Toxicogenomics: New strategies for ecotoxicology studies in autochthonous species

I. A glade in the entangled path towards the “fingerprint” of environmental impact?

Abstract

The search of early and sensitive biomarkers pointing to toxic effects of contaminants over organisms, has led the state of art to the molecular mechanisms of signalling, target and response. The trial to obtain more specific biomarkers of response to toxicants was in general biased by the finding of multiple crosstalks between signalling and the transcription factor regulation on gene expression. This may result in virtually impossible application in field ecotoxicological studies and biomonitoring, when native or local species must be used. Molecular biomarkers of toxicity generally show higher sensitivity and precocity in the response, while classical biomarkers such as cholinesterases in organisms exposed to anticholinesterasic agents may show “inconsistent” responses. The use of non-model aquatic organisms in general defies the application of commercially available antibodies and implies the design of primers for gene expression studies from model species sequences. The application of transcriptomics has opened a new way to gene annotation and for the design of adequate molecules as tools for the research in new biomarkers in autochthonous species.

Keywords: biomarkers; aquatic toxicology; gene expression regulation; transcriptomics

1 Introduction

There has been an increasing concern in the diverse sectors of the society about the impact of the chemicals that are released in the environment, since the second half of the past century when the dramatic effects of the organochlorine DDT and other pesticides were made visible (Carson, 1962). Ecotoxicology grew as a new branch of Toxicology to determine the deleterious actions of chemicals on the biota and establish the “secure levels” of any particular compound to which a species or a group of them could be exposed in the ecosystem. A series of conservative and predictive probabilistic models have been developed and refined, to link exposure concentrations and acute or chronic effects in the organisms. The concept of a diagnosis of the “Environmental Health” gained importance among ecologists, toxicologists and government environmental agencies. It was quickly realized that other tools than direct chemical analyses on the different matrices and organisms were needed to assess chemical impact on the ecosystem. If sentinel organisms may point to toxicants impacts in the environment, it still results very hard or even impossible to determine the nature of these contaminants by a sole approach. The potential or actual history of contamination coming from anthropogenic origin, chemical detections or identification at some points and time of episodes, soil/ sediment matrix, aerial dissemination, hydrology, bio-dynamics in webs, etc, all contribute as a sort of clinical history to risk or impact analysis. Biochemical and molecular

tools clearly contributed in the way, and the challenge is to determine which levels of the different contaminants may be considered secure, taking in mind all the possible interactions in this complex scenario (Figure 1).

Then, the notion of biomarker appeared to complete the overview, integrating in time, flow of contamination currents, combined exposure to multiple contaminants and recovery/ delayed effects (Adams et al., 2001). Starting at the level of individuals, biomarkers function as signals of the exposure, studied on a sentinel species, spanning from acute toxic effects to physiological impairments, biochemical alterations on targets involving membrane lipids, proteins or DNA, and molecular regulatory events including signalling and gene expression regulation. It is classically recognised that the deeper insight in mechanisms is gained i.e. into the molecular level, the earlier and more sensitive results the selected biomarker to determine that the organisms are exposed or affected by toxicants. However, it may be arguable at this point that these organisms are really damaged or at risk unless late or chronic effects are developed. On the other side, acute effects observed in organism physiology, behaviour or even lethality may clearly indicate the impact of toxicants but also with a heavy probability of serious risks that may become totally unacceptable at population or community levels (Figure 2). We were able to demonstrate the relationship between the probability of exceedence of the toxic metalloid Arsenic in waters from Argentina and the depth of biomarker level affected in anurans up to molecule, finding some concern even at environmental concentrations within the regulatory limits (Mardirosian et al. 2015; 2016 in press).

2 Biomarkers: attributes, advantages and pitfalls.

Several attributes have been established for endpoints to be considered as appropriate biomarkers. The proportionality of the response with the dose or concentration of the toxicant and the exposure duration is one of them. Nevertheless, it is difficult to find this situation in the environment on an extended or adequate range, because the dynamics of contamination may proceed in pulses, multiple entry points, recovery from exposures, etc. The specificity of the response or effect is also desirable for biomarkers. Certain biomarkers may act as specific targets of a particular class of toxicant; a classical example is the inhibitory response of cholinesterases following the exposure to organophosphate or carbamate insecticides. Other biochemical biomarkers rather respond to a wide variety of contaminants, reflecting the stress situation and/or the trial of response of the organism. Many substances may directly or indirectly generate oxidative stress in target or detoxifying organs, and elicit an antioxidant response through molecular signalling related to reduced glutathione (GSH). However, even for biochemical targets supposed to be specifically affected by one type of contaminant as the cholinesterases, it has been frequently described the effects of other unrelated toxicants, i.e. mercury and other metals, diverse therapeutic drugs such as β-adrenergic blocking agents, etc. It has been also described odd dose-related effects on classical specific biomarkers such as hormesis and the induction of gene expression after low and/or brief exposures. We were able to determine such effects on cholinesterase activity after organophosphate exposure in toad embryos (Sotomayor et al., 2015) (Figure 3).

