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CONTRIBUTION 2

Biomarker applications in ecotoxicology: bridging the gap between toxicology and ecology

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ABSTRACT

Biomarkers have been used increasingly to investigate the adverse impacts of pollutants on different aspects of the structure and function of the ecosystem. In this manuscript, a conceptual overview and general evaluation of the present biomarker research in the rapidly expanding area of ecotoxicological research is presented. In general, ecotoxicological studies can be divided into 2 basic approaches, largely due to the historical backgrounds and the intrinsic differences in the merging sub-disciplines, namely the "toxicological" versus the "ecological" oriented approach. At present, this discrepancy largely hampers the application of biomarker techniques in environmental risk assessments. In order to use these effect- and/or exposure-based biomarker criteria in an applied ecological context, quantitative relationships between the sub-organismal effects and effects occurring at higher levels of biological organization need to be developed. The potential of certain energy-based biomarker criteria to bridge the existing gap between both types of research is illustrated for the waterflea *Daphnia magna*. It is demonstrated how short term (*in vitro*) digestive enzyme-based effects as well as Cellular Energy Allocation measurements allow estimation of threshold concentrations which are indicative of adverse effects at the (supra) organismal level.

THE USE OF BIOMARKERS IN ECOTOXICOLOGY: A HISTORICAL PERSPECTIVE

Over the last 3 decades biochemical, physiological and histological measurements have been used increasingly to assess the effects of toxic exposure on biota (Depledge, 1990; McCarthy and Shugart, 1990; Peakall, 1992). These criteria are generally indicated by the term "biomarker". Although, at present, several definitions of the term biomarker exist (NRC, 1989; Depledge, 1993; Rand, 1995; McCarty and Munkittrick, 1997), in the present context, we refer to the following description: "Biomarkers are biochemical, physiological and histological changes as well as aberrations in organisms used to estimate either exposure to chemicals or their resultant effects" (Huggett *et al.*, 1992).

Based on increasing knowledge of the modes of action of chemicals and their residues in biological systems, several "clinical" measurements have been proposed as diagnostic tools in ecotoxicological research (Mehrle and Mayer, 1980). Whereas originally much research effort was devoted to the identification of specific chemicals based on biomarker analysis, nowadays, more emphasis is being placed on the evaluation of biological effects based on biomarker measurements (McCarty and Munkittrick, 1997). Biomarker techniques are

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receiving increasing attention because they offer a number of advantages compared to conventional ecotoxicity tests which generally use lethality as an endpoint. In general, biomarkers can be considered as measures of the initial changes caused by toxicological interactions between the chemical and the (biological) receptor site. This interaction induces a cascade of events starting at the sub-cellular level (e.g. disturbance of gene transcription, interference with metabolic pathways) and ultimately leads to adverse effects at higher levels of biological organization. The effects which are normally studied in conventional toxicity tests (impaired growth, reproduction or survival) can thus be considered as the final result of accumulating damage at the sub-organismal level.

Historically, biomarkers originate from biomedical research and toxicology studies where the main concern was to identify the pharmacological dose of a compound causing a certain type of effect on humans. This mechanistic approach was focussed on the health of a human individual rather than on population level health effects. A basic theorem of this pharmacological approach is the fact that a biological response is caused by the interaction of a chemical with the site(s) of action in/on the organism and that the magnitude of the response is related to the target-dose. As a result of this central role of the biochemical response in the toxicological interaction, many studies have focussed on the assessment of subtle biochemical responses caused by toxic exposures (NRCC, 1985). One of the main differences between biomedical research on humans or on a limited number of surrogate organisms and ecotoxicology studies, is that in the latter a wide range of species must be considered.

