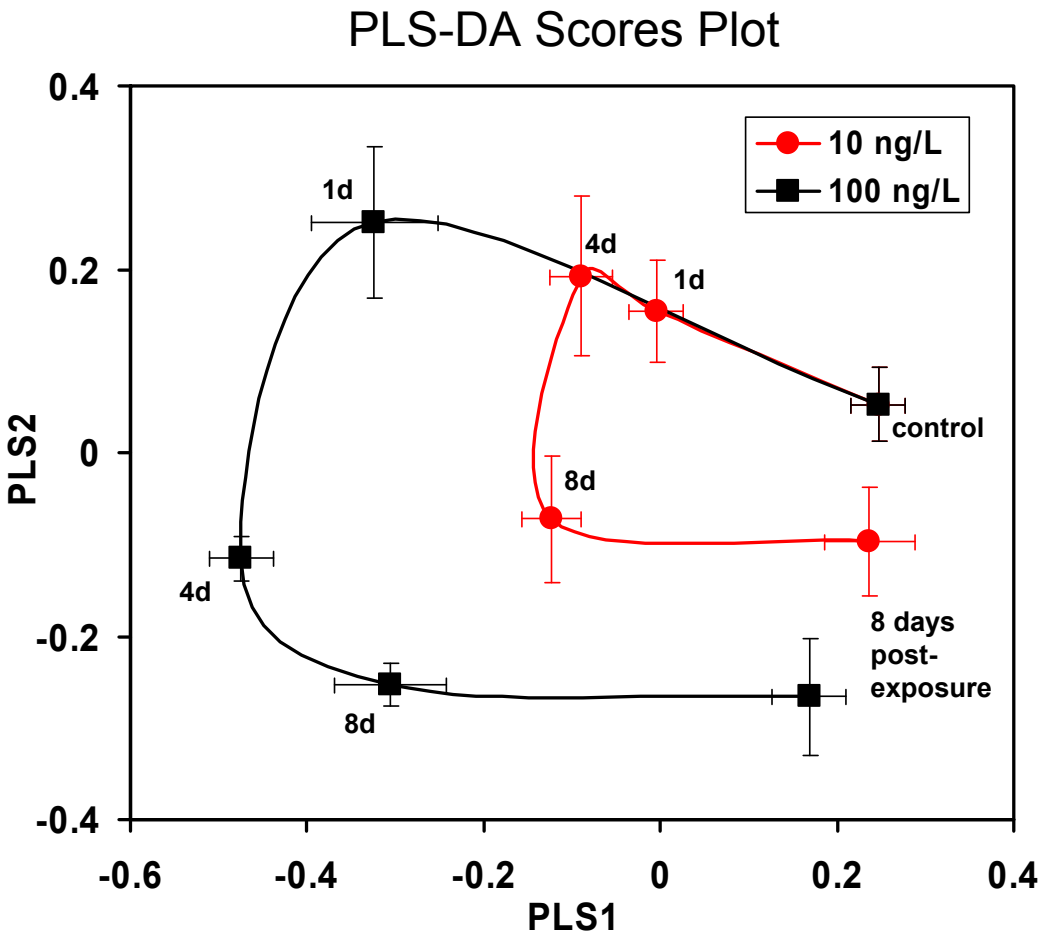
Impact of exposure is greater for males

Males are more like females after exposure

Can We See Compensation and Recovery in Males ?

