Metodologie ecotossicologiche: nuove sperimentazioni e criticità applicative

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cibm
CENTRO INTERUNIVERSITARIO BIOLOGIA MARINA ED ECOLOGIA APPLICATA

Current approach in environmental assessment by European legislations

Law limits for single chemicals

- Quantification of about **100-150** chemicals
- Comparison of concentrations with **safety limits**

Current approach in environmental assessment by European legislations

However more than **280.000 substances** are registered as **toxic** by the American Chemical Society

Examples of **emerging pollutants**:

- nanoparticles
- pharmaceuticals
- body care products
- drugs
- etc…

Limits of the chemical approach

- It's impossible to quantify **all potentially toxic substances**

- It's difficult to infer toxic effects due to **mixture of pollutants**
  (e.g. additive, synergistic, etc…)

- It's impossible to evaluate the biovulnerability of all the different chemicals for the organisms.

Coupling biological and chemical data

- Assessment of the **ecological risk** of a site

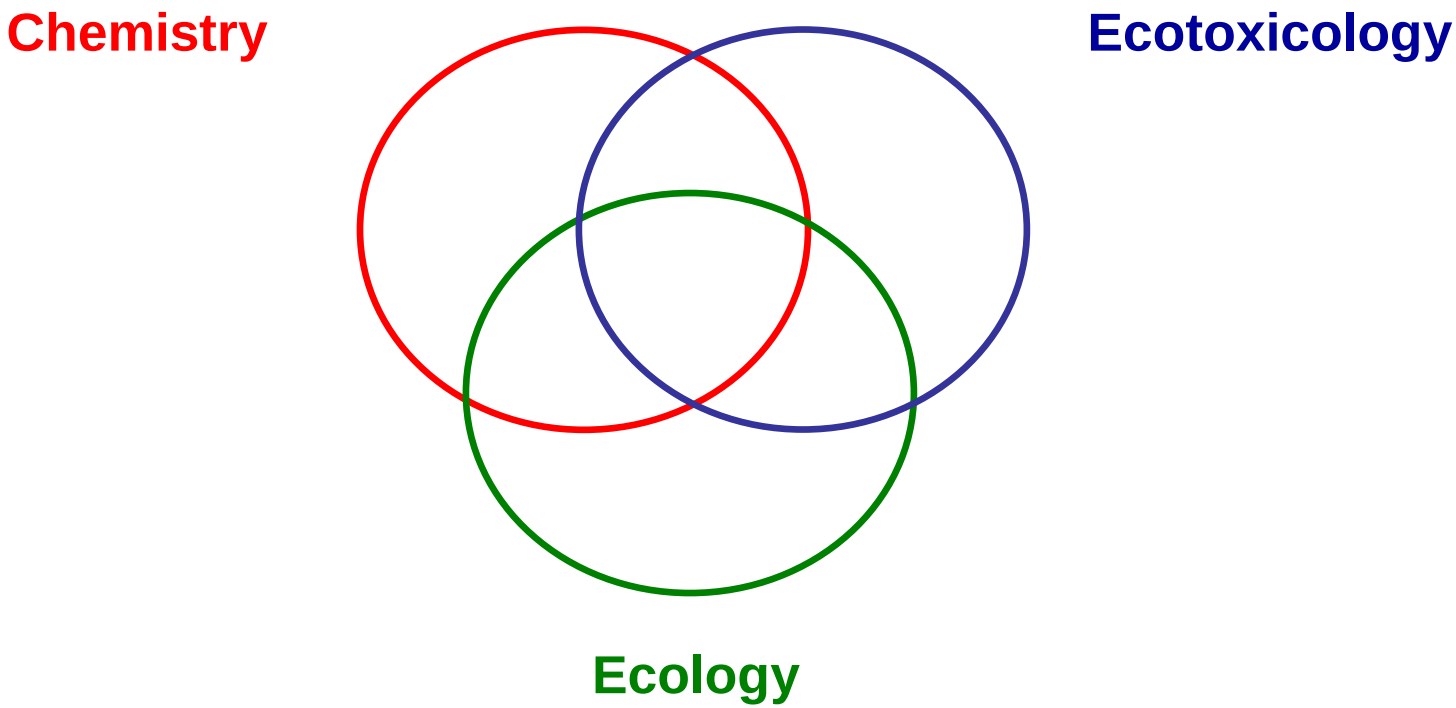
  → through a weight-of-evidence **Triad approach** (i.e. integration of chemical, ecotoxicological and ecological data)

- Evaluation of the **quality** of an environmental matrix, such as sediment and water

  → through a 2-legs **Duad approach** (i.e. integration of chemical and ecotoxicological data)

Crucial for a **correct environmental management** (e.g. dredging activities of dams, remediation, etc…)

The Triad approach



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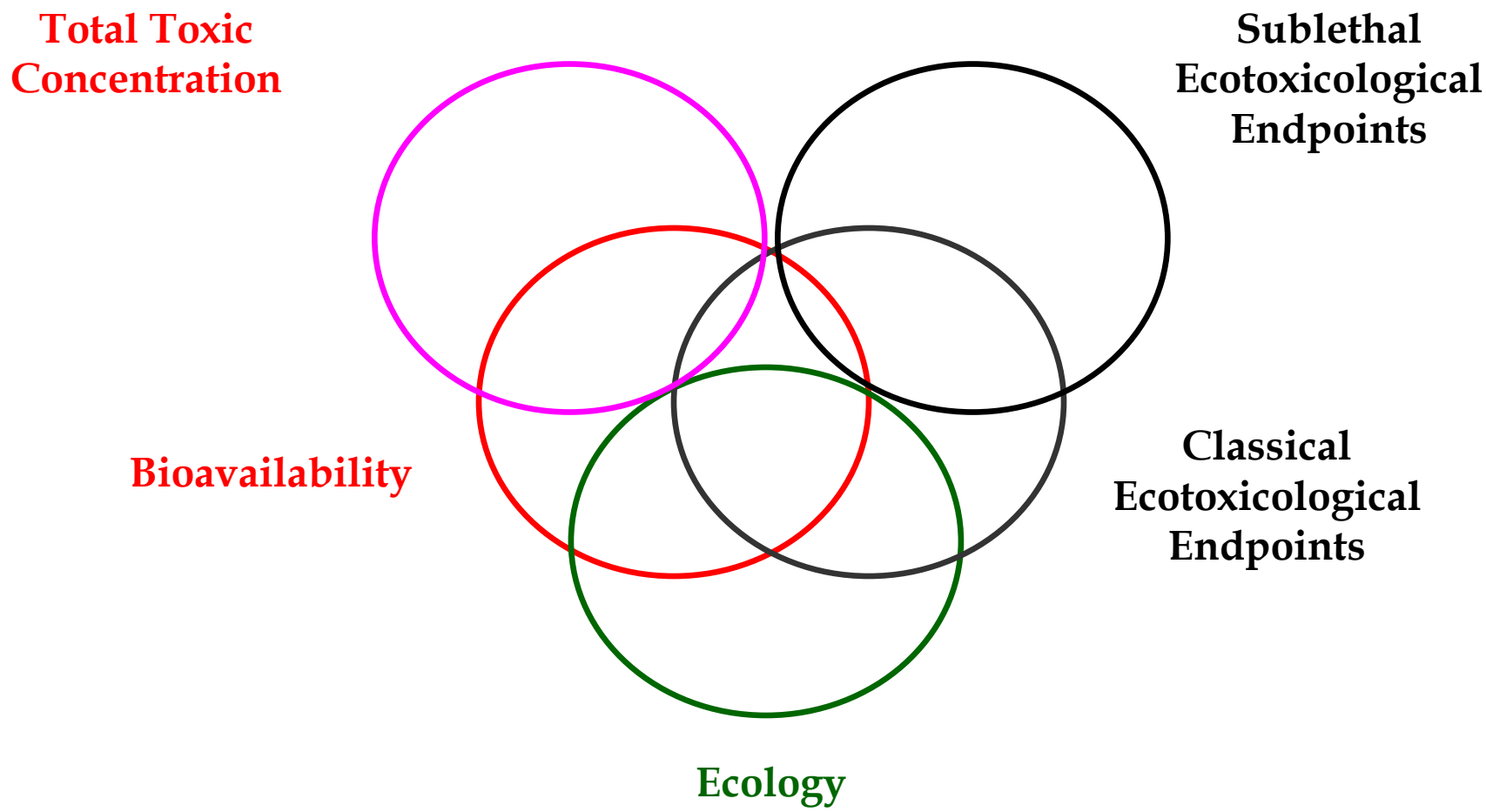
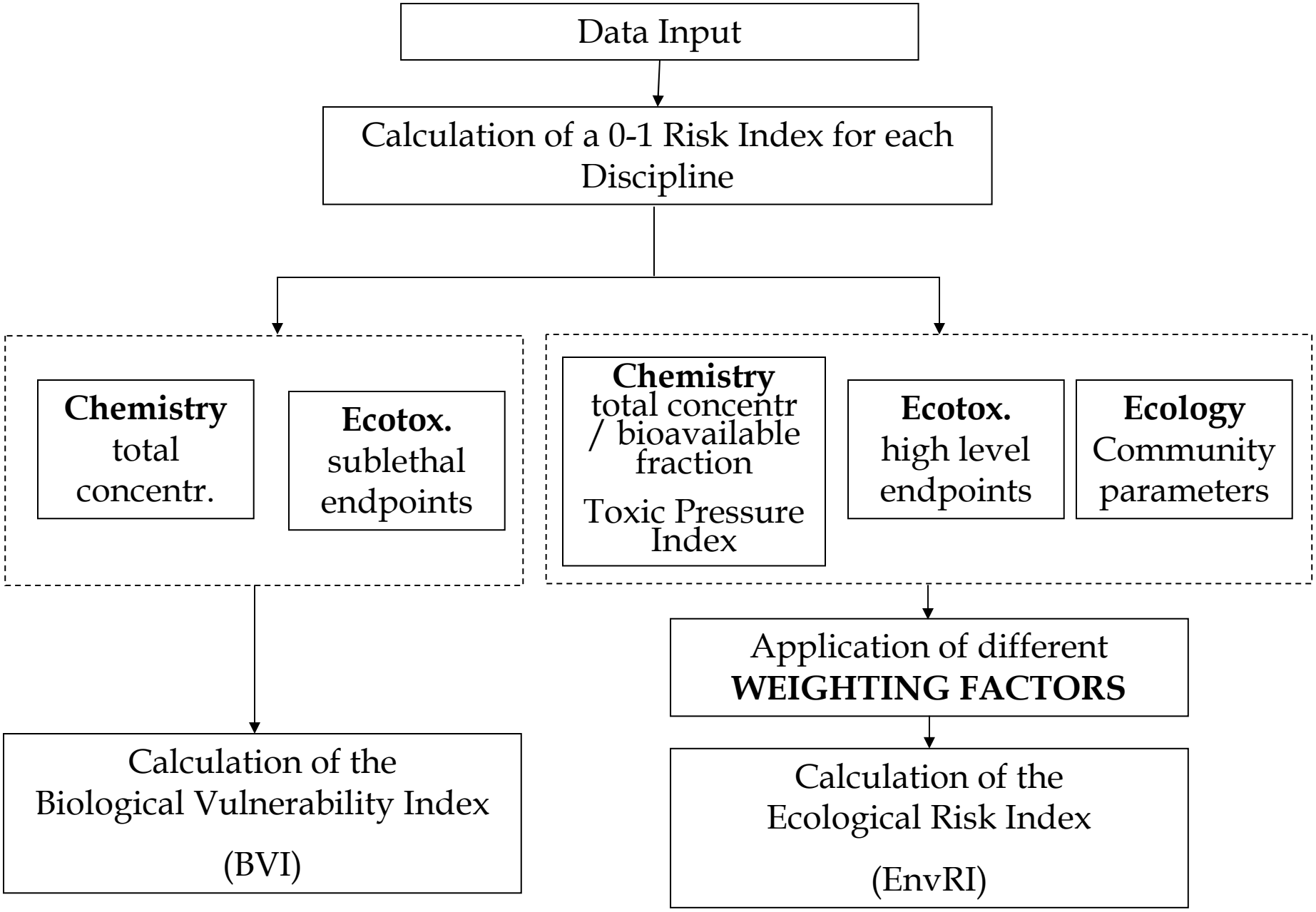
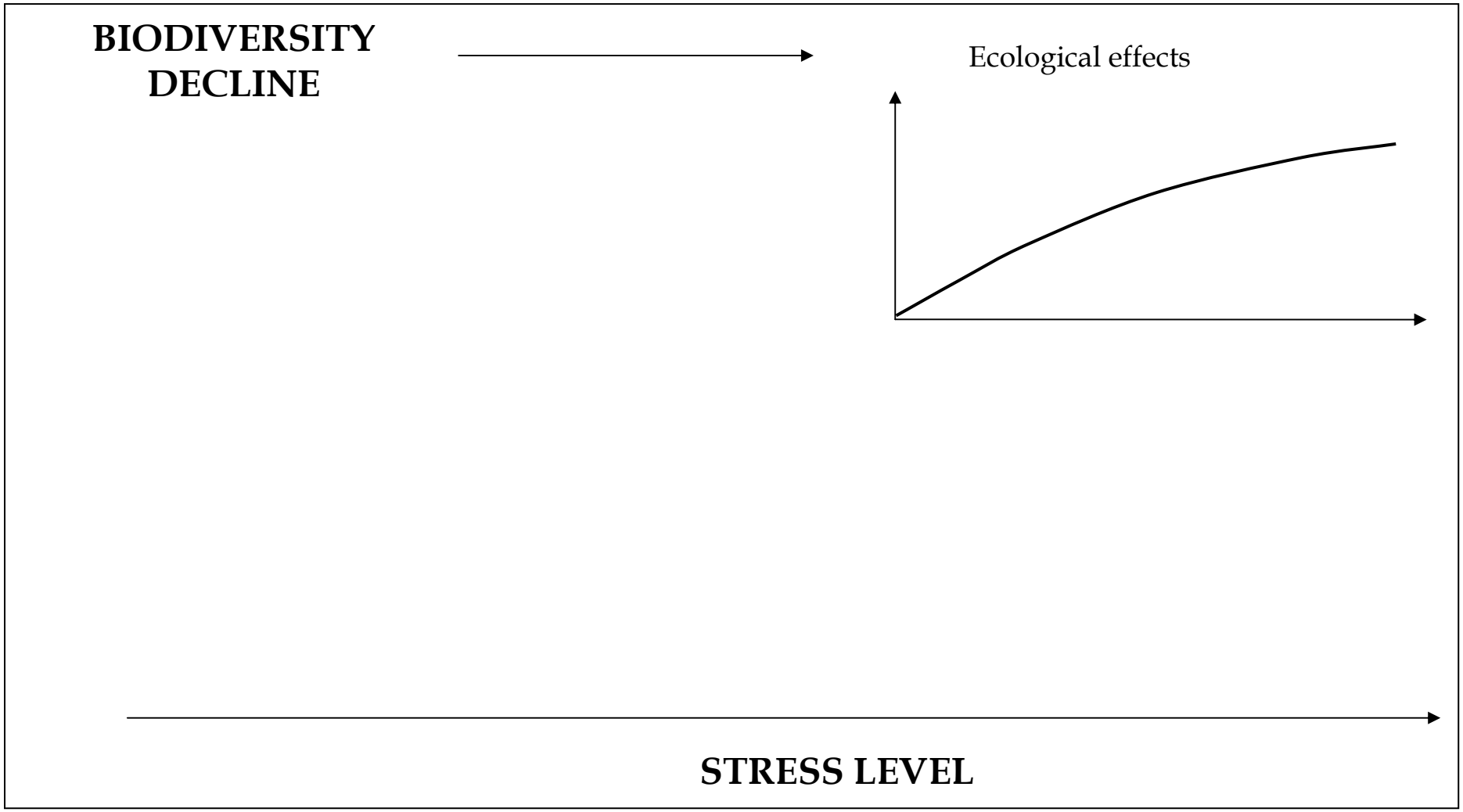