In the context of the present manuscript, a modified version of Brett's (1958) definition of stress is used: "Stress is a state produced by an environmental or other factor which extends the adaptive responses of the organism beyond the normal range or which disturbs the normal functioning to such an extent that the chances of survival and/or reproduction are significantly reduced". The general biochemical basis of stress response was first described by Selye (1950) but was only applied in aquatic toxicity studies during the 1970s. Selye described the stress response of an organism in 3 distinct phases: alarm, compensation and exhaustion. This concept was later adapted for use in an ecological context (Mazeaud *et al.*, 1979; Pickering, 1981). The 3 phases of Selye (1976) were translated into effects at the primary (i.e. neuro-endocrine), secondary (i.e. physiological) and tertiary (i.e. individual responses) level of organization within the individual. Primary responses are rapid reversible responses, are generally short-lived and include neuro-endocrine releases of hormones. Secondary responses are generally physiological changes, which are less transitory than the primary responses and which are the result of the neuro-endocrine reaction. Tertiary responses are the least reversible and remain the longest (e.g. reduced reproductive performance, impaired growth). This "bottom-up" concept has been modified and illustrated by other authors. Depledge (1989), for example, proposed an interesting rationale to classify early indicators of toxic stress, based on the hypothesis of Hatch (1962). This concept was mainly formulated for physiological indicators, but in the context of the present study we would like to expand this concept to every level of biological organization. Distinctions are made based on the degree of impairment of the normal biological function and the disability or impaired health consequences which are associated with it (Figure 1).

Initial perturbation caused by toxic exposure will generate responses within the homeostatically regulated mechanism of the unit of study (cell, individual,...). These toxicant-induced sub-organismal alterations are biologically irrelevant if they do not lead to impaired characteristics at higher levels of biological organization (Versteeg *et al.*, 1983). With a gradual increase of toxic insult, compensatory adjustments are made, leading to deviations from the "healthy" condition. Within this compensatory phase, the organism's survival and reproduction capacities might start to be affected as the organism has less capacity to withstand additional stress. Eventually, a situation is reached were further repair and/or compensation is impossible and the organism dies. However, the possibility remains that if the conditions improve and repair processes are able to restore the compensatory mechanisms, a diseased organism might return to a healthy stage. Although the quantitative nature of this relationship is uncertain, this concept underlines the relevance of biomarker analysis as they offer the possibility to determine where the organism is situated in this continuum and can indicate early deviations of the "normal" functioning of biota (Versteeg *et al.*, 1983; Depledge, 1989). The resulting effects of increased stress-induced disturbance at different

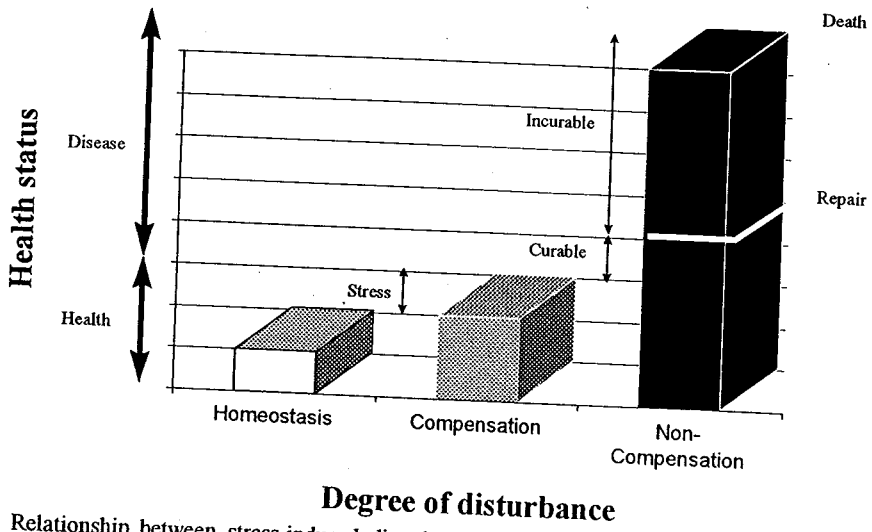

Figure 1. Relationship between stress-induced disturbance and the subsequent health impairment (based on Versteeg *et al.*, 1983 and Depledge, 1989).

levels of biological organization are illustrated in Figure 2. Subtle changes occurring at the sub-organismal level lead to effects within the homeostatic capacity of the organism, resulting in no adverse effects on growth, survival and reproduction. In an increasing time/concentration continuum, accumulative toxicant-inflicted impact eventually causes loss of species diversity within the communities and finally results in total loss of ecosystem structure and function.

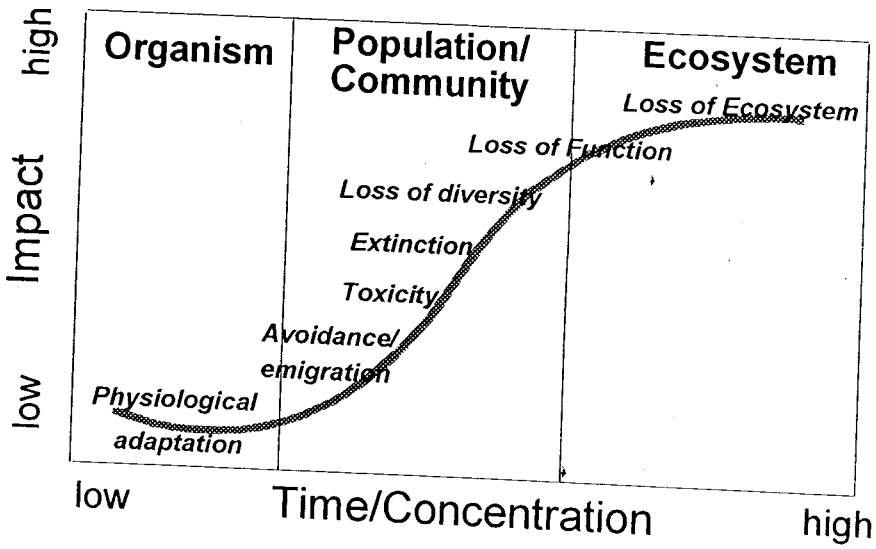

Figure 2. Effect of increasing toxicant-induced stress perturbation on the different levels of biological organization.

From an ecological point of view, however, effects of toxic stress are more descriptive in nature. Ecologists tend to describe the effects occurring at the individual, population, community and ecosystem level, usually, without prior knowledge of the causing agent.

Rather than giving a mechanistic picture, they try to gather a holistic and integrative overview (Munkittrick *et al.*, 1991).

Although at present, the importance of linking both approaches is recognized, their different approaches regarding the biological continuum of organization has lead to the "linear ecotoxicological paradigm" (Munkittrick and McCarty, 1995). In this concept, it is recognized that toxic exposure will interfere at the biochemical level and ultimately will give rise to effects at the individual level, however, it is impossible to identify *a priori* which specific sub-organismal mechanism will be linked to the individual changes. The organism integrates the most important biochemical effects which eventually become manifest in reduced ecological characteristics such as growth, reproduction and survival. With every increase of biological organization, the effects of the toxic exposure are translated into characteristics which are non-existing at the lower level of biological organization. Those lower-level effects which are most likely to be related to biologically relevant changes are those parameters which influence growth, reproduction and survival, i.e. the main determinants of the population-level changes. The "impairment-disability" concept explained higher is also applicable to higher levels of biological organization. At the ecosystem level, for example, compensatory adjustments might also occur which, however, do not affect the structure and the function of the ecosystem (Webster *et al.*, 1975). This "ecosystem resistance" might lead to situations where several species are lost due to toxic exposure but which are replaced by other species taking over the same role (Giesy and Odum, 1980; Kelly and Harwell, 1989). However, when key species are lost or affected, the point of "ecosystem resistance" is exceeded leading to impaired ecosystem functioning.

The link between the different levels of biological organization which can be studied in ecotoxicology and their degree of disturbance are illustrated in Figure 3. Within every increase in hierarchical level, a unit with higher ecological relevance is reached. At every level, however, different types of reponses can be distinguished. These range from

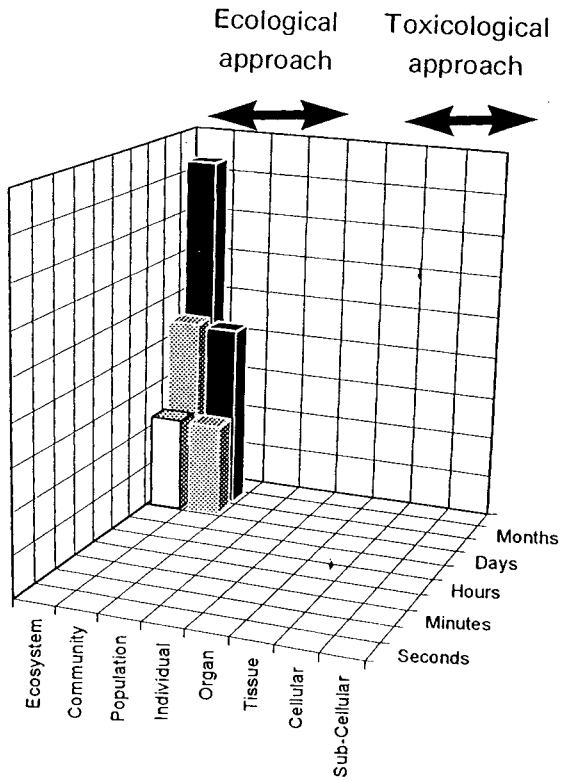

Figure 3. Illustration of the different hierarchical levels of biological organization which are studied in ecotoxicology. Each level has characteristic homeostatic (white bar), compensatory (grey bar) and non-compensatory responses (black bar).