Time Course Data for Both Concentrations

polar liver extracts males only ^{1}H-NMR spectra

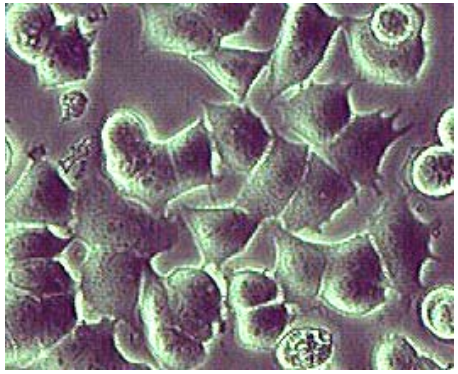


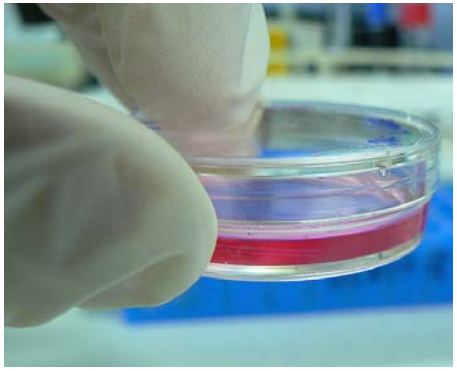
Fish appear to partially compensate by Day 8

Fish appear to partially recover after EE2 is removed

Recovery is more-complete at the low concentration

Cell Culture-Based Metabolomics

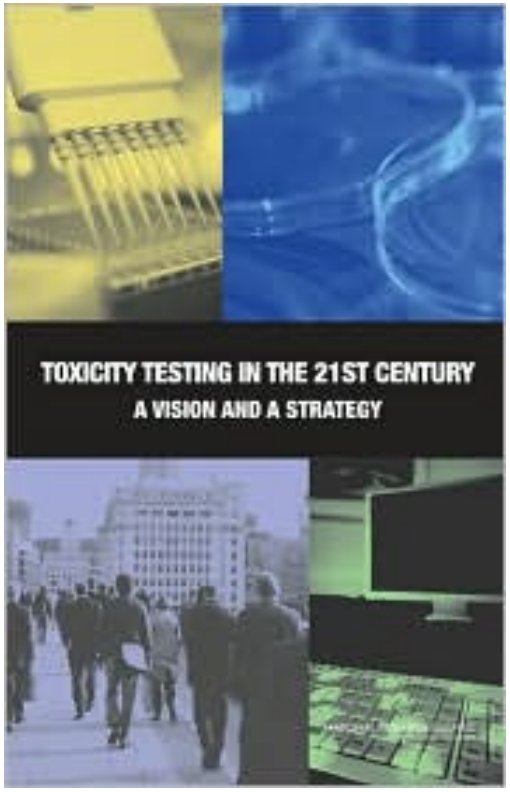




- Can reduce the use of animals (but, effective extrapolation to whole organism responses is required).
- Can be very rapid, inexpensive and highly automated.
- Facilitates cross-species comparisons.
- Human cell lines can be employed.

We are developing a method for measuring metabolites in living cells in real time !

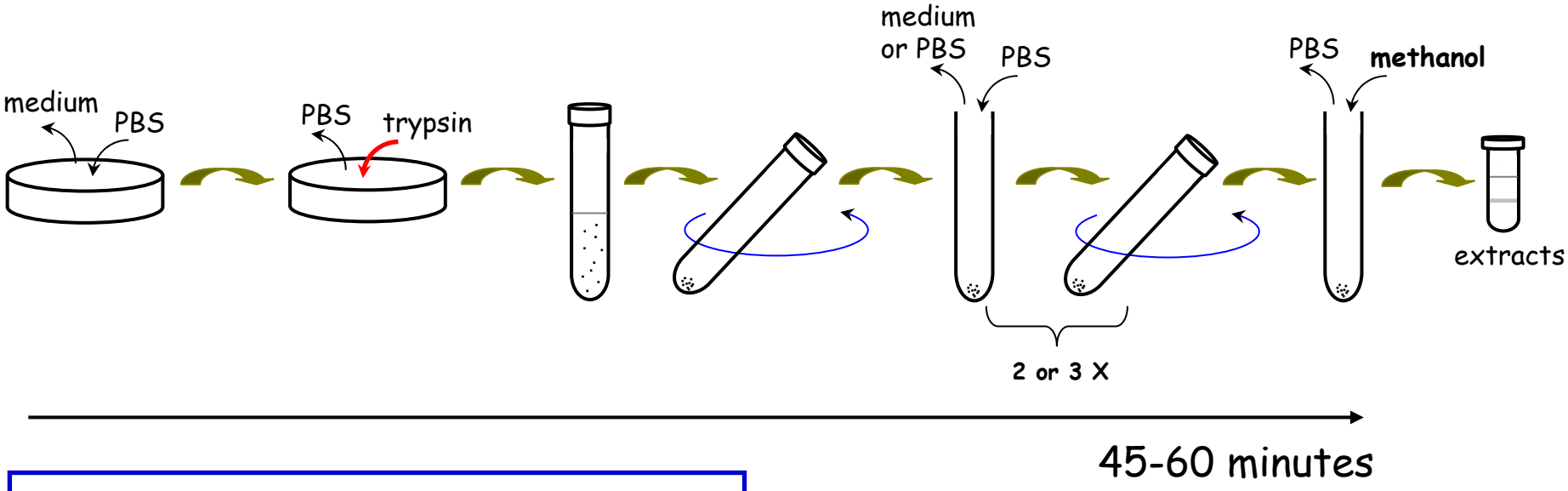
Toxicity Testing in the 21st Century
(a big role is envisioned for cell-based assays)



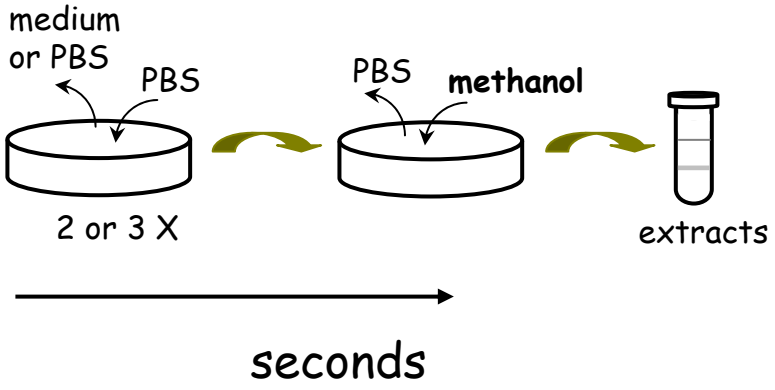
"Transform toxicity testing from a system based on whole-animal testing to one founded primarily on *in vitro* methods that evaluate changes in biologic processes using cells, cell lines, or cellular components,..."

National Research Council (2007)

First Step – Develop Cell Quenching Method Suited for Profiling Intracellular Metabolites



Our Direct Quench Method



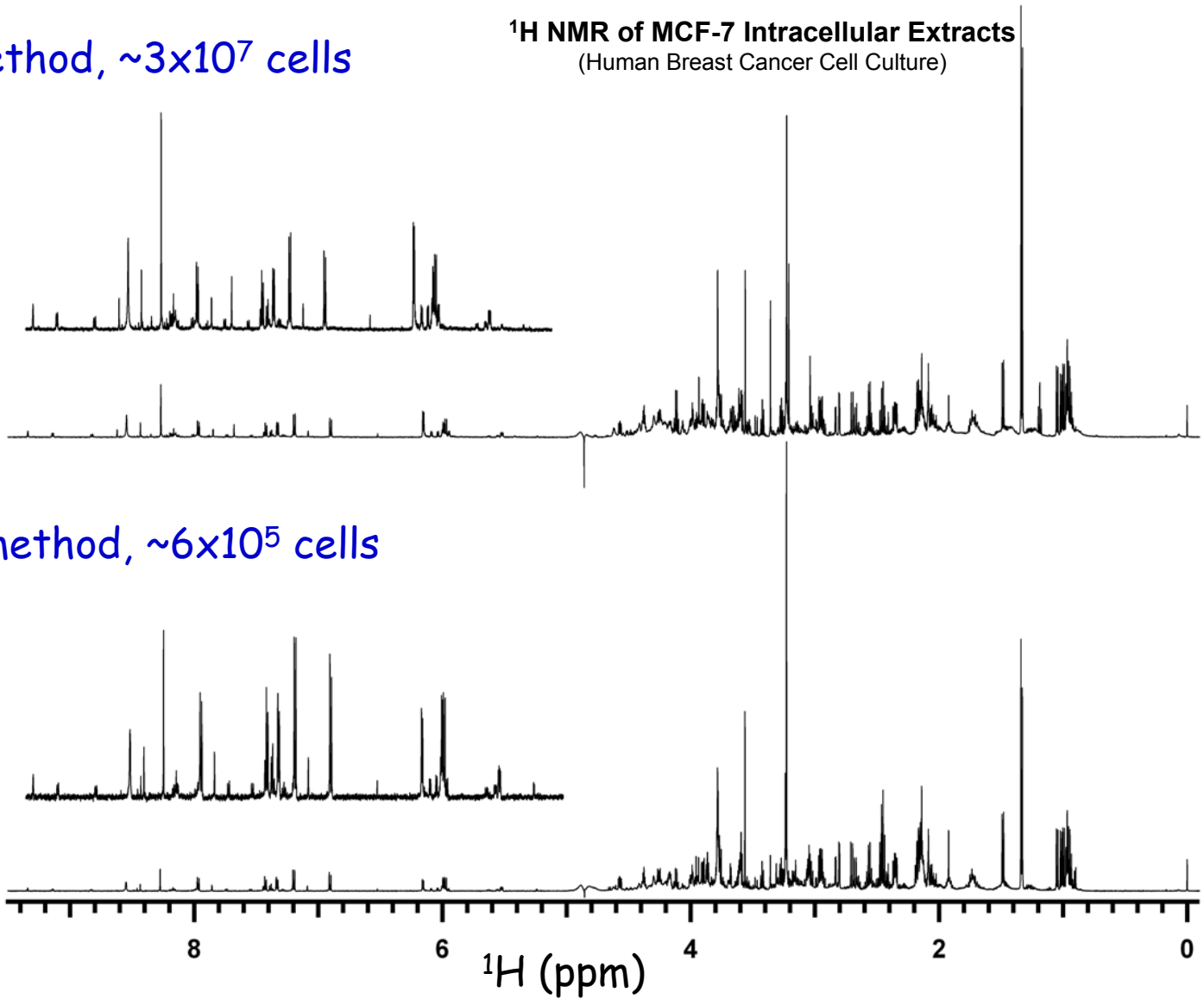
Conventional Method –

- **Some metabolites lost into solution during trypsinization and wash / centrifuge steps.**
- **Metabolome changing due to fast turn-over of some metabolites (seconds to minutes).**

Direct Quench Method – 50 X Recovery

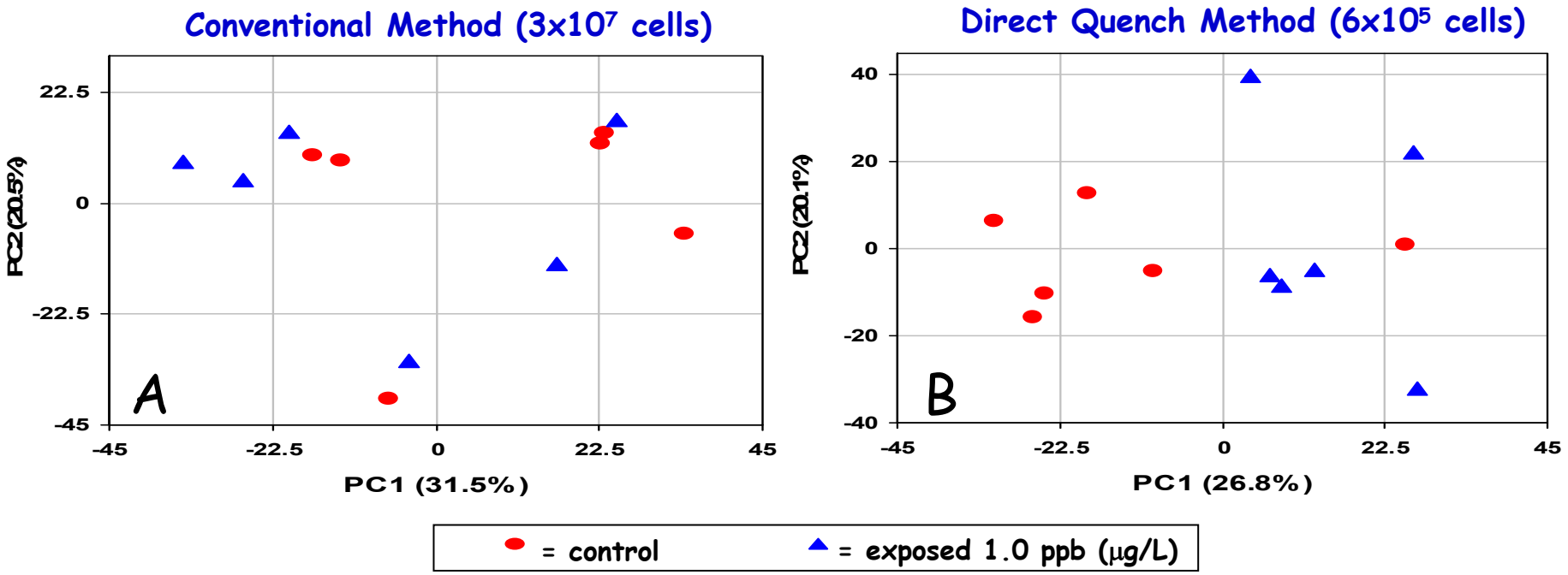
Conventional method, ~3x10^7 cells

Direct quench method, ~6x10^5 cells

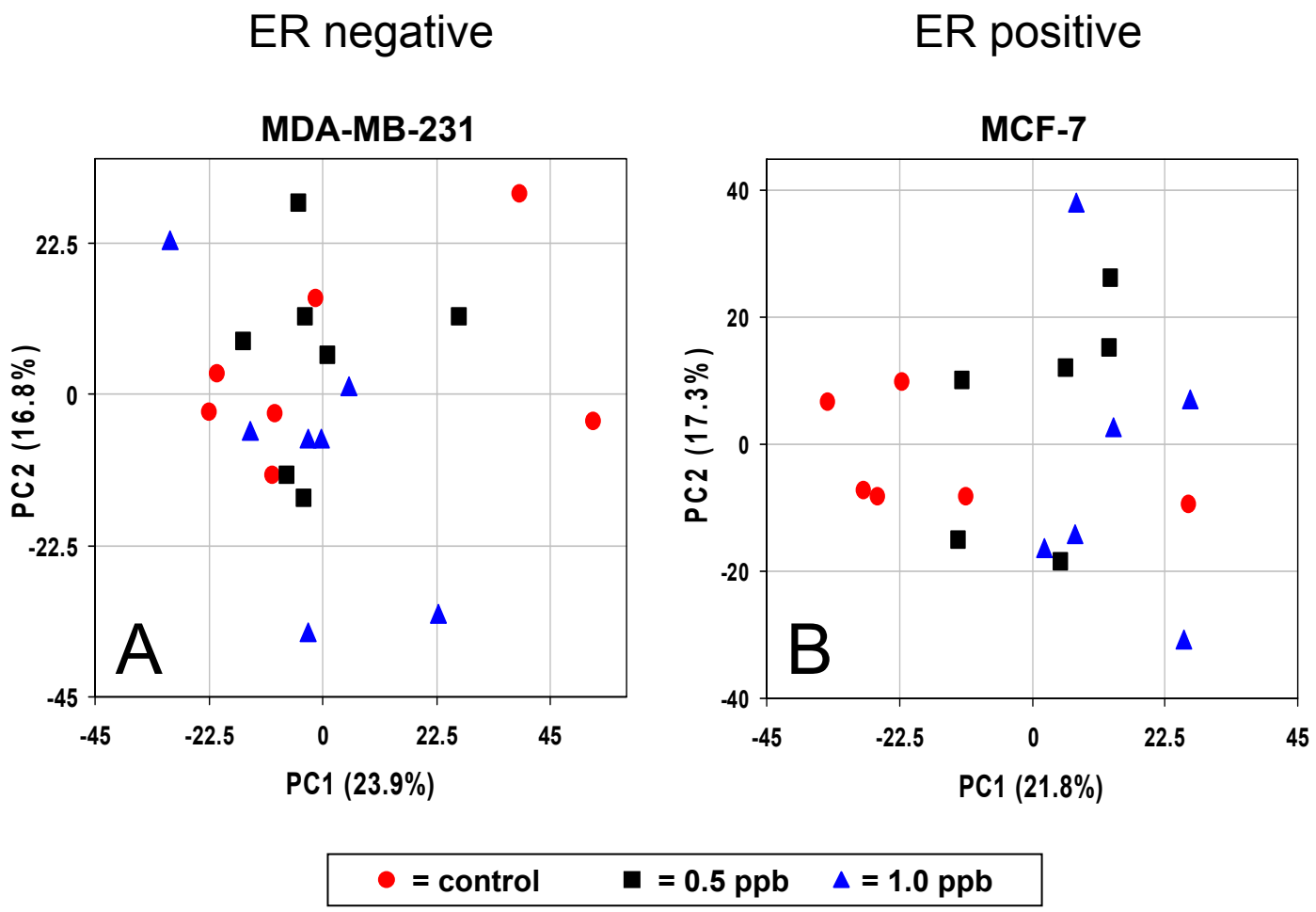


Direct Quench Method – More-representative Metabolite Profile

Exposure of a human breast cancer cell line (MCF-7), which is estrogen-receptor positive, to EE2

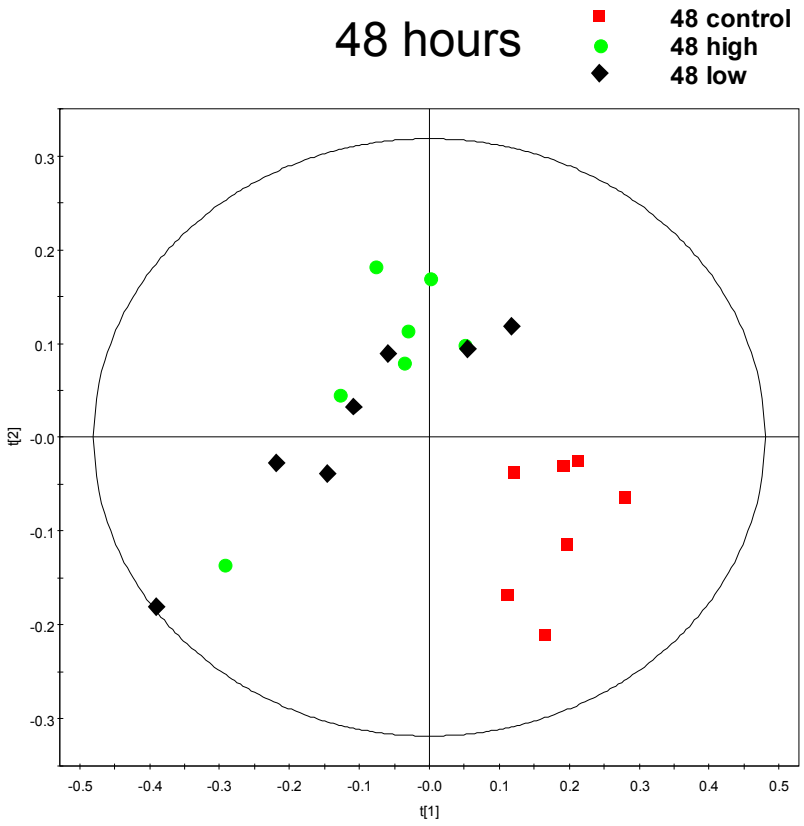


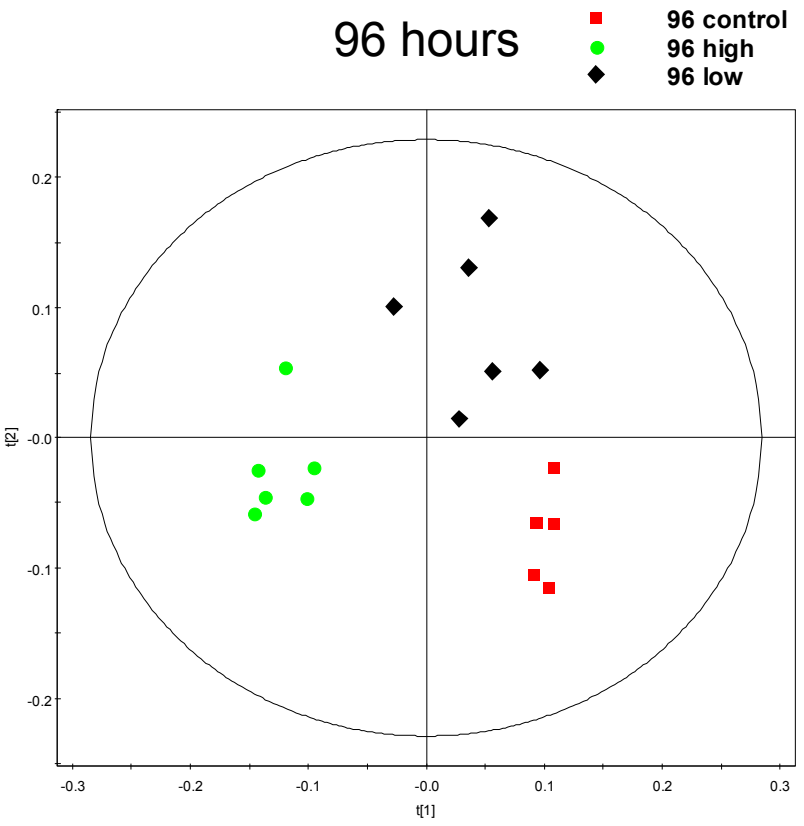
Exposure of a human breast cancer cell line to EE2



Zebrafish Liver Cell Line
exposed to 17α-Ethynylestradiol (EE2)

PLS-DA scores plots





two different concentrations (0.5 or 5.0 ppb) for 48 or 96 hours

In Closing .....

Metabolomics is a powerful technique in the molecular toolbox for studying aquatic contaminants

- particularly with non-model species (no need for sequenced genome)
- potential for high-throughput (low per sample cost)
- useful for distinguishing true long-term harm from temporary alterations
- well suited for cell-culture exposures

Coworkers / Collaborators

Athens Metabolomics Group Members:

Drew Ekman
Matthew Henderson
Quincy Teng
Kim Ralston-Hooper
Wenlin Huang

US EPA, Duluth, MN.

Gary Ankley
Elizabeth Durhan
Kathy Jensen
Mike Kahl
Elizabeth Makynen
Dalma Martinovic
Dan Villeneuve

Other Government, Academics, and Private Organizations

Various other EPA labs, Army Corp of Engineers, UFla, OHSU,.....