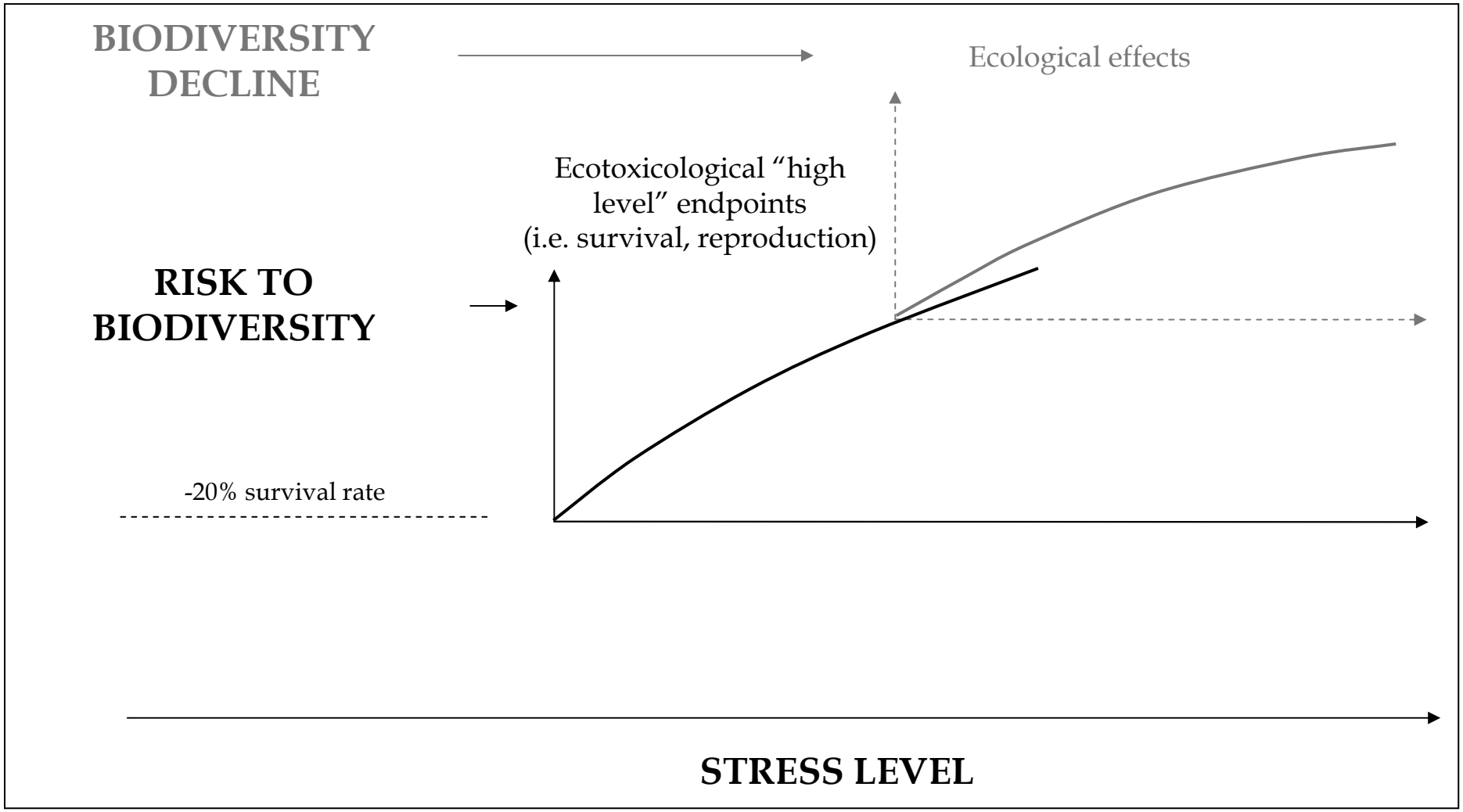
Integration procedure



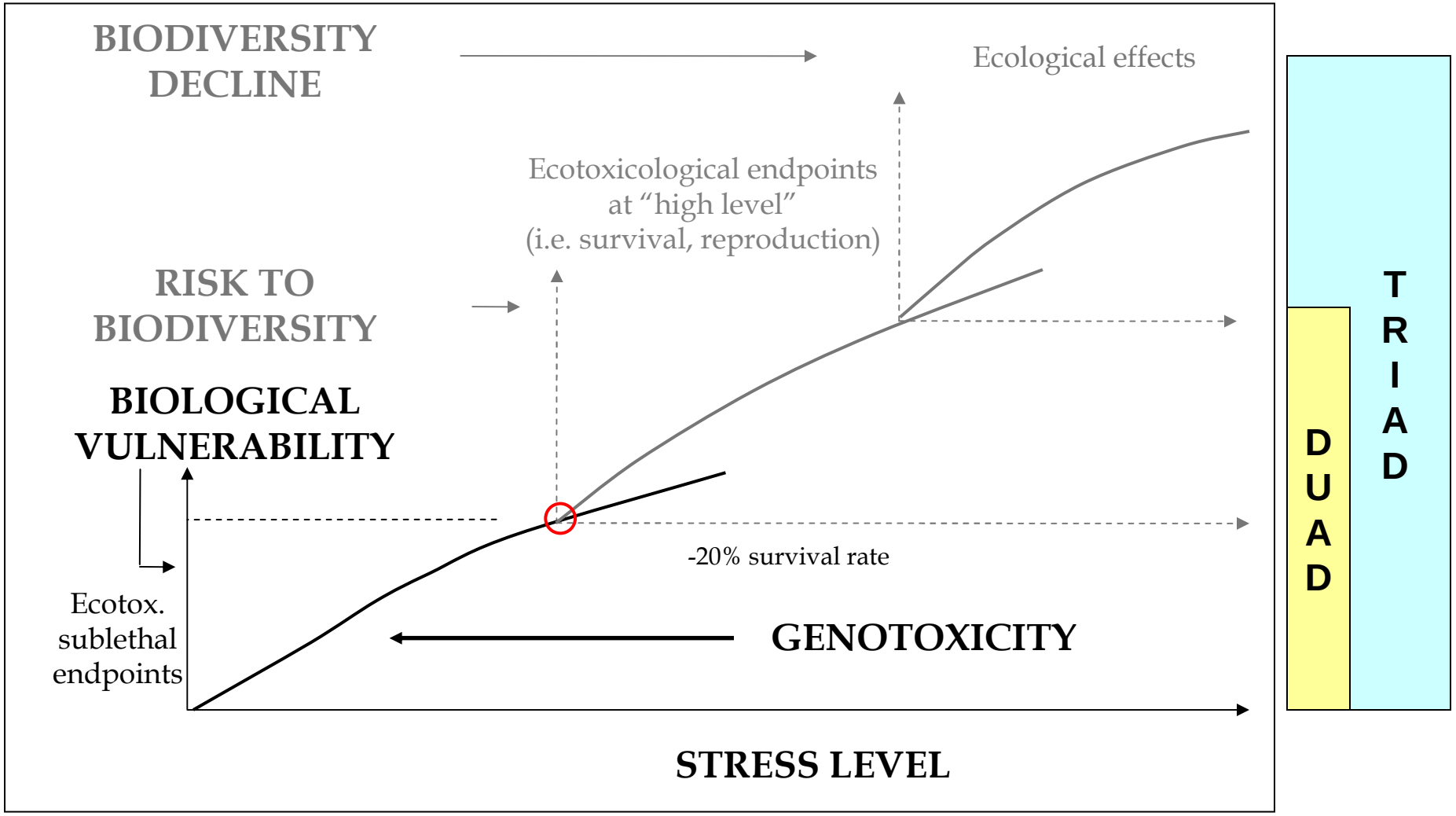
Stress level and biological response



Stress level and biological response



Stress level and biological response

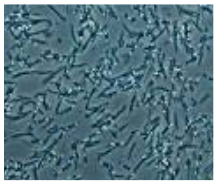


Community structure effects

Aquatic ecosystem

Benthic macroinvertebrates

Extended Biotic Index (different microhabitat)



Microbial community

Bacterial biomass, DGGE

Diatom community

EPI-D

Terrestrial ecosystem

Soil microarthropods

QBS index

Nematode community

Maturity index

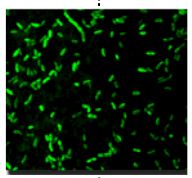
Microbial community

Bacterial biomass, DGGE

"High level" ecotoxicological endpoints
Toxicity of the different matrices (Duad chemicals/biological data)

WATER AND SEDIMENT **SOIL**

Vibrio fischeri



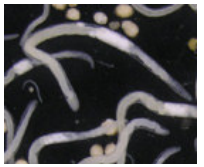
Chiromons tentans

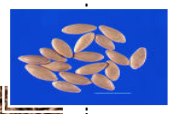
Caenorhabditis elegans

Eisenia andrei

Dictyostelium discoideum

Enchytraeus crypticus

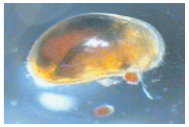


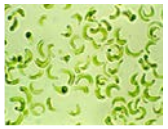


Phytotest

Daphnia magna Straus

Heterocypris incongruens





Folsomia candida

Pseudokirchneriella subcapitata

Assessment of sublethal effects
Test with *Pisum sativum*

Germination rate

DNA damage

(Comet assay)

Root growth

Mitotic index

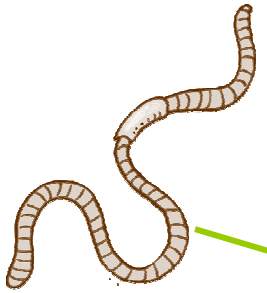
Micronuclei frequency

Mitotic anomalies

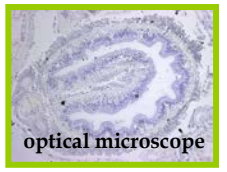
Assessment of sublethal effects
Test with earthworms

Eisenia andrei

Lumbricus rubellus

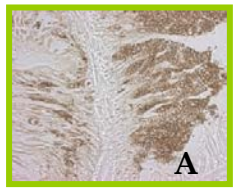


Histology



Earthworm's digestive system

Cytochemistry



Plasmembrane CaATPase activity:
A – chloragogen tissue, B – intestinal epithelium

Stress biomarkers

- **Lysosomal membrane stability**
- **Lipofuscine and neutral lipids accumulation**
- **Ca^{2+}-ATPase activity**
- **Lisosome/cytoplasm – Tissue damage**

Exposure biomarkers

- **MT: heavy metals response**
- **Peroxisomes proliferation**

Genotoxicity biomarkers

- **DNA damage (Comet assay)**
- **Micronuclei frequency**

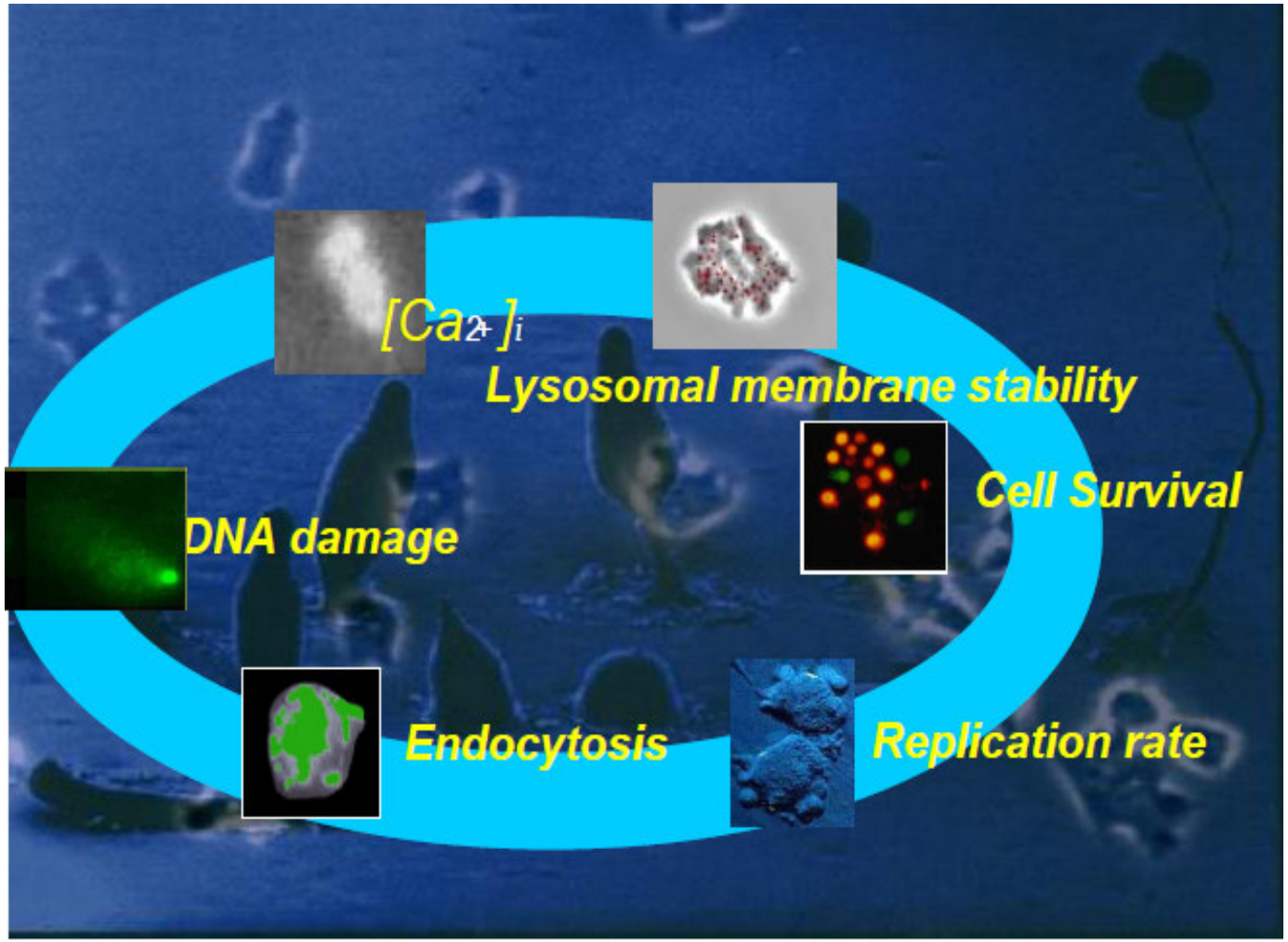
Assessment of the **health status**

applying an

Expert System

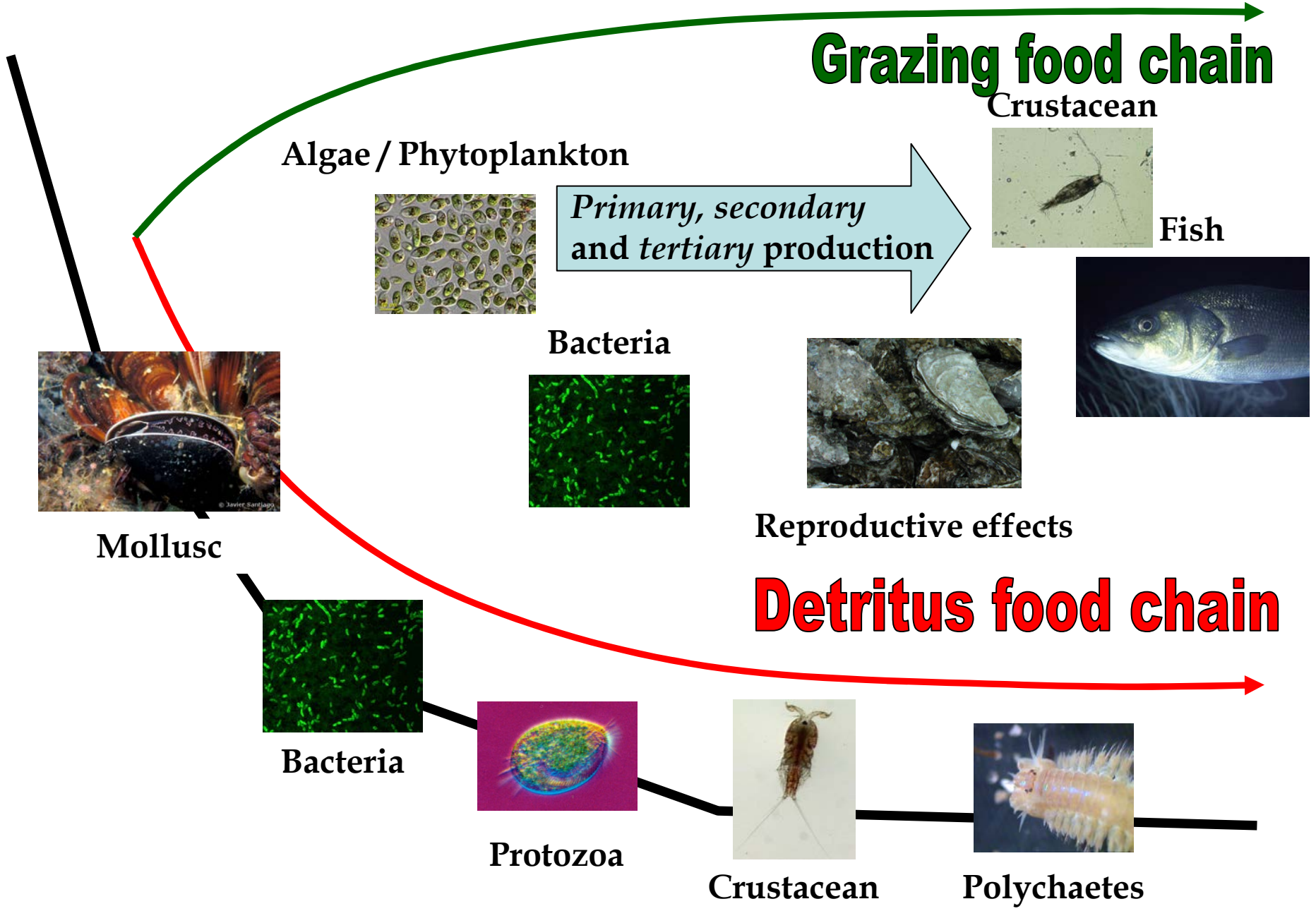
Dictyostelium discoideum as a model in ecotoxicology

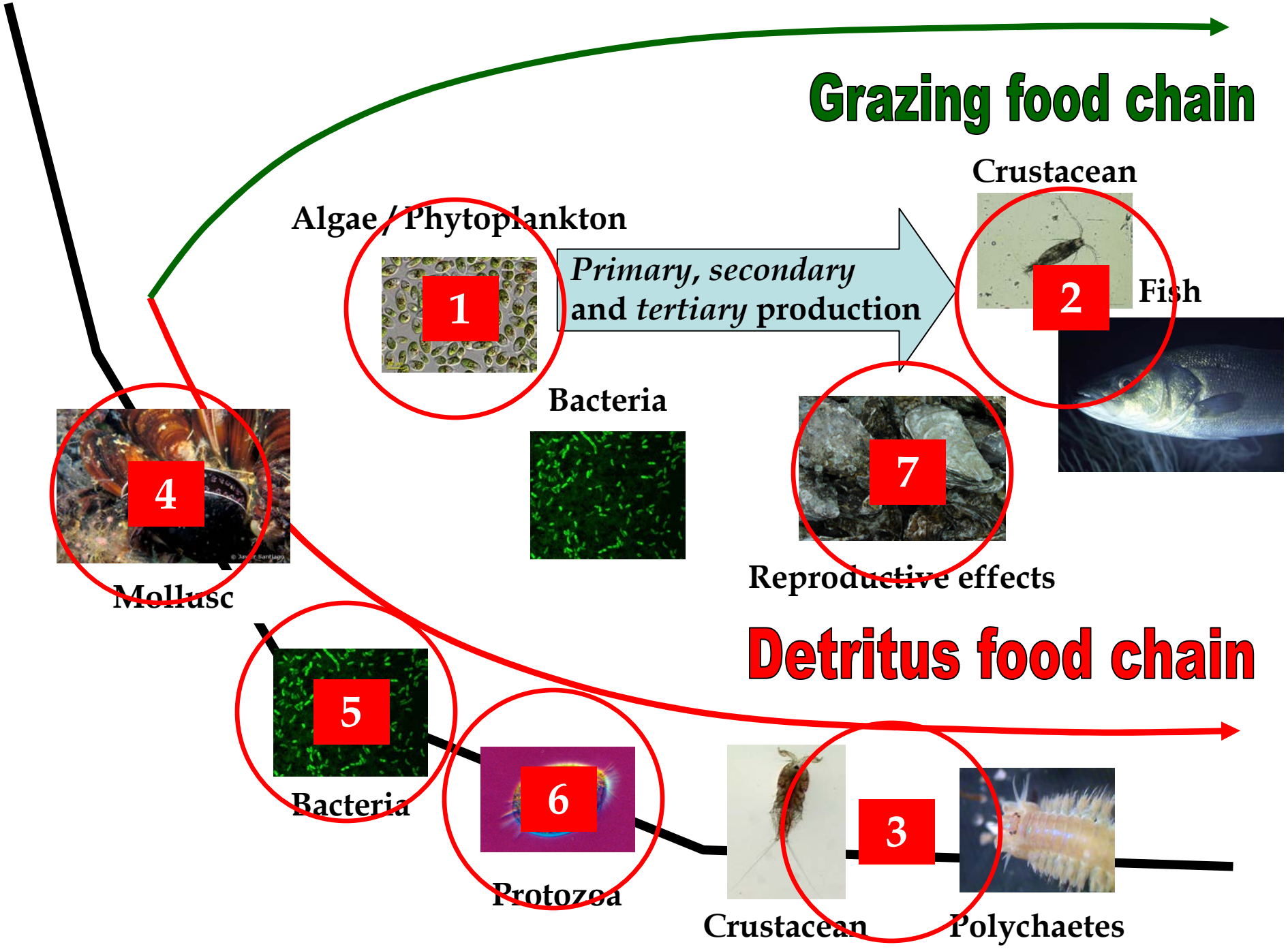
The bioassay consists of a battery of biomarkers able to evaluate the effects of toxic environmental contaminants



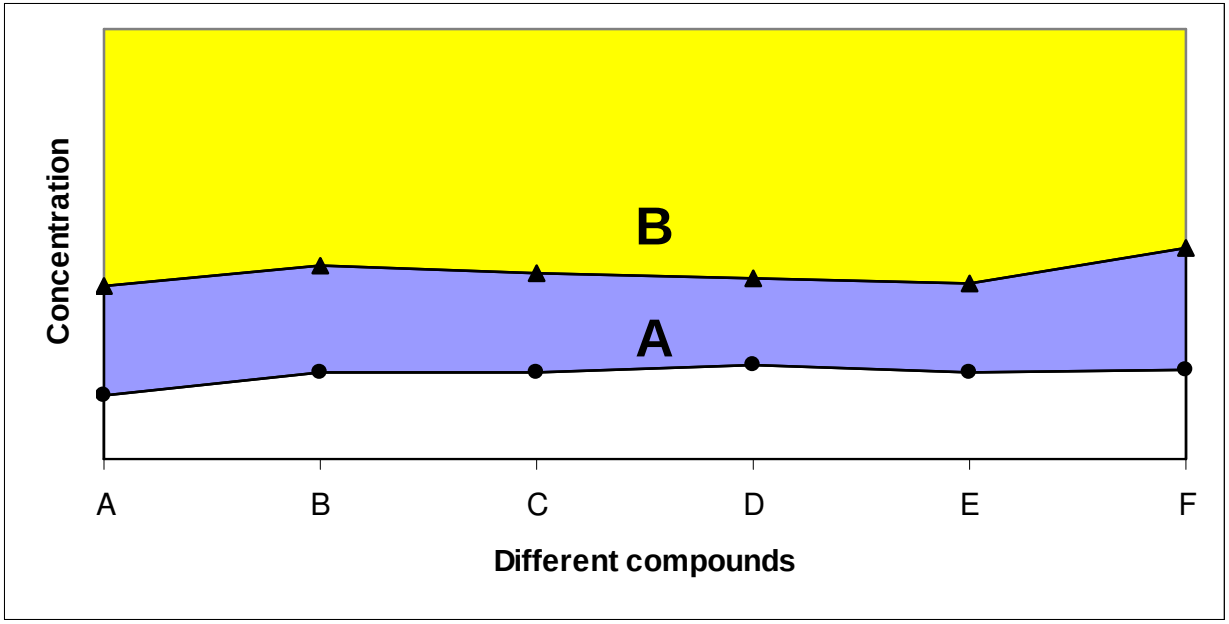
Dondero et al., Comp. Biochem. Physiol., 2006.

Marine Coastal Environmental I example




Grazing food chain
Crustacean
Algae / Phytoplankton
1
Primary, secondary and tertiary production
2
Fish
Bacteria
4
7
Mollusc
Reproductive effects
Detritus food chain
5
Bacteria
6
Protozoa
3
Crustacean
Polychaetes

The evaluation of the fresh water sediment toxicity II Example



In collaboration with
ISPRA - Ing. Angelo Felli

A screening bioassay to evaluate possible to effects of toxic chemicals on the organisms.

B evaluation of the level of the effects of pollutants on living organisms utilising a full duad approach.

Proposed ecotoxicological bioassays

Organism	Systematic group	Method	Sample		
			Elutriate	Pore water	Sediment
Daphnia magna	Cladocera	OECD 202 (1984) OECD 211 (1998)	+	+	+
Pseudokirchneriella subcapitata	Algae	OECD 201 (1984)	+	+	
Lumbriculus variegatus	Oligochaeta	OECD 225 (2007)	+	+	+
Chironomus spp.	Chironomidae	OECD 219 (2004)	+		+
Microtox (*Vibrio fischerii*)	Bacteria	Standard method not available	+	+	+
Dictyostelium discoideum	Protozoa	Standard method not available	+	+	
Phytotest		UNI 10780 (1998)	+	+	+

The application of these tests follows the indications of US-EPA (2007)

Bioassays: a correct mix of different tests and end points

Different tests: depending on the studied ecosystem with emphasis on the grazing/detritus food chain

End points

High level: mortality: usually the less sensitive

reproduction (chronic tests): usually high by sensitive

Sublethal end points

1) Early warning

2) Able to follow the development of the pollutant induced stress syndrome from initial effects at molecular/cellular level to mortality.

3) Responses able to link effects on the ecosystem to the risk at human population level:

   a) genotoxicity effects

   b) effects on the immunosystem

   c) endocrine disruption

QA (Quality Assurance) Program

- Preparation of cultures and distribution of the model organisms utilized in the different bioassays: same specie, same genotype, same level of organism development;
- Distribution of a manual for bioassay utilization;
- Diffusion of a video showing bioassays methodologies;
- Organization of Training Courses to prepare the researchers to participate to the oncoming biomonitoring programs;
- Organization of an “Intercalibration Program”.

Molecular Highthroughput Techniques and The Systems Toxicology Approach

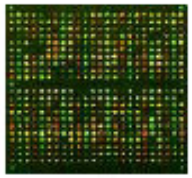
i.e. an integration of

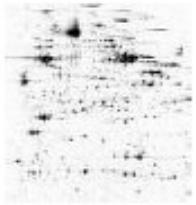
Transcriptomics

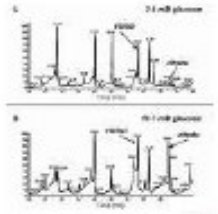
Proteomics

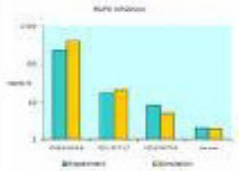
Metabolomics

biochemical / cytochemical / functional data (physiomics)









To explain mechanistic effects of pollutants in ecotoxicological relevant species

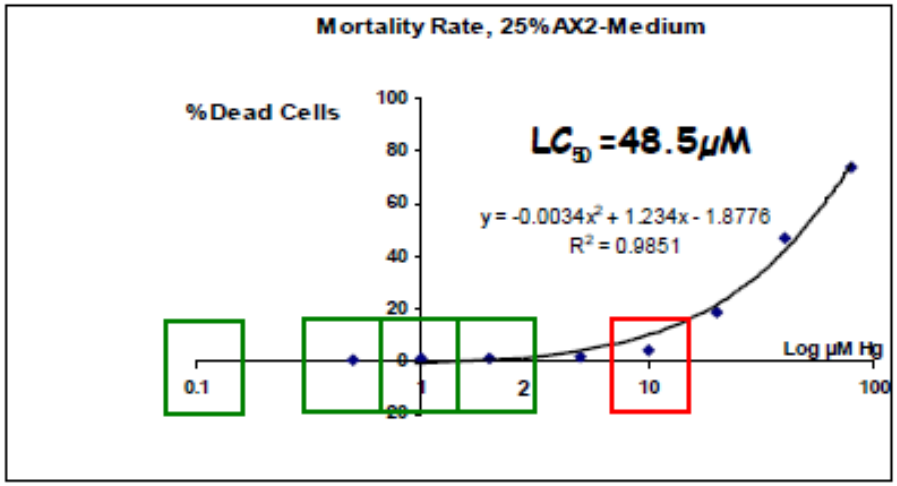
Two different exposure levels: different physiological states

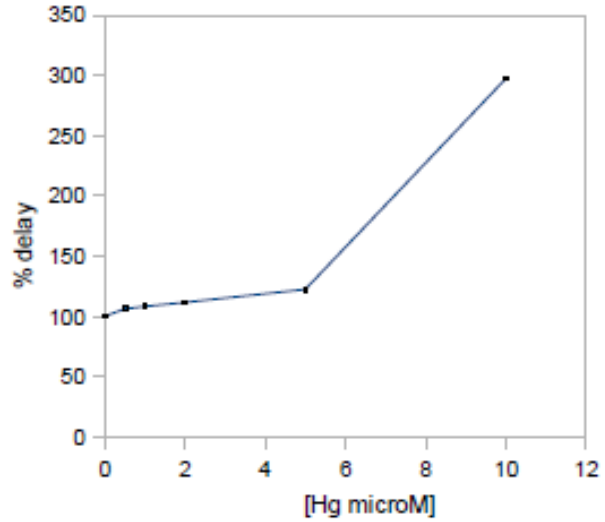
NoEC

2 µM Hg: No effect on mortality and cell replication

LoEC

10 µM Hg: starting effects on mortality and clear effects on replication

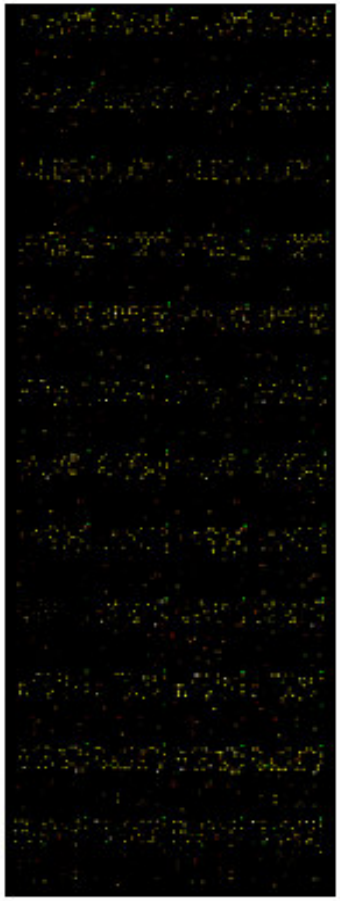




The Wellcome Trust Sanger Institute

First informational level:

Transcriptomics: 10K microarray



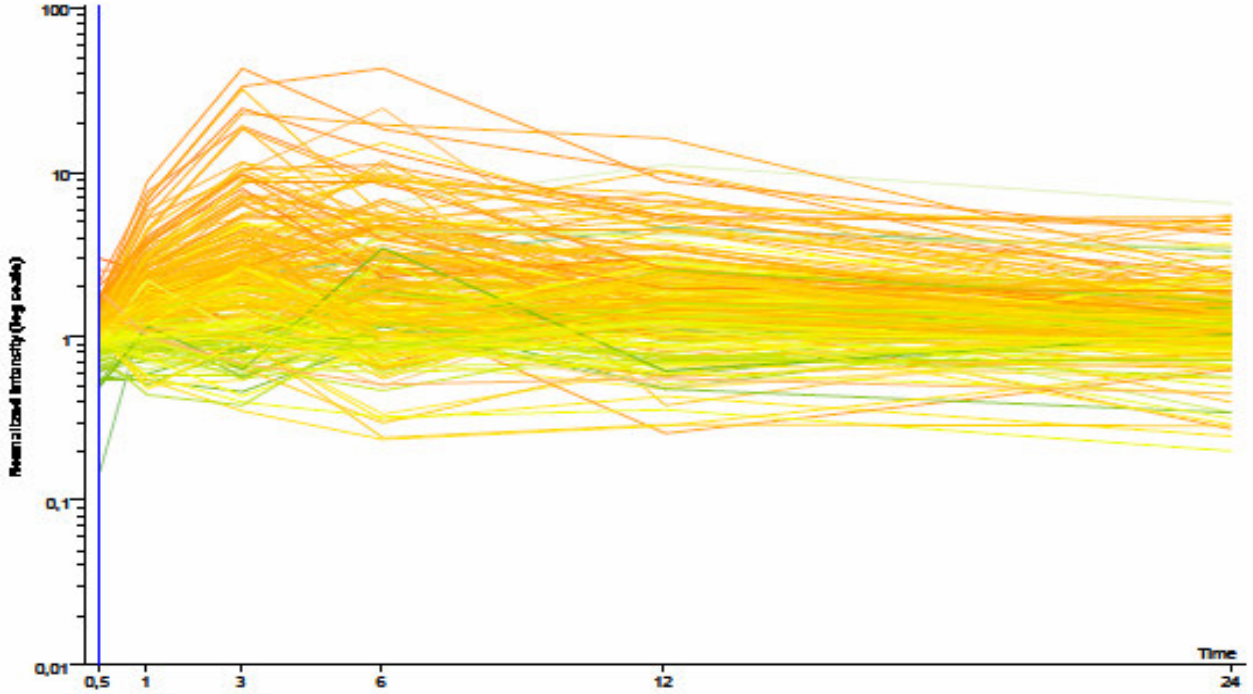
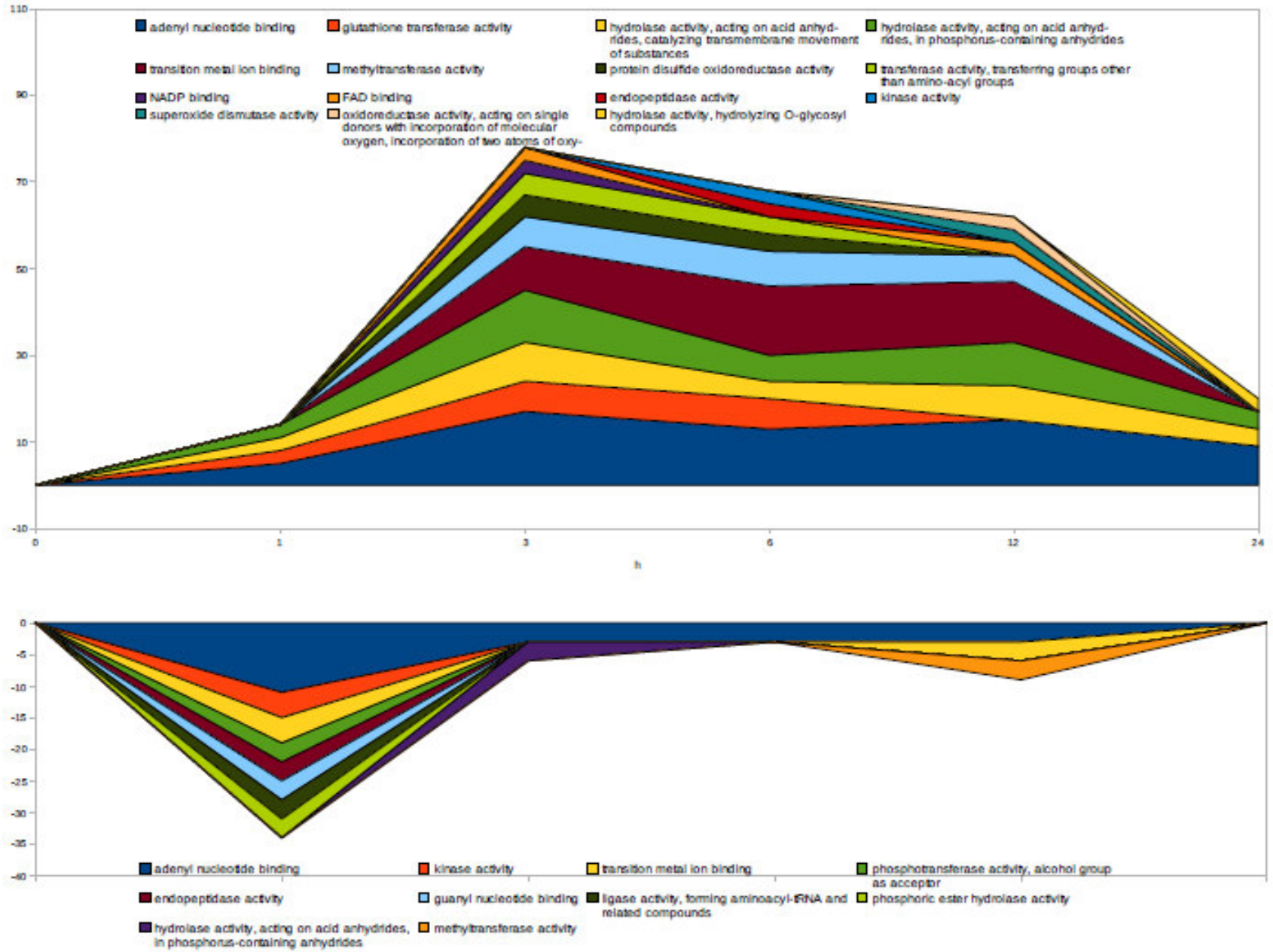


FIG2

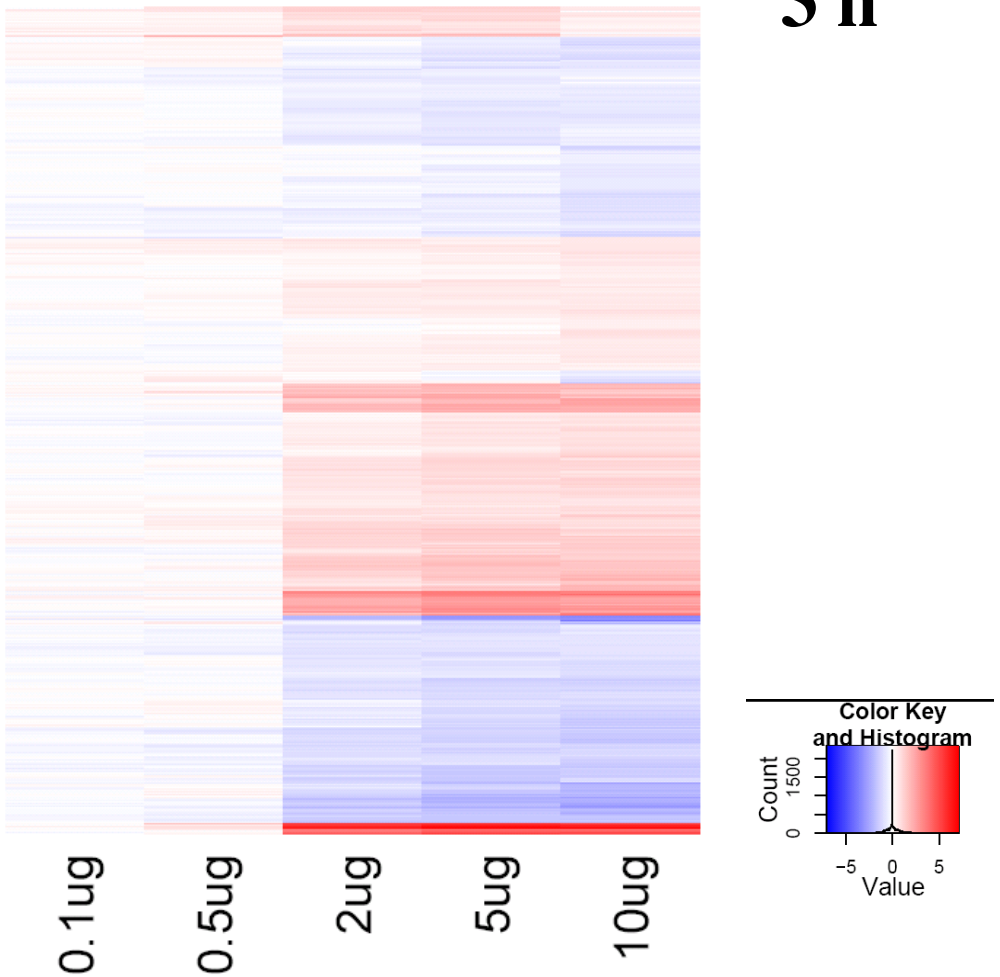

adenyl nucleotide binding
glutathione transferase activity
hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
transition metal ion binding
methyltransferase activity
protein disulfide oxidoreductase activity
transferase activity, transferring groups other than amino-acyl groups
NADP binding
FAD binding
endopeptidase activity
kinase activity
superoxide dismutase activity
oxidoreductase activity, acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxy-
hydrolase activity, hydrolyzing O-glycosyl compounds
110
90
70
50
30
10
-10
0
1
3
6
12
24
h
0
-5
-10
-15
-20
-25
-30
-35
-40
adenyl nucleotide binding
kinase activity
transition metal ion binding
phosphotransferase activity, alcohol group as acceptor
endopeptidase activity
guanyl nucleotide binding
ligase activity, forming aminoacyl-tRNA and related compounds
phosphoric ester hydrolase activity
hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
methyltransferase activity

Genomics: dose dependent effects

Heat map 1

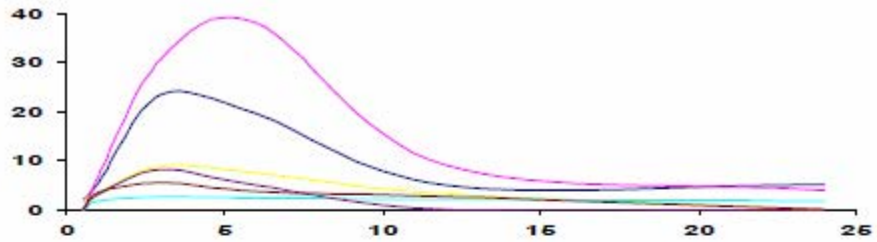
3 h

An increasing number of genes is significantly affected (more than 1000) and their intensities higher

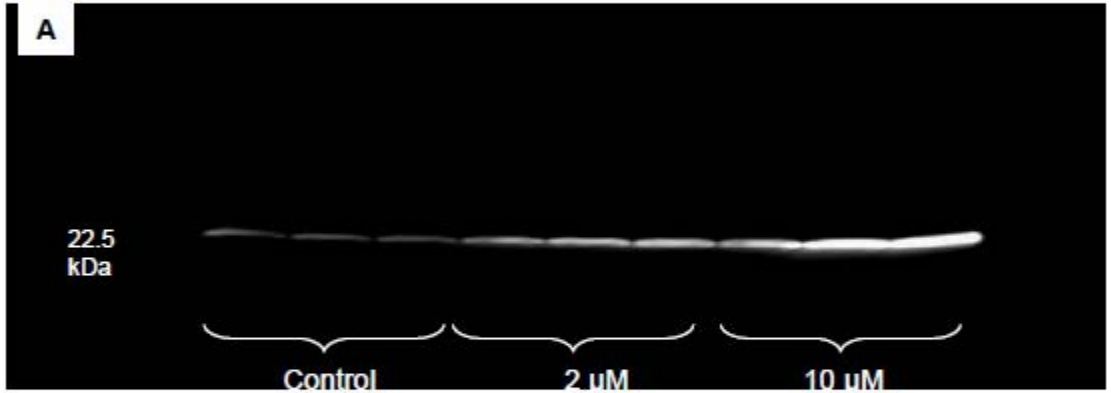


Detoxification processess: GST

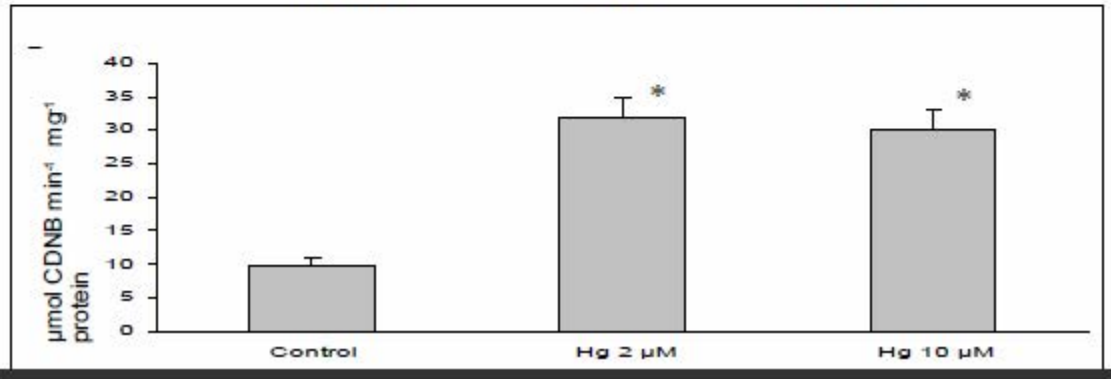
6 out 16 gst genes are induced



WB (anti-GST-sigma)

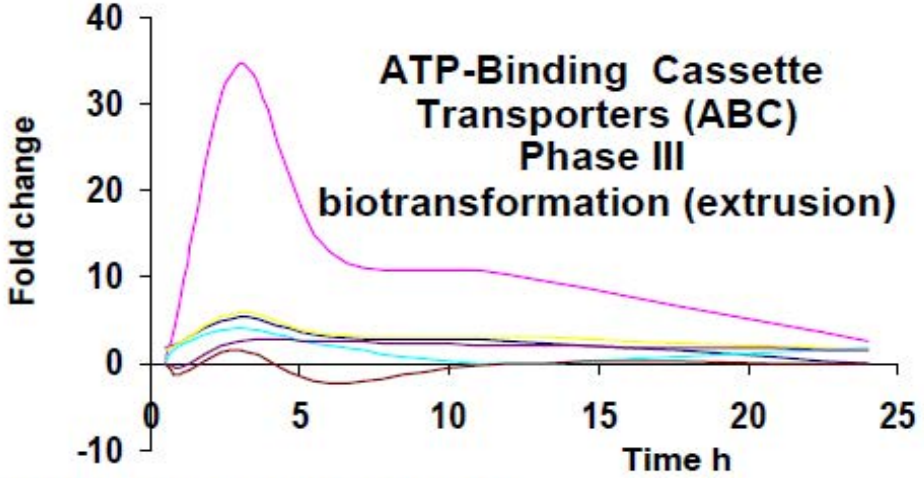


Enzymatic activity



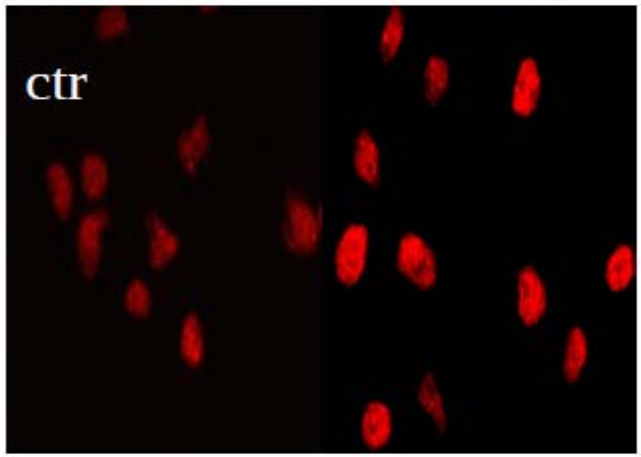
Phase III detoxification activities: ABCs

8 out of 66 ABC-Trp genes were up-regulated Peaking at 3 hours and dropping after 6 hours

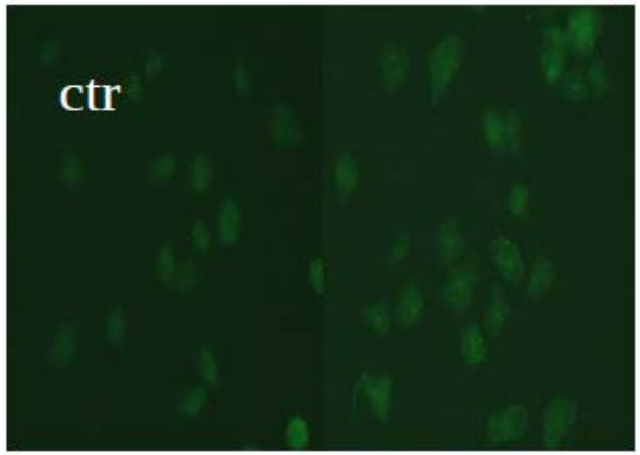


Increase of ABCs at protein levels

ABC-b1

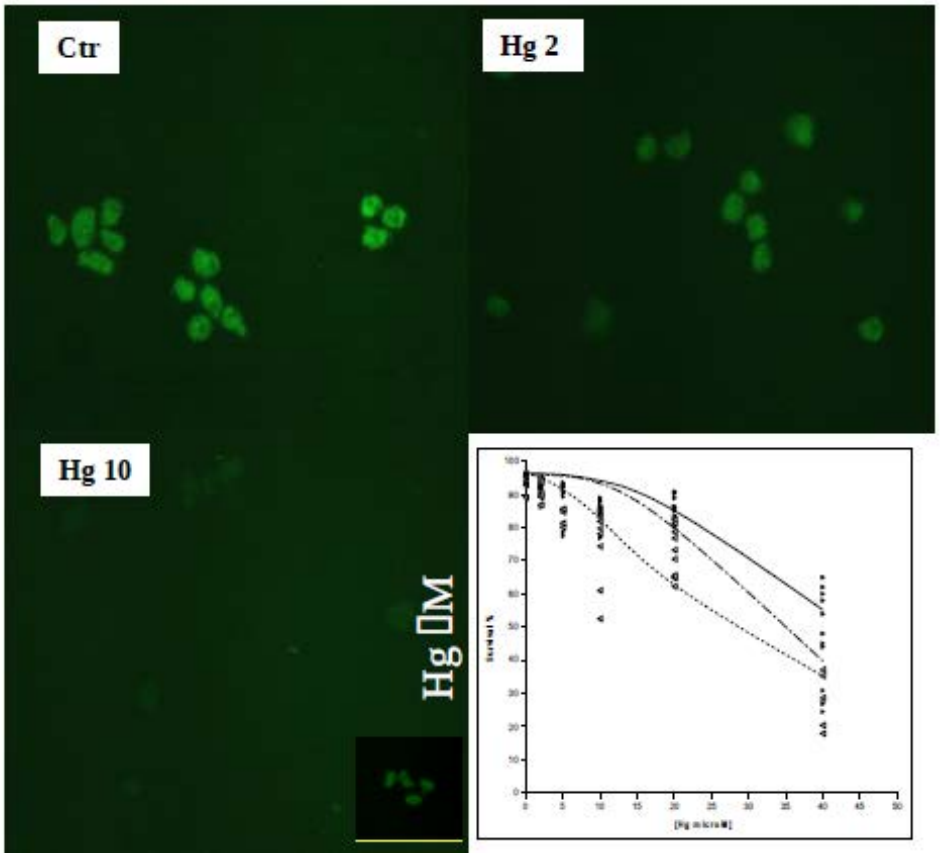


ABC-G17



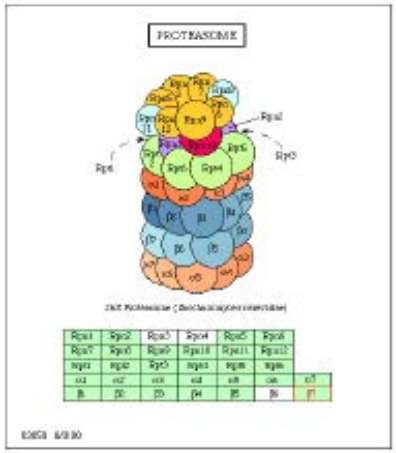
Phase III detoxification activities is critical for cell survival

Transport across the membrane was tracked using a fluorescent dye (**1,2,3 rhodamine**) and proved to be dose-dependently stimulated by Hg pressure

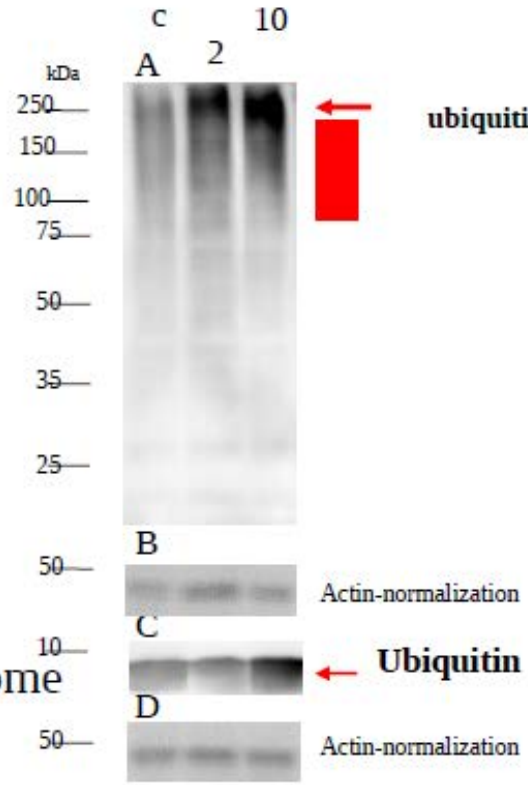


Inhibition (MK571) of this transport system caused a severe drop of survival in Hg challenged cells

Increased catabolic processes

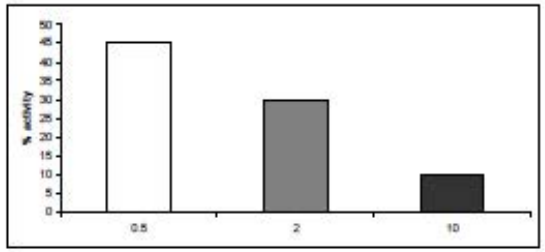


Almost all genes belonging to the proteasome complex were overexpressed at 10 μM Hg



Molecular: protein expression

Lysosomal membrane stability

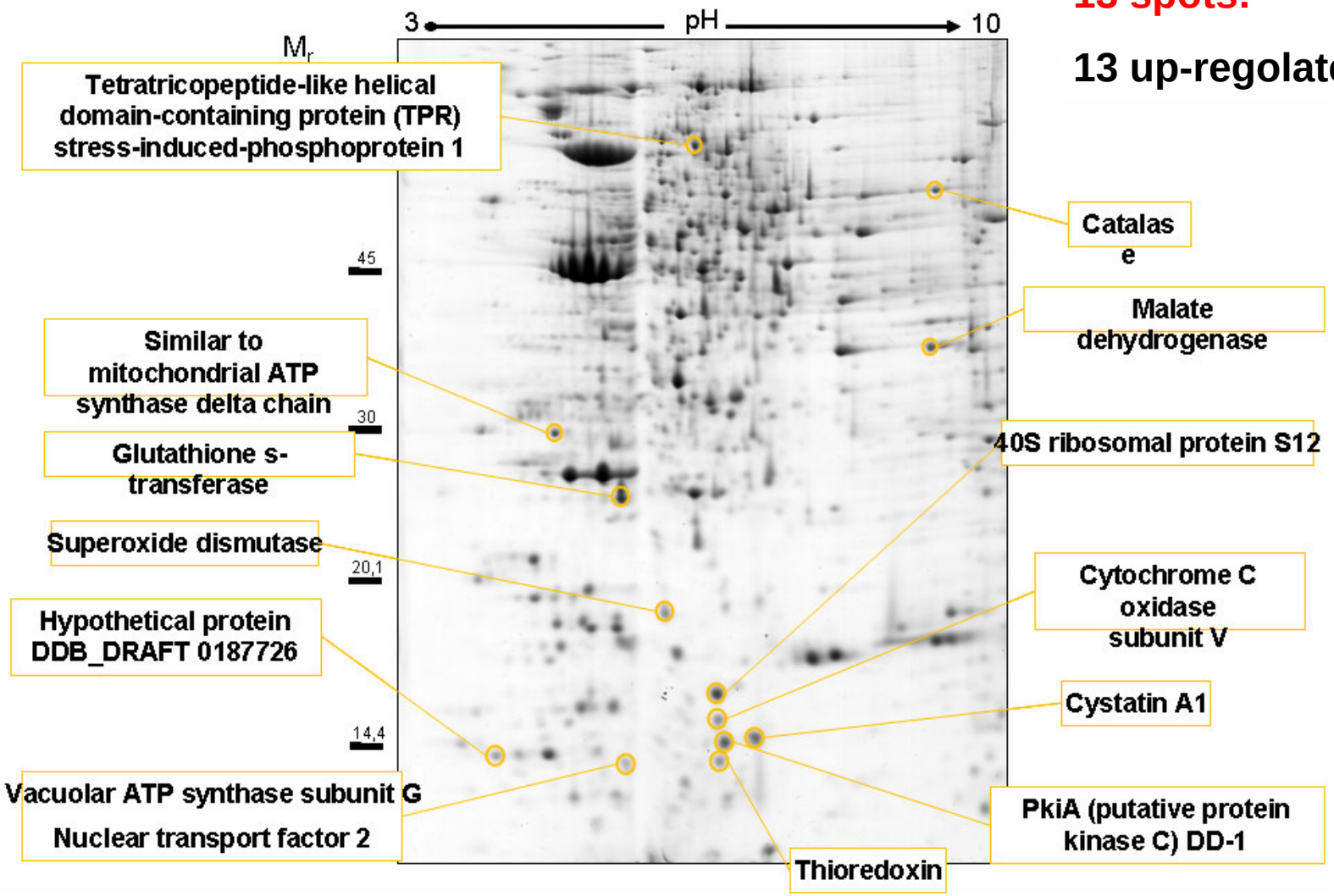


Increase of autophagy

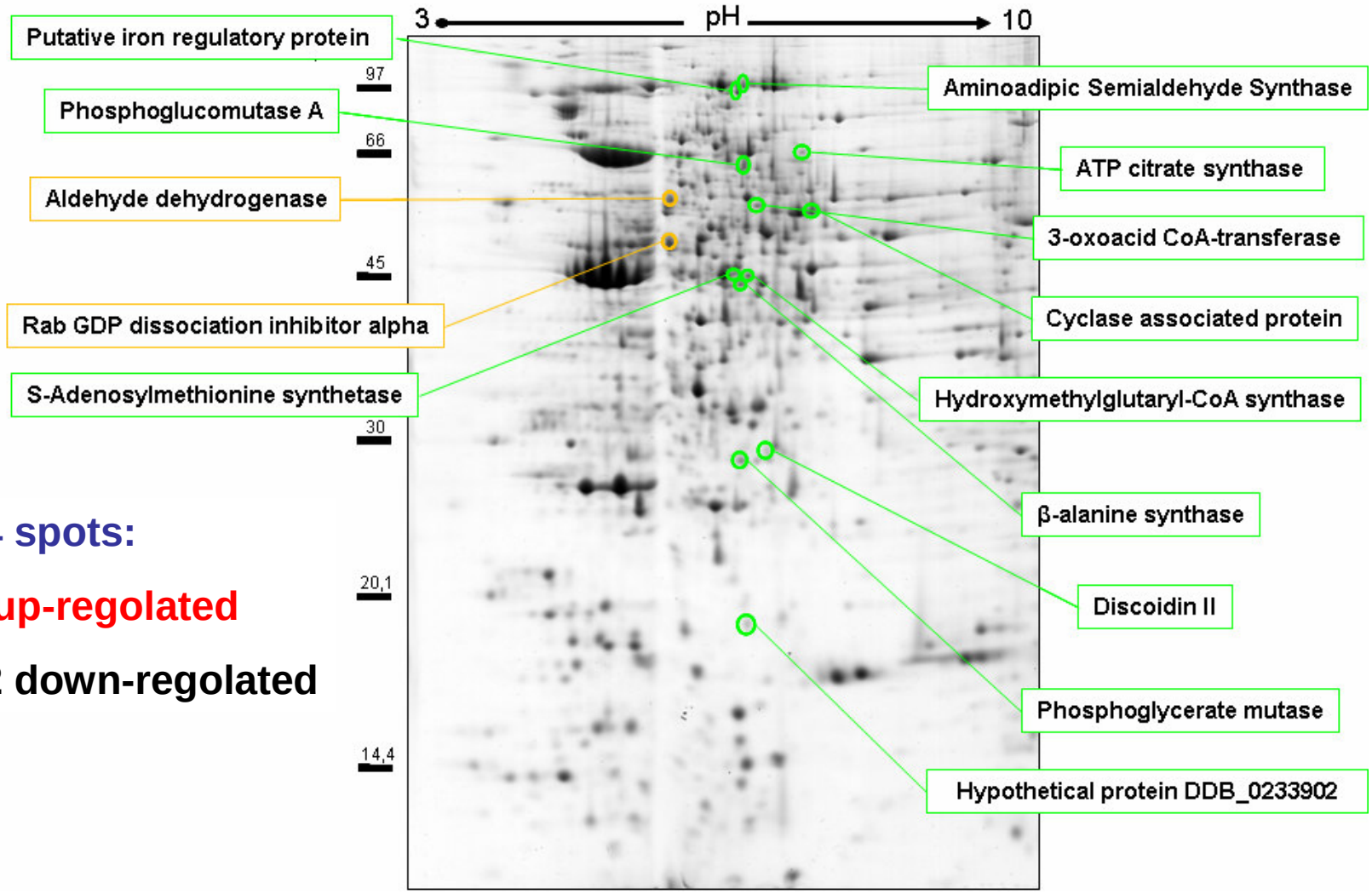
Dictyostelium discoideum – Hg 2 µM

13 spots:

13 up-regolated



Dictyostelium discoideum – Hg 10 µM



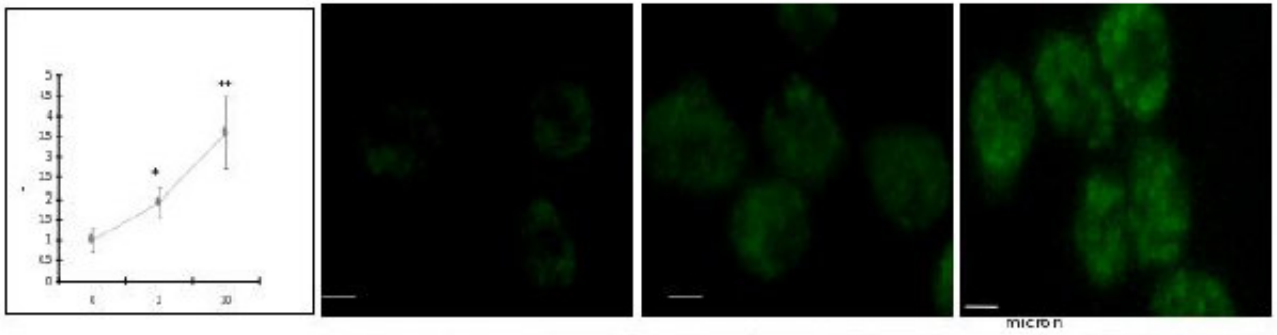
14 spots:

2 up-regolated

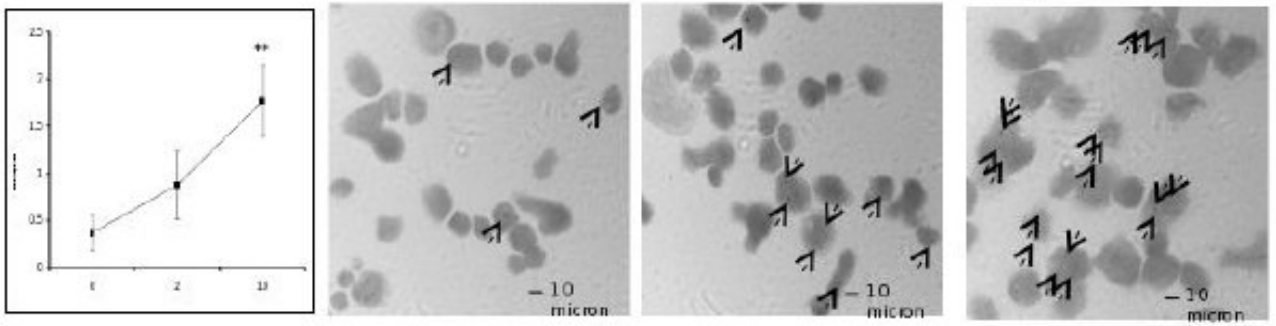
12 down-regolated

OXIDATIVE STRESS RISED

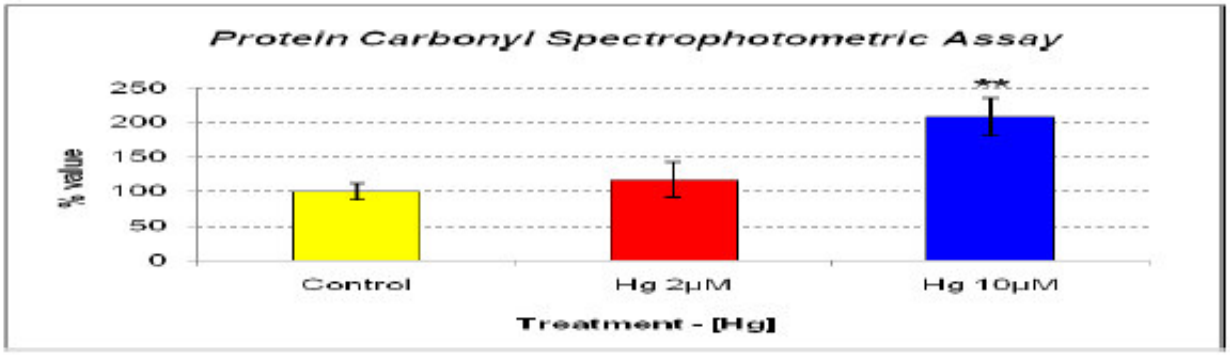
ctr 2 µM Hg 10 µM Hg



ROS



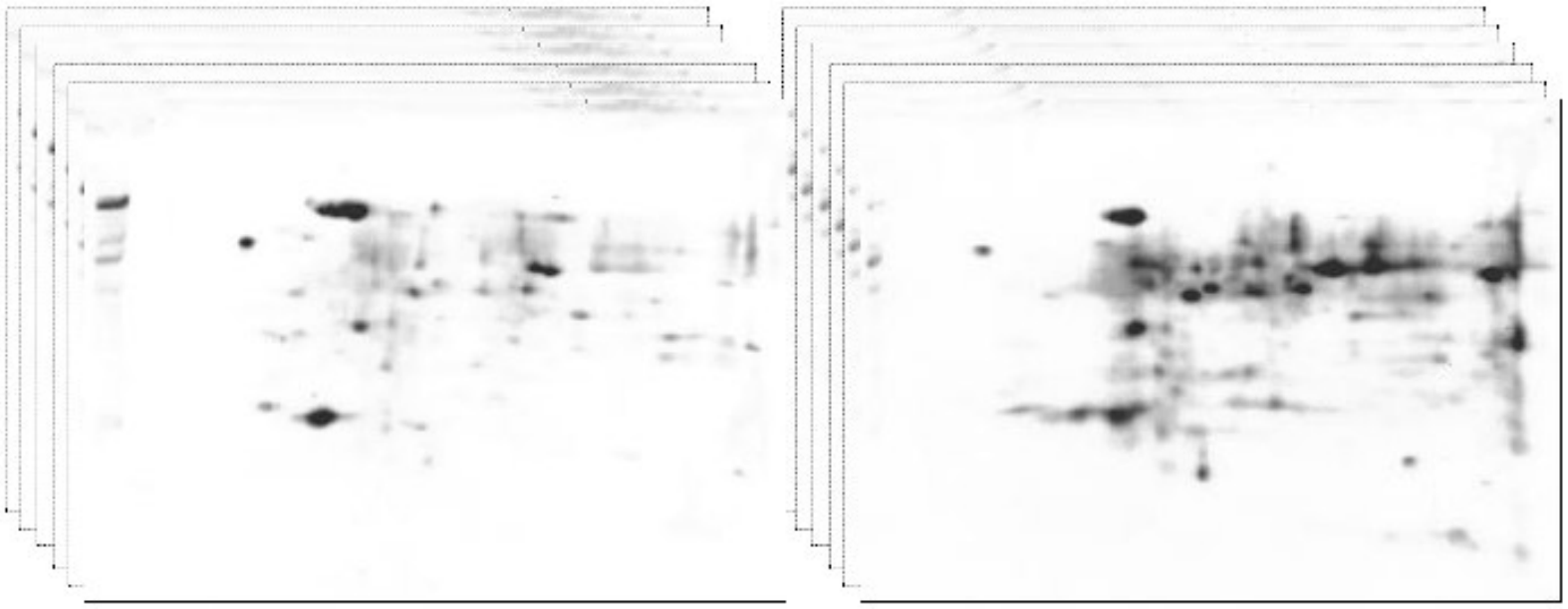
Lipofuscin



Carbonylated proteins

Dictyostelium discoideum – *Hg 10 µM*

Protein Carbonyl Assay - immunoblotting method (2-DE)

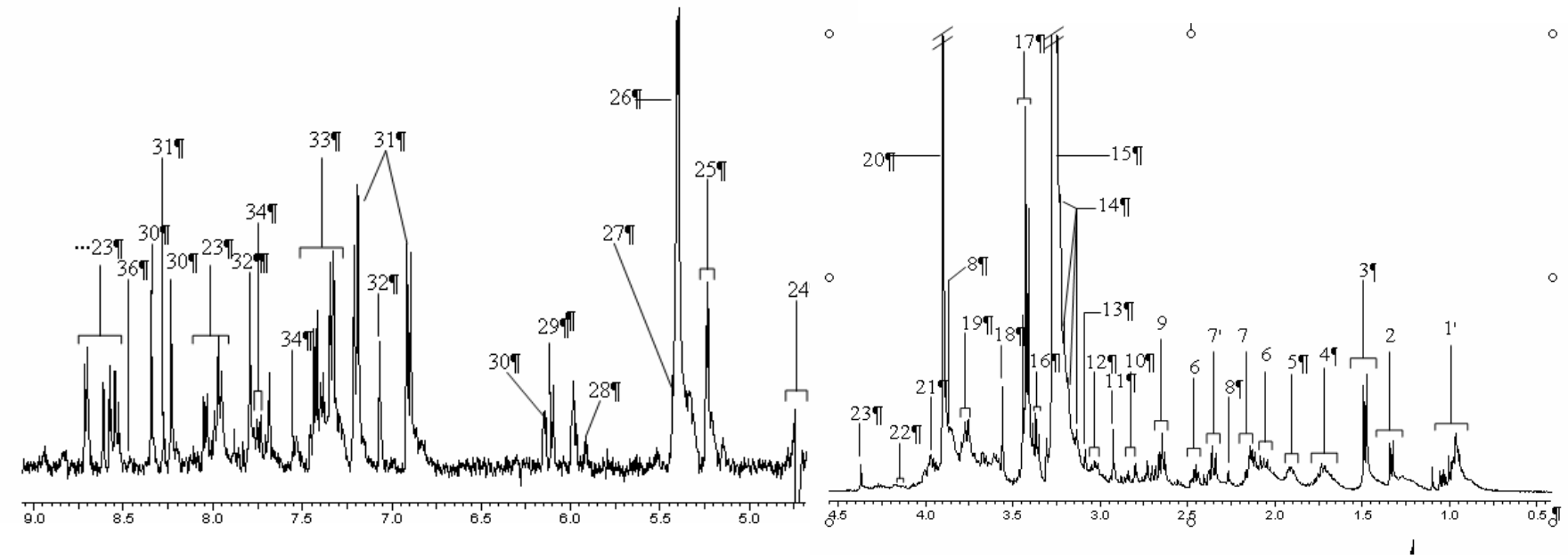


Control *Treated*

Dictyostelium discoideum – Hg 10 µM Protein Carbonyl Assay - Protein ID

Spot No	Protein ID	gi
1	Hypothetical protein DDBDRAFT_0216235	gi|66803106
2	Heat-shock cognate protein 70; Hsc70	gi|2564920
3	*under identification*	/
4	Hypothetical protein DDBDRAFT_0184451	gi|66801147
5	*under identification*	/
6	Hypothetical protein DDBDRAFT_0187704	gi|66806497
7	Isocitrate Dehydrogenase (NADP+)	gi|66823857
8	Cystathionine Beta-Synthase	gi|66828293
	6-Phosphogluconate Dehydrogenase	gi|66816225
9	Glyceraldehyde-3-Phosphate Dehydrogenase	gi|66820500
10	*under identification*	/

H NMR analysis of cellular metabolites



Key = 1) Isoleucine, Leucine and Valine, 2) Lactate, 3) Alanine, 4) Arginine, 5) Lysine and Arginine, 6) Glutamate, 7) Glutamine, 8) Succinat, 9) Methionine, 10) Aspartate, 11) Dimethylglycine, 12) Lysine, 13) Malonate, 14) Overlapping resonances from choline, acetyl choline and phosphocholine, 15) TMAO, 16) Hypotaurine, 17) Taurine, 18) Glycine, 19) Phenylacetylglycine, 20) Betaine, 21) Hippurate, 22) Proline, 23) Homarine, 24) Water peak (suppressed), 25) Alpha glucose, 26) Allantoin (and some Glycogen), 27) Glycogen, 28) Guanine nucleotides, 29) Adenosine nucleotides, 30) ATP and ADP, 31) Tyrosine, 32) Histidine, 33) Phenyl alanine, 34) Tryptophan, 35) Uracil nucleotides and 36) Formate

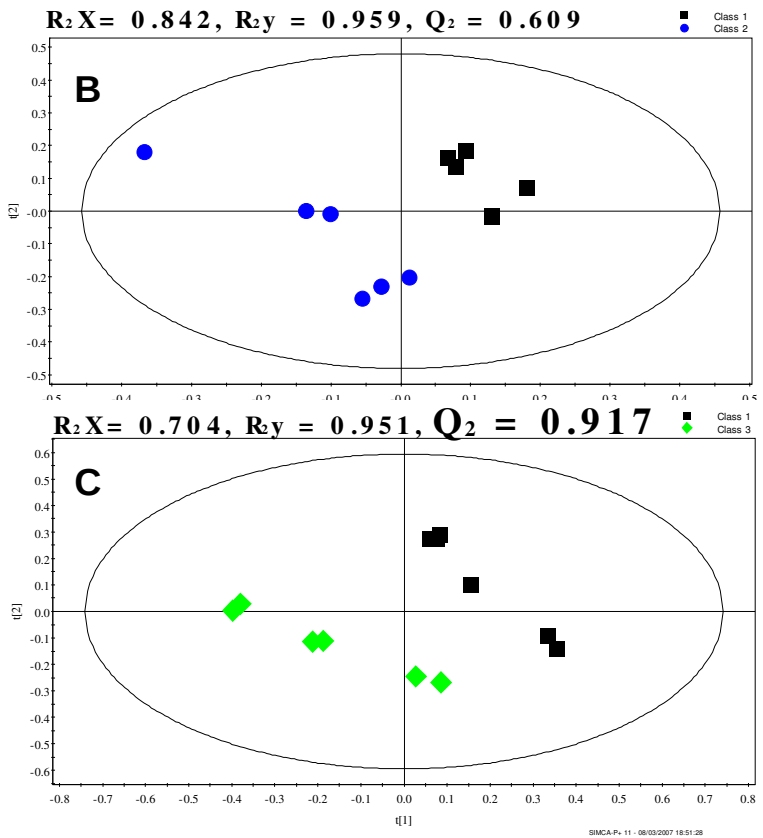
Metabolomics

UNIVERSITY OF CAMBRIDGE

Jules Griffin

Oliver Jones

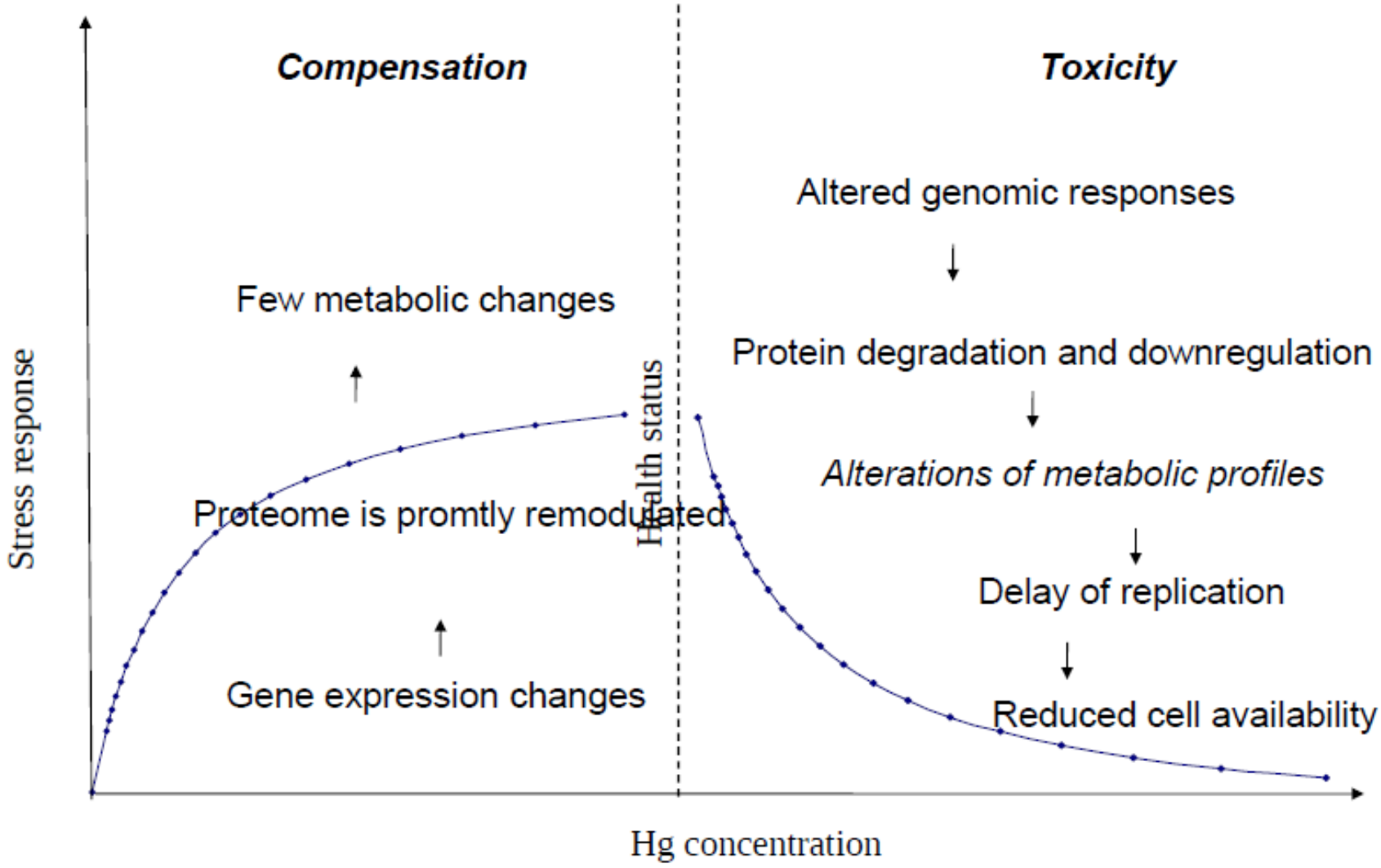
Distinct patterns have been evidenced



Driving metabolite: increased levels of reduced GSH

Driving metabolites: drop of many aminoacids (serine/glycine; valine, leucine, asparagine

The systems toxicology approach suggested:



Thank you for your attention!

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