homeostatic responses (white bars), to compensatory adjustments (grey bar) and eventually non-compensatory responses (black bars). Effects at lower levels can be detected in a much shorter time-span and will ultimately affect higher levels. However, in order to extrapolate effects between different levels of biological organization, a currency has to be identified which links both in a qualitative and quantitative way, the different levels. One of the most commonly used currencies to describe this hierarchical transfer are parameters of the energy metabolism. As mentioned higher, the complementary action of the top-down ecological approach with that of the bottom-up approach of fundamental toxicology should be able to identify which lower-level effects might be relevant to predict the direct consequences of biomarker responses for the individual or the population. At present, however, large uncertainties are involved in the different extrapolations which are performed between the different levels of biological organization (Hastings and Huggins, 1994; Munkittrick and McCarty, 1995). An illustration of the errors which might occur when extrapolations within a bottom-up approach are made is given in Figure 4. If a theoretical relationship is established between a biomarker criterion and effects at the community level, for example, the largest errors are to be expected near the bottom and the top of the concentration-response relationship. Near the lower end of the curve the species redundancy and community resilience might counteract the expected adverse effect (Giesy and Odum, 1980), whereas, at the higher end of the curve interactive effects between species, populations and communities might aggravate the expected impact.

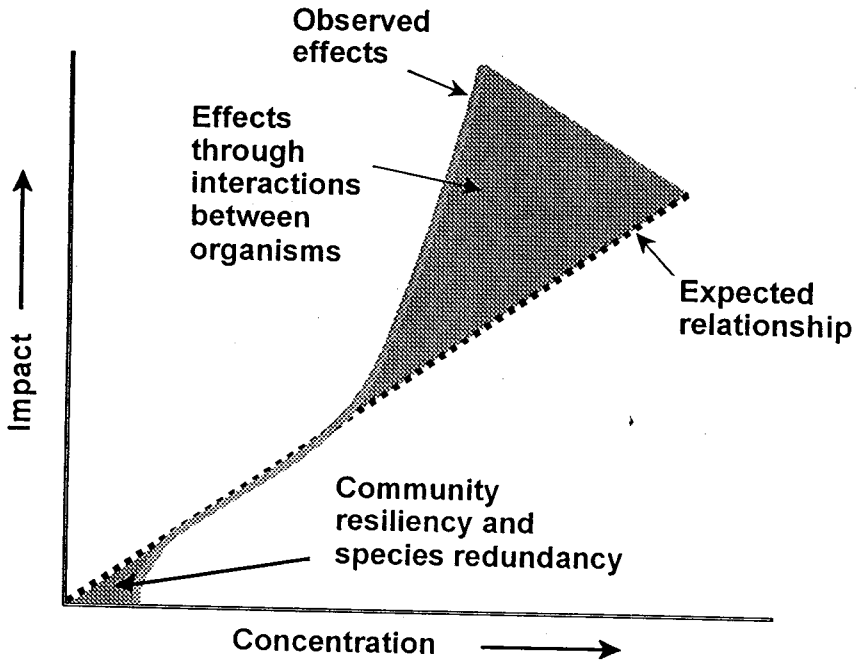

Figure 4. Illustration of the possible errors involved in the bottom-up extrapolation between different levels of biological organization.

BIOMARKER APPLICATIONS IN ECOTOXICOLOGICAL RESEARCH: STATE-OF-THE-ART

In general, biomarkers can be classified as either indicators of effects and/or exposure to a natural or man-made stressor. Biomarkers of effects are measurements indicative of the occurrence of a stress situation in the organism, without pinpointing the responsible agent. Examples include biomarkers of the energy metabolism, changes in DNA/RNA ratios, protein synthesis, etc. Effect biomarkers are usually applied as "clinical" health indicators of the test

organism. Biomarkers of exposure, on the other hand, can be used to identify the specific classes of agents to which an organism has been exposed. However, changes in the activity of these classes of biomarkers do not give a straightforward indication on the adverse health effects on an organism. Examples of biomarkers of exposure are cytochrome P450 detoxicification activity as an indication of exposure to polyaromatic hydrocarbons (PAHs), polychlorobiphenyls (PCBs), dioxins and benzofurans (Stegeman, 1981; Sanderson *et al.* 1996). Metallothioneins are another well known example as biomarkers of heavy metal exposure (Bracken and Klaassen, 1987; Gagné and Blaise, 1993).

To illustrate the current tendencies between both types of biomarker research, we depicted the number of publications dealing with cytochrome P450 and energy metabolism in environmental toxicology sciences over the last 20 years. Publications on CYP450 have increased almost exponentially, whereas a much lower number of publications have addressed the effects of toxicants on the various aspects of the energy metabolism (Figure 5). More specifically a 55-fold increase was noted for the former, whereas the latter category showed only a marginal 4-fold increase. These figures indicate that within the "toxicological/ecological framework" of the overall ecotoxicological research, much more attention is payed to biomarkers which tend to provide insights into mechanistic and toxicological processes, rather than on biomarkers which can, at least on a theoretical basis, be extrapolated to higher levels of biological organization. Indeed, bio-energetics or physiological energetics, in general, offer the advantage to provide information on key processes in the organism's energy acquisition and expenditure, possibly also elucidating the mode of action of the toxicant. Moreover, based on the "metabolic cost hypothesis" (Calow, 1989; Sibly and Calow, 1989; Koehn and Bayne, 1989; Calow and Sibly, 1990), changes in the energy metabolism, in general, will ultimately influence the future life characteristics of an organism. Responses to contaminants are considered to be a metabolic cost for an organism.

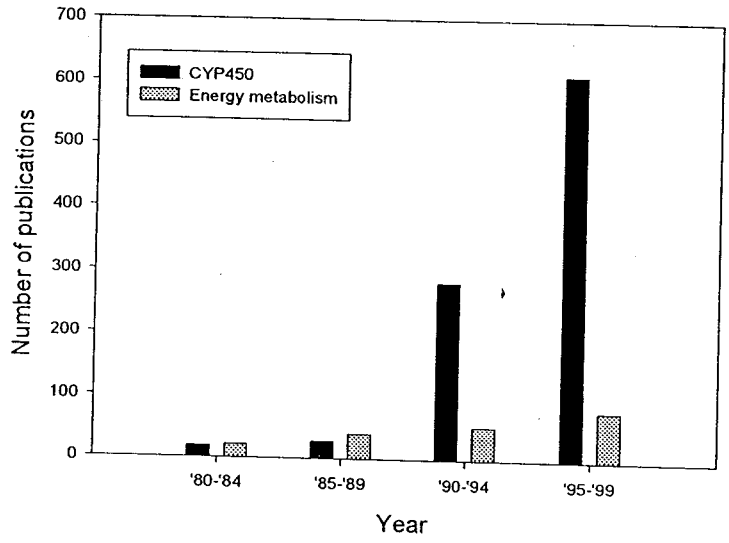

Figure 5. Overview of the number of publications dealing with Cytochrome P450 and energy metabolism in environmental toxicity research. (Source: Biological Abstracts, Silverplatter).

As under normal conditions the specific amounts of energy are allocated to the basal metabolism, growth and reproduction, from a theoretical perspective, changes in the metabolic turnover and specific allocations will be linked to effects at higher levels of ecological organization. This theoretical basis has been the onset of several studies which indicate that impaired energy metabolism as a result of toxic exposure, is the most likely type of biomarker which, in principle, could be linked to the future health of an individual.

In the context of the metabolic cost hypothesis, every response of an organism towards a toxic insult will influence the total energy budget and consequently the energy allocation towards

growth and reproduction (Koehn and Bayne, 1989). To date, several physiological and biochemical measurements have been used to study the effects of toxic exposure on the energy metabolism of aquatic organisms. The most commonly used technique to assess metabolic expenditure has been oxygen consumption measurements, which, according to some authors, should increase with increasing toxicant concentrations (Hatch, 1962; Calow, 1989). However, at present is believed that the relationship between this endpoint and toxicant stress can vary in all possible ways (Calabrese *et al.*, 1977; McKim *et al.*, 1987). As the total metabolism can be devised into different components (e.g. standard metabolism, active metabolism) these might not all respond in the same way to toxic stressors. This was illustrated by Baird *et al.*, (1990) who failed to detect increased O_2 consumption in chronically exposed daphnids but did demonstrate a reduction in food uptake, indicating a differential shift in the overall metabolism.

Various other methods have been proposed to characterize various aspects of an organism's energy metabolism and have been advocated as diagnostic tools to assess the organismal health. Among those, Scope for Growth (SfG - Warren and Davies, 1967), which measures the net amount of energy available for growth and reproduction, by subtracting the amount of energy respired and excreted from the amount of energy ingested. This parameter has been used both in laboratory and field conditions and has been shown to decrease with increasing stress levels. However, these reduced SfG values are usually mainly caused by a decreased food uptake rather than by an increased O_2 consumption, which means that the reduced energy budget is not always due to increased metabolic costs (Widdows and Page, 1993). Other energy related biomarkers such as the oxygen/nitrogen ratio (Bayne, 1975), Adenylate Energy Charge (AEC) (Giesy and Dickson, 1981; Giesy *et al.*, 1983), RNA/DNA ratio and protein synthesis (Brachet, 1960; Fiszer-Szafarz and Szafarz, 1984) have been used to quantify the effects of toxic stress in aquatic organisms. Except for the Scope for Growth parameter no efforts have been reported to quantitative relate the observed sub-organismal effects with effects occurring at higher levels of biological organization.

In general, biomarkers directly related to growth measurements are of high "ecological relevance" as impaired growth effects are directly interpretable in a broader ecological context. In this context, 3 biomarkers show particular promise for routine application: the RNA/DNA ratio, the protein synthesis and the free amino acid content. As increased growth rates are correlated to increased RNA concentrations, and the total DNA content per cell remains relatively constant, the RNA/DNA ratio can be a useful indicator of cellular growth rates. Studies with daphnids and salmonids, however, have shown that although this parameter was affected after short-term exposure periods, it was not as sensitive as the conventional growth and reproduction measurements (McKee and Knowles, 1986; Knowles and McKee, 1987; McKee *et al.*, 1989). Studies with fathead minnows on the other hand, demonstrated that the short-term effects on RNA concentration might be predictive of long term effects. Protein synthesis, measured by means of radioactively labelled amino acid incorporation *in vivo* or *in vitro*, has been suggested as a sublethal stress indicator in molluscs and fish (Viarengo *et al.*, 1980; Smith, 1981). Both methods have considerable potential as sensitive and predictive techniques for assessing growth under field conditions, however, the influence of several environmental factors (such as food supply, temperature, etc.) remain to be evaluated.

The free amino acid pool (FAA) represents another energy reserve fraction related to important biochemical and physiological processes in the organism. In general, increasing toxicant concentrations will cause a decrease in the total and individual free amino acid concentrations. Free amino acids are important biomolecules as they play crucial roles in the osmoregulation and the protein metabolism of the organism. Both in freshwater and marine organisms this parameter has been shown to change due to toxic stress (Kasschau *et al.*, 1980; Graney and Giesy, 1986a, 1986b, 1987, 1988). Whereas for most of these energy-related criteria, at present, sufficient information is available on the potential application of this biomarker, both under laboratory or field conditions, little exact data have been generated to illustrate the relationships with effects at higher level of biological organization.

EXAMPLES OF BIOMARKER VALIDATION: LINKING SUB-ORGANISMAL PROPERTIES TO HIGHER LEVELS OF BIOLOGICAL ORGANIZATION

As the majority of the biomarker research in aquatic toxicology has been carried out on fish, it is not surprising that little studies have attempted to link short-term biomarker effects with effects emerging after prolonged (chronic) exposures. Due to their relatively long life-cycle, adverse effects on survival, growth and reproduction can only be quantified after several weeks or months. Although invertebrates have - in comparison to fish - largely been overlooked in biomarker research, they offer several (practical) advantages: their relatively short life-cycle and their small body size, makes the performance of (multi) generation as well as population studies possible within much shorter time periods.

To illustrate the advantages of invertebrate species in biomarker applications, we present 2 examples of biomarkers which were investigated in the waterflea *Daphnia magna*, which is one of the most commonly used zooplankton organisms in aquatic toxicity studies (Baudo, 1987; De Coen and Janssen, 1998a). In a first example we will demonstrate how enzymatic *in vitro* inhibition studies with this micