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Chronic measures of toxicant-induced effects on fish

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Biomarkers can be a powerful tool to detect the exposure of sublethal concentrations of xenobiotics to fish and are useful in studies of chronic toxicity. However, evaluation of the results should be done with caution, especially if results are extrapolated to population- and ecosystem-level effects. If a biomarker can not be connected to clear adverse effects, on at least an individual level in the ecosystem, it is not useful in an ecotoxicological perspective. Still if the biomarker has a high specificity, it can be used as an integrated measure of exposure to certain xenobiotics and replace direct measurements of chemical concentration. In field the interactions of the investigated chemical, with both abiotic and biotic factors are very important and determines the ecological effect. Seasonal variations of the bioavailability of the toxic chemicals are evident, and so are the physiological status of the individuals in the ecosystem. The step from toxicology to ecotoxicology is long, and the use of biomarkers in ecotoxicological studies must never replace the ecological on-site investigation.

1. Introduction

Information on chronic toxicity concerning single aquatic species can usually not be directly used for predictions about effects at the ecosystem level. On the other hand, they generally give better approximations of those effects than results from acute toxicity tests. The difference in usefulness between data from acute and chronic toxicity testing can be said to be that the acute tests ought to be used for toxicity ranking of chemicals, while the chronic tests can give a first approximation of the concentrations in the environment that can produce harmful effects. One of the reasons for this is that the experimental, laboratory determination of chronic toxicity can be performed using realistic concentrations, i.e. concentrations that can be expected to occur in the environment. Further, the endpoints in chronic tests are various, not only lethality, whereby several signs on adverse effects can be detected (Landner 1987).

To classify a toxicity test as "chronic" principally demands that the test organism is exposed during the whole life cycle or a significant part of it. Thus, tests with long-living organisms or organisms that reach sexual maturity late will take a long time and be costly to perform. As a result, efforts have been made to construct short-term tests focusing on particular sensitive life stages to obtain information, more rapidly and at a lesser cost, on chronic toxicity. These short-time tests have traditionally investigated survival of embryo- and

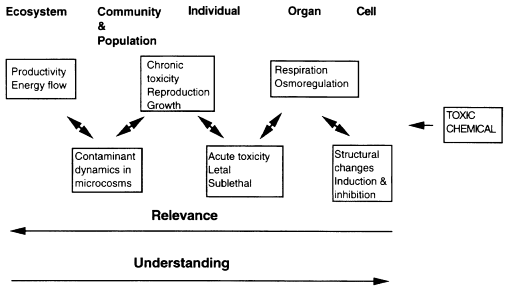

Fig. 1. Effects of toxicants occur at all levels of biological organisation. Toxic effects are best understood at the cell and organ level where they also are most easily studied, while the ecosystem and community levels are least understood although most relevant. Slightly modified from Haux & Förlin 1988.

larval-stages since it has been presumed that they are the most sensitive life-cycle stages (McKim 1977). However, it has been observed that, in several cases, reproduction is a more sensitive functionality than survival of embryos or larvae (Mount 1968, Kimball et al. 1978). In some cases, the early stages of reproduction, such as gamete maturation in older fish, has been considered as the most sensitive, at least to some types of stressors (Landner et al. 1985).

In ecotoxicology, toxicity testing of chemicals is, in most cases, performed with an intent to extrapolate the results to population-, community- or ecosystem-levels of organisation and predict effects. With these ambitions chronic toxicity tests may, even though they may last longer and require more intensive measurements than acute toxicity tests, still have approximately the same disadvantages in predicting ecosystem effects as acute tests. However, when only population level effects are concerned the tests are often adequate (Fig. 1).

2. Measurements of chronic toxicity

Problems with chronic toxicity testing with fish are the same as for acute tests, i.e. mainly repeatability and standardisation. The possibility for standardisation increases with the understanding of the mechanisms of the observed effect. In Fig. 1, the "understanding" arrow would also indicate the "possibility of standardisation". Since many of the steps taken to decrease variation in test results, both in intra- and inter-calibrations, tend to reduce the test, moving it to the right on the "understanding arrow", they also often decrease the possibility to extrapolate results to the environment. The reduction increases the estrangement of the test relative to higher levels of organisation.

Some of the endpoints used to measure chronic toxicity are biochemical. The use of these biochemical responses, often referred to as biomarkers, to predict potential for adverse effects on survival, growth and reproduction of individuals is based on the belief that a non-acute effect at the cellular or organ level can result in an effect in the integrated organism functions. Or defining biomarkers in another way "By definition, a chemically induced change in biochemical systems represents an effect of the chemical" (Stegeman et al. 1992).

The use of biomarkers in predicting chronic toxicity is expanding. Biomarkers are easily measured and thereby allow a ranking of different exposures or, if the investigation is performed in the field, a ranking of different sites. Effects revealed with biomarkers are also absolute and can therefore more easily be applied to regulatory decision-making and environmental management.

Theoretically, any measurable biochemical function of fish can be used as a biomarker. However, using these functions as a measure of stress must be accompanied by knowledge about the importance of the biochemical function on a higher organisation level. The biomarker response can be said to be an effect by definition, but iwhether this effect is relevant at a population or community level is another question. Pigmentation of our skin is correlated to sunlight exposure but this biomarker is not directly connected to any adverse effects. It is a normal, physiological response to a sometimes dangerous, exposure to UV light.

3. Biochemical and physiological endpoints

Most endpoints measured during chronic toxicity testing are biochemical in nature. This is probably due to the numeric character of the generated results, making the evaluation of test results easier, as well as comparisons with other, previously performed, tests.

One of the most extensively used endpoints is the induction of the mixed-function oxidase (MFO) system. The monooxygenase reactions are, in mammals mediated by a family of over 100 different enzymes referred to as cytochrome P450. The synthesis of certain types of cytochrome P450 can be induced by exposure to some classes of toxicants. These enzymes can thus be used as "biomarkers".

A number of more or less specific substrates can induce expression of enzyme activity. As a result, the rate of chemical transformation catalysed by these enzymes is altered. Cytochrome P450 induction can also serve as an indicator of an organism's toxic burden, or the extent to which it has been exposed to chemical inducers in the environment. This induction generally involves synthesis of a new messenger RNA and, subsequently, a new enzyme protein. Cytochrome P450 enzyme activity, protein, or mRNA might be measured to detect induction (Stegeman et al. 1992).

One type of cytochrome P450, purified from the livers of several fish species, has been identified as the single P450 isoenzyme primarily induced by PAH and planar chlorinated aromatic hydrocarbons. The three most well-studied fish proteins of this type to date are scup P450E, trout $P450LM_{4b}$, and cod P450c. These three P450 isozymes are structurally similar and are generally referred to as P4501A1, but because of the potential for cross-reactions with P4501A2 they are sometimes just called P4501A. P4501A is the enzyme responsible for the 7-ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) catalytic activities (Stegeman et al. 1992). The induction of EROD and AHH-activity is mediated by the binding of the substrate to a specific receptor, the Ah-receptor. This receptor protein mediates the response to certain aromatic hydrocarbons (Whitlock 1989). Tests using EROD and AHH-activity as endpoints have high specificity. However, the effects of contaminants not having this special "aromatic character" (planar, diaromatic, halogenated hydrocarbons or polycyclic aromatic hydrocarbons) are not detected. Depending on the test objects, specificity can be regarded as both an advantage and disadvantage.

Metallothioneins (MTs) are cytosolic, low molecular weight, metal-binding proteins. In the same manner as cytochrome P450, MT induction is due to transcriptional activation of MT genes. The induction of MT in fish has been demonstrated for zinc, copper and cadmium (Haux & Förlin 1988). However, the biological function of metallothioneins is not fully understood. Therefore it has not been possible, to date, to clearly link changes in the levels of metallothioneins to injury at cellular or organism level (Stegeman et al. 1992).

In a comparative study of several biomarker responses in fish exposed to contaminated sediments, both in the field and in the laboratory, Theodorakis et al. (1992) concluded that the field and laboratory responses were similar, but that the field response was stronger. This could be due to:

1) Species-specific differences between the Bluegill Sunfish used in the laboratory and the indigenous Sunfish studied in the field.
2) Lifetime toxicant exposure.
3) Natural selection.
4) Population and community interactions.
5) Abiotic mediating factors in the field.

The duration of laboratory exposure requires consideration, since different biomarkers show different trends of response over time. Responses also vary with animal sex, age, nutritional status and season. Choosing a biomarker that is appropriate for the time course of the study is an important consideration. Certain biomarkers are affected by xenobiotics but remain stable over time for example, intestine and gill ATPase activity (Theodorakis et al. 1992). Hence they should provide comparable field and short-term laboratory data. Some biomarker responses fluctuate over time, like brain ATPase activity, total liver cytochrome P450 and NADPH content, stress proteins, chromatin proteins and DNA strand brakes. Still other biomarkers, such as EROD activity and DNA adducts show a steady time-dependent increase. For these biomarkers, only long-term laboratory data may be comparable with the field. Overall, blood and organs at the site of exposure seem to respond quicker and with greater magnitude than internal organs. Internal organs seem to be more indicative of chronic exposures (Theodorakis et al. 1992).

4. Examples of histological and organ level endpoints

The liver is the most extensively studied organ. It is one of the principal organs exposed, and its

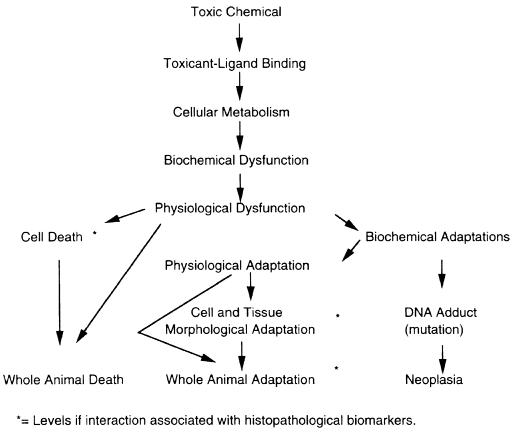

Fig. 2. Integration of histopathological biomarkers with physiological and biochemical approaches. Slightly modified from Hinton et al. 1992.

function as the primary site of metabolism for most chemicals makes the liver the first organ to be exposed for the metabolites produced. One of the classic measures of exposure to toxic chemicals is the liver somatic index (Andersson et al. 1988) which is still used and still valid. In this simple index, the liver weight is compared with the body weight.

With direct histopathology, several histological changes can be detected (Wester & Vos 1994). Among other morphological changes, structural modifications like rounding up of the cell nuclei, reduction of the rough endoplasmatic reticulum, etc. are studied (Braunbeck & Völkl 1991). Further, carcinogenesis may be studied. For example, in the yolk sac, microinjection assay of rainbow trout. In this test solutions of the studied chemical or extracts for example of sediments, are injected into the yolk sac of fry. After 12 months, the fish livers are surveyed for evidence of neoplasia (Metcalfe et al. 1990).

Histological changes are precided by biochemical and physiological responces (Fig. 2). As endpoints, histological changes are not as easily and objectively assessed as the biochemical markers and may require greater experience. But, since the damages are not only an indication of exposure to toxic chemicals, but also clear and indisputable evidence of an adverse effect, test results concluding histological damages at sublethal exposure of chemicals are very difficult to dispute.

Blood is a special organ which is quickly exposed to the bioaccumulated chemical. Generally, blood seems to be a good indicator of stress, and has the advantages that it is easy to collect and data can be recorded from live specimens. Thus, data can be collected from one fish over several points in time, which may reduce individual variability in time-course experiments. For field studies, animals do not need to be permanently removed from the ecosystem (Theodorakis et al. 1992). Blood, in most cases, is the medium for signals in the animal. Disturbances in integrated functions can be detected, or strongly indicated, with rather simple analysis of blood parameters. Blood ion composition and content can be used for detecting disturbed osmoregulation, and blood cell counts can give a general health measurement in this respect (Södergren et al. 1989). Other parameters such as lipid content (Gill et al. 1991, Singh & Singh 1992), hormone levels (Munkittrik et al. 1991, 1992), and immuno response (Dunier & Siwicki 1994) are often studied.

5. Testing integrated functions

One method of detecting chronic effects in fish is to analyse integrated functions. The integrated functions that are investigated can be divided into two categories; somatic and behavioural. Examples of somatic endpoints are growth rate and scope for growth. Scope for growth is a measure of the energy status of an organism at a particular time. It is based on the concept that energy, in excess of that required for basal maintenance, will be available for the growth and reproduction of the organism. The same character of endpoint is glycogen content of the liver. Both increases and decreases in glycogenolysis can occur due to toxicant-induced stress, which results in either depletion or accumulation of glycogen (Mayer et al. 1992).

Behavioural measurements can also be considered as integrated functions. Three examples are: "Preference-avoidance tests", where the animals ability to detect and avoid a chemical is investigated (Carr et al. 1990); studies of foraging behaviour, mainly focusing on effectiveness of capturing prey (Morgan & Kiceniuk 1990, 1991), and disturbances of prey fish schooling behaviour (Myllyvirta & Vuorinen 1989).

In some cases, behavioural endpoints have the problem of being a somewhat subjective measure. The tests often require much work and take time to perform, but can, on the other hand, give very valuable information on interactions between species that can not be elucidated with any other methods.

To be protective of populations, chronic tests must use animals in the most sensitive stage of life. It is also useful that the effect is easily detected during that life stage. There are several standardised early life stage (ELS) tests. For aquatic toxicology, the most used species are Zebra fish (Görge & Nagel 1990), and different *Oncorhynchus* (Hamilton et al. 1990) and *Salmo* (Van Leeuwen et al. 1990) species. As endpoints, embryo lethality and malformations are the most common parameters. These ELS-tests can also be conducted in the field (Karås et al. 1991). The possible risk of ELS-tests is that chemicals affecting other life stages, for example, sexual maturation, would not be detected as toxic. The ELS-tests are, by definition, not chronic toxicity tests but tests for toxicity of sublethal concentrations during embryo and larval stages, and should, therefore, not completely replace a full lifetime study.

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