Normally, the use of a battery of biomarkers may overcome these pitfalls, pointing to an effect of the contaminants when they cover different pathways of action and response (Rosenbaum et al., 2012). In particular, those considered as biomarkers of exposure are able in general to accomplish the expected attributes, when they correspond to the direct measurement of the toxicant or any of its metabolites in the organism or in its excretions. Certain contaminants such as toxic metals may cause proportional effects when metal bioaccumulation is measured as biomarker of exposure; however, the ratio with the exposure levels (i.e. the bioconcentration factors BCF) is not necessarily maintained (Figure 4). In these situations, the challenge is to associate the levels of bioaccumulation of any xenobiotic or element above physiological levels, with adverse consequences for the organisms to decide if there is any risk or impact.

Other aspect that must be attained for ecotoxicological risk evaluation is the exposure to multiple contaminants, added to this already complex scenario. Sentinel organisms may integrate these multiple episodes and mixtures, showing varying responses of biomarkers that reflect the pulses of exposures and recoveries. At this point, it is important to note that the integrated analysis of these biomarkers will prompt that *there is* an impact due to environmental stressors, but it is also probable that it will remain unknown *what* is causing the impact unless other information is available. Some biomarkers are also able to indicate long term effects or reflect chronic exposures. This is an important point for environmental risk assessment, as contaminant limits are commonly settled from acute or chronic toxicity in the whole organisms. In turn, biochemical biomarkers may in general anticipate to these probably irreversible effects and should be considered as more effective endpoints to set the permitted environmental limits (Figure 5). Moreover, the study of a battery of biochemical biomarkers may particularly reveal some aspects of the toxic mechanisms on different organisms differing in their responses to any particular toxicant (Guerreño et al., 2016).

3 Molecular biomarkers.

The approach to the molecular basis of toxicity is the first level from which biomarker application starts. It includes the proteins targeted by xenobiotics, which are preceding the activities of the affected enzymes and metabolites formed/ accumulated by results of their induction or inhibition. Thus, their responses may be considered as more “stable” and may coincide with the enzyme and metabolite profiles or not, depending on the cellular response on the corresponding gene expression, protein degradation, etc. Although molecular biomarkers most currently used include mRNA and protein molecules, others related to signalling and regulatory mechanisms in gene expression must also be considered. There are well defined pathways induced by contaminants generating oxidative stress and an antioxidant response, involving the signalling and activation of transcription factors recruited on Antioxidant Response Elements (ARE) in the promoter regions of a battery of genes regulated by this signal. Other xenobiotics act inducing the expression of genes involved in their metabolisation and detoxification, involving the Aryl Hydrocarbon receptor (AhR) and a Dioxin- or Xenobiotic Response Element (DRE or XRE) in the promoter regions of genes (Venturino and

Pechen, 2005). More recently, there is an increasing body of information related to epigenetic changes caused by chronic exposure to toxicants that may be engaged to crucial gene regulatory effects on later generations.

Our own experience in the use of molecular effectors as biomarkers has been variable. Two technical obstacles challenge the massive application for native species that are eligible for environmental risk assessment. The first is the necessity of designing or finding the appropriate antibodies working for specific proteins in such species. The other is the lack of information about most of gene sequences or genomes for closely related species that enable the design of DNA primers and probes, although in this case there are also many informatic tools for comparing sequences in databases and obtain the most conserved ones as candidates. We were able to detect the response of some highly conserved protein molecules, such as protein kinases PKC, MAPK cascade including JunK, ERK, MEK, in amphibian embryos exposed to insecticides or to Arsenic. These cannot be considered as specific responses because these signalling pathways are involved in many cellular processes, but their alteration might be in fact indicating the first stages of response or even damage due to toxicant exposure. Transcription factor responses in toad embryos exposed to organophosphates or Arsenic, such as cJun and cFos that are related to proliferation, also reinforce this notion (Sotomayor et al., 2015). We were able to detect some of the enzyme proteins regulated downstream of the kinase cascades, i.e. glutathione-S-transferase, catalase and superoxide dismutase, responding to Arsenic exposure in toad embryos in parallel with enzyme activities. In turn, mRNA expression analysis led to some degree of success in local species from our country, such as the toad *Rhinella arenarum*, the fishes *Jenynsia multidentata* and *Odontesthes hatcheri*, and the introduced fish *Onchorhynchus mykiss*, with economical and sportive importance. Different approaches were tried, i.e. BLAST design of primers starting from gene sequences available in databases for related species, direct use of published sequences of primers, or design of degenerated primers when the results were not satisfactory. The genes tested for expression varied from detoxifying enzymes, transporters of the ABC type, cholinesterase, and enzymes related to polyamine metabolism as growth factors (Table 1).

At this stage, it is evident that the use of molecular biomarkers in autochthonous species may be hard but not impossible. Some pathways related to cellular processes such as proliferation, arrest or apoptosis, or oxidative stress, may be suggesting an incoming effect resulting *a posteriori* into troublesome damage. Other molecules may be considered as specific for a type of toxicant, with the same restrictions as above, i.e., the detection of a resilient isoform of acetylcholinesterase that is overexpressed after the exposure to anticholinesterase agents (Kaupfer et al., 1998).

4 The aid of “omic” approaches.

Since the arrival of massive sequencing techniques, preceded by high-resolution methods and the possibility of managing massive databases, it was viable to evaluate on a single approach was it is happening in a whole organism, tissue or cell concerning its metabolites, proteins or gene expression products. The transcriptomic approach is indicative of the status of activation or repression of gene expression, particularly in mRNAs, that will be traduced into proteins at any time, which is observed in the proteome. Then, from the active proteins at the precise moment of the study, the profiles of different metabolites are observable in the metabolome, as a photograph of all the effects and responses triggered by any source. When the driving

force is the exposure to toxicants or contaminants, this approach has been named "toxicogenomics", although it initially applied to the part of the genome associated to toxicity and detoxification. There is then a gradation of approaches increasing in the precision of what is effectively occurring as a consequence of the exposure to contaminants, but it also implies that this precise image is highly dynamic and submitted to regulatory changes in protein activity, content, and finally in the mRNA expressing the genome regulation (Figure 6). The advantages of omic approaches in autochthonous species are immediate: no *a priori* knowledge in nucleic acid sequences or protein structure is required for the work.

We focus next in the transcriptomic approach to the ecotoxicological study of contaminant impact. One of the goals is the acquisition of a complete profile of mRNA expression after exposure, which becomes a sort of fingerprint for any tissue, organ or whole organism. Nevertheless, although relatively stable, this expression profile is dependent with time of/ from exposure, recovery, triggered cellular fate depending of the magnitude of exposure, etc. In native species, gene annotation requires additional computational work than model species whose genome is already sequenced. A sort of cDNA library is generated, which may be gradually completed from other sequencing work with the same species. These databases are in turn a valuable source of information to develop primers for any particular gene. This enables to confirm the expression level of known target genes in the exposure to toxicants and also identify and verify the effects over genes not previously identified.

5 Concluding remarks.

The use of biomarkers as tools in the environmental impact evaluation of contaminants, particularly at early stages preceding irreversible damage, has demonstrated the necessity of analysing a battery of them to cover the complex diversity of field exposure. The molecular biomarkers are more stable as responses to contaminant exposure, more sensitive and generally early anticipating possible damage to the organism. The Toxicogenomic approach envisages the possibility of using response patterns as fingerprints in environmental impact evaluation, with the aids of Bioinformatics. Transcriptomics opens a very interesting variety of possibilities in autochthonous or "non-model" species with not sequenced genomes, from specific gene annotation.

References

Adams, S.M., Giesy, J.P., Tremblay, L.A. and Eason, C.T. (2001) 'The use of biomarkers in ecological risk assessment: Recommendations from the Christchurch conference on Biomarkers in Ecotoxicology' *Biomarkers*, Vol. 6, pp.1–6.

Carson, R. (1962) *Silent Spring*, Houghton Mifflin Co., Boston.

Guerreño, M., López Armengol, M.F., Luquet, C.M. and Venturino, A. (2016), 'Comparative study of toxicity and biochemical responses induced by sublethal levels of the pesticide azinphosmethyl in two fish species from North-Patagonia, Argentina', *Aquatic Toxicology*, Vol. 177, pp.365-372.

Kaufer, D., Friedman, A., Seidman, S. and Soreq, H. (1998), 'Acute stress facilitates long-lasting changes in cholinergic gene expression', *Nature* Vol. 393 pp.373–377.

Mardirosian, M.N., Lascano, C.I., Bongiovanni, G.A. and Venturino, A. (in press) 'Chronic toxicity of arsenic during Rhinella arenarum toad embryonic and larval development: biomarkers of oxidative stress and antioxidant response', *Environmental Toxicology and Chemistry* DOI: 10.1002/etc.3693.

Mardirosian, M.N., Lascano, C.I., Ferrari, A., Bongiovanni, G.A. and Venturino, A. (2015) 'Acute toxicity of Arsenic and oxidative stress responses in the embryonic development of the common South American toad *Rhinella arenarum*', *Environmental Toxicology and Chemistry*, Vol. 34, pp.1009-1014.

Rosenbaum, E.A., Duboscq, L., Soleño, J., Montagna, C.M., Ferrari, A. and Venturino, A. (2012), 'Response of biomarkers in amphibian larvae to in situ exposures in a fruit producing region at North Patagonia, Argentina', *Environmental Toxicology and Chemistry*, Vol. 31, pp.2311-2317.

Sotomayor, V., Chiriotto, S., Pechen de D´Angelo, A.M. and Venturino, A. (2015) 'Biochemical biomarkers of sublethal effects in *Rhinella arenarum* late gastrula exposed to the organophosphate chlorpyrifos', *Pesticide Biochemistry and Physiology*, Vol. 119, pp.48-53.

Venturino, A. and Pechen de D'Angelo, A.M. (2005), 'Biochemical targets of xenobiotics: Biomarkers in amphibian ecotoxicology', *Applied Herpetology*, Vol. 2, pp.335-353.

Table 1. Primer design for mRNA amplification in autochthonous species.

Primer (source for design)	Gene	Tested Species	Starting sequence Species	Results
Detoxifying enzymes				
GSTκ (Primer-BLAST)	GSTκ	*O. mykiss* (rainbow trout) *O. hatcheri* (Patagonian silverside)	*O. mykiss* (NM_001165133.1)	(+) liver and intestine (-)
GSTπ (references)	GSTπ	*O. mykiss* *O. hatcheri* *R. arenarum* (larva)	*Salmo salar* (BT059949.1)	(+) liver and intestine (-) (-)
GSTπ (Primer-BLAST)	GSTπ	*R. arenarum* (adult)	*X. laevis* *X. tropicalis*	(+) liver
GR (Primer-BLAST) (degenerated)	GR	R. arenarum (adult) *R. arenarum* (larva)	*X. laevis* *X. tropicalis*	(+) liver (-)
ABC Transporters				
Mrp2 (references)	Mrp2 (Abcc2)	*O. mykiss* *O. hatcheri*	*O. mykiss* (NM_001124655)	(+) liver and intestine (-)
Pgp (references)	Pgp (Abcb1)	*O. mykiss* *O. hatcheri*	*O. mykiss* (AY863423.3)	Variable (+)(-) (-)
Mrp4 (references)	Mrp4 (Abcc4)	*O. mykiss*	*O. mykiss* (BX911853.3)	(-)
Bcrp (references)	Bcrp (Abcg2)	*O. mykiss*	*O. mykiss* (NM_001124683.1)	(+)
Cholinesterases				
ACHE (Primer-BLAST)	ACHE	*R. arenarum* (larva)	*X. laevis* *X. tropicalis*	(+) weak signal
Polyamine metabolism				
SSAT1 (Primer-BLAST)	SSAT1	*R. arenarum* (larva)	*X. laevis* *X. tropicalis*	(-)
ODC1 (references)	ODC1	*R. arenarum* (larva)	*X. laevis*	(-)
ODC2 (references)	ODC2	*R. arenarum* (larva)	*X. laevis*	(-)

The approaches and results of several trials developed at CITAAC (National University of Comahue, Neuquén, Argentina) are shown for several local species and one introduced (rainbow trout). Acronyms stand for: GST, glutathione-S-transferase; GR, glutathione reductase; Mrp, multidrug resistance-associated protein; Pgp, P-glycoprotein; Bcrp, breast

cancer resistance protein; ACHE, acetylcholinesterase; SSAT, spermidine/ spermine acetyl transferase; ODC, ornithine decarboxylase.

Figure Captions

Figure 1. Ecotogicology and Environmental Health. Different levels of information about contaminants and their effect to evaluate risk or impact and establish environmental limits.

Figure 2. An example of relationship between the level of toxicant in water and biomarker response in an aquatic organism. Levels of Arsenic in water (logarithmic scale) were related with the probability of exceedence (logistic model), and the levels at which different types of biomarkers were triggered in autochthonous species in Argentina were denoted.

Figure 3. Differential response of cholinesterase activity to organophosphate insecticide in early toad embryos. Unpredicted responses of the specific and classical biomarker Cholinesterase are found in the exposure to the anticholinesterase agent chlorpyrifos: at 24h of development, the activity remains unaffected; at 48h of development, an induction of the activity is observed at low concentrations, while the classical inactivation response is observed at high concentrations, showing a hormetic variation.

Figure 4. Relationship of metal bioaccumulation with concentration and duration of exposure. Data are measures in toad embryos exposed to a wide range of Arsenic concentrations in water. While the bioaccumulation increases with the level of metal in water, the bioconcentration factor may not, as saturation effects are possible at high levels in the media.

Figure 5. Biomarkers and the molecular mechanisms of toxicity. Different autochthonous species show differential responses of biomarkers, indicating main mechanisms associated with the effects elicited by the organophosphate azinphos methyl, and others with secondary importance in the toxicity. It can also be noted that the ranges of toxicant for the response of biomarkers have a different distance to lethal concentrations in both species.

Figure 6. Omic flux diagram in Ecotoxicology.

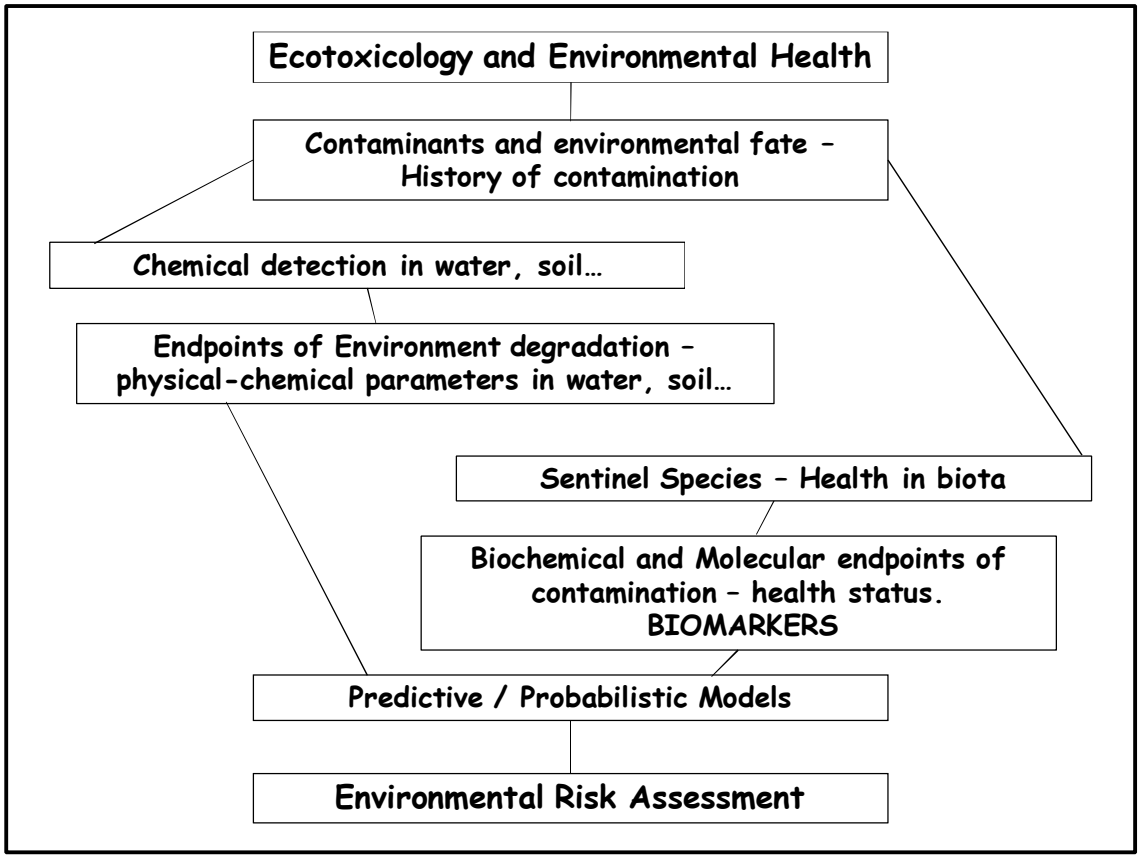
Ecotoxicology and Environmental Health
Contaminants and environmental fate – History of contamination
Chemical detection in water, soil…
Endpoints of Environment degradation – physical-chemical parameters in water, soil…
Sentinel Species – Health in biota
Biochemical and Molecular endpoints of contamination – health status. BIOMARKERS
Predictive / Probabilistic Models
Environmental Risk Assessment

Figure 1

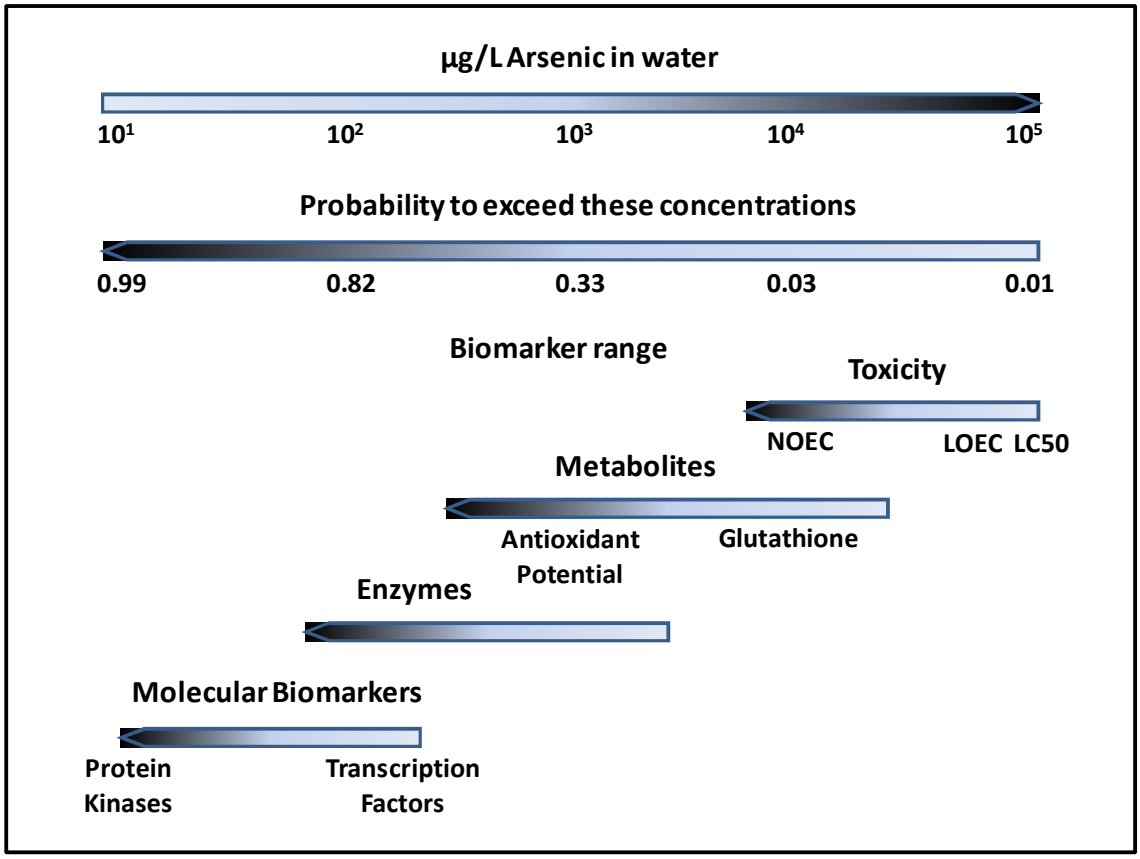
µg/L Arsenic in water
10¹
10²
10³
10⁴
10⁵
Probability to exceed these concentrations
0.99
0.82
0.33
0.03
0.01
Biomarker range
Toxicity
NOEC
LOEC LC50
Metabolites
Antioxidant Potential
Glutathione
Enzymes
Molecular Biomarkers
Protein Kinases
Transcription Factors

Figure 2.

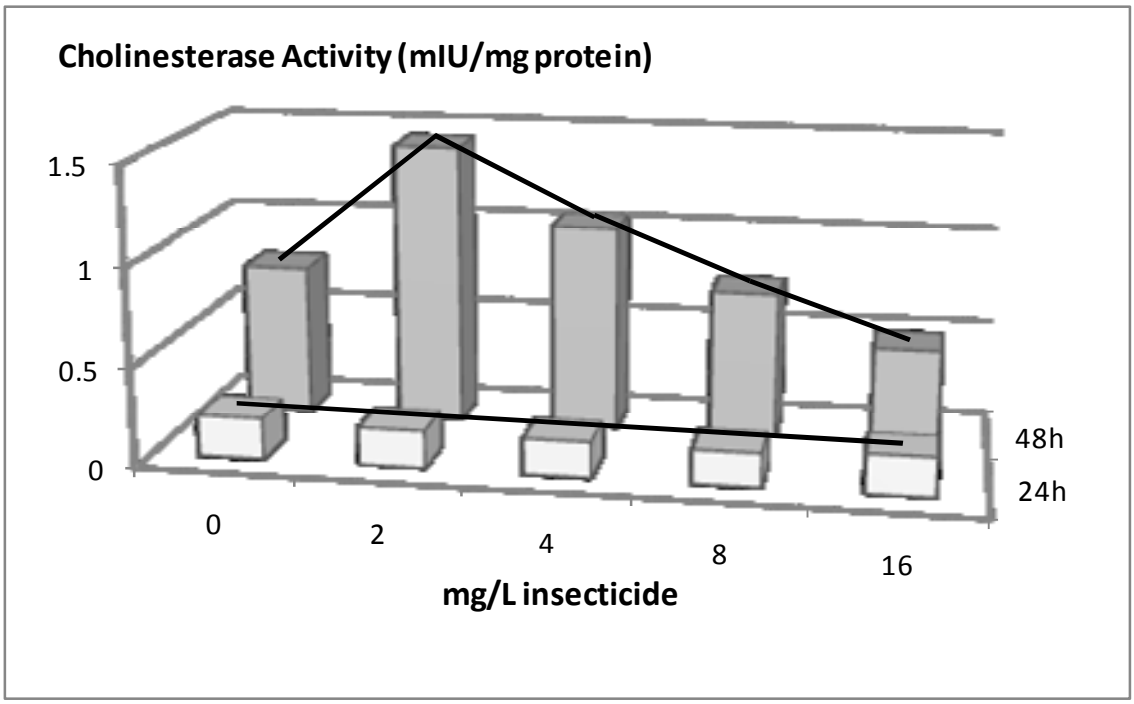
Cholinesterase Activity (mIU/mg protein)
1.5
1
0.5
0
0
2
4
8
16
mg/L insecticide
48h
24h

Figure 3

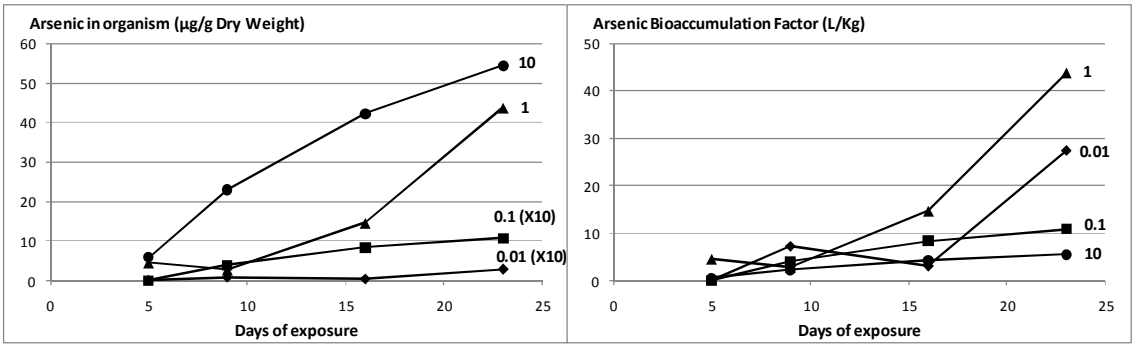
Arsenic in organism (µg/g Dry Weight)
60
50
40
30
20
10
0
0
5
10
15
20
25
Days of exposure
10
1
0.1 (X10)
0.01 (X10)
Arsenic Bioaccumulation Factor (L/Kg)
50
40
30
20
10
0
0
5
10
15
20
25
Days of exposure
1
0.01
0.1
10

Figure 4.

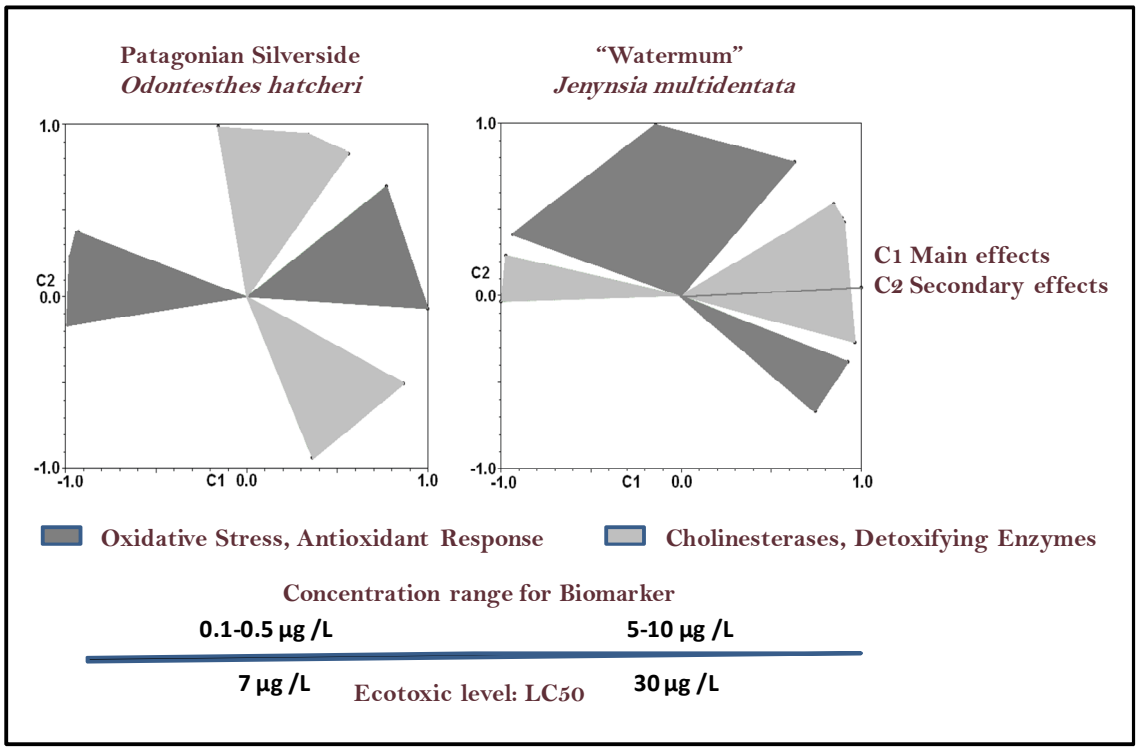
Patagonian Silverside
Odontesthes hatcheri
"Watermum"
Jenynsia multidentata
1.0
C2
0.0
-1.0
-1.0
C1 0.0
1.0
C1 Main effects
C2 Secondary effects
Oxidative Stress, Antioxidant Response
Cholinesterases, Detoxifying Enzymes
Concentration range for Biomarker
0.1-0.5 µg /L
5-10 µg /L
7 µg /L
Ecotoxic level: LC50
30 µg /L

Figure 5.

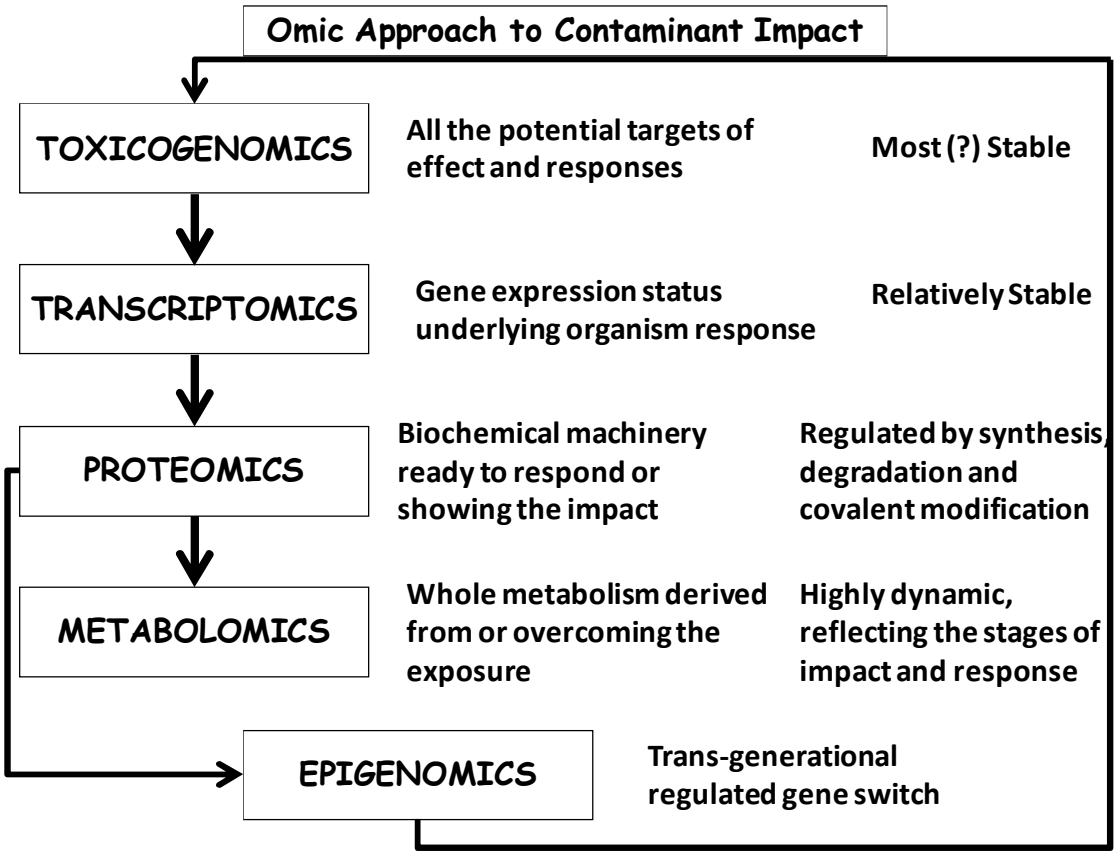
Omic Approach to Contaminant Impact
TOXICOGENOMICS
All the potential targets of effect and responses
Most (?) Stable
TRANSCRIPTOMICS
Gene expression status underlying organism response
Relatively Stable
PROTEOMICS
Biochemical machinery ready to respond or showing the impact
Regulated by synthesis, degradation and covalent modification
METABOLOMICS
Whole metabolism derived from or overcoming the exposure
Highly dynamic, reflecting the stages of impact and response
EPIGENOMICS
Trans-generational regulated gene switch

Figure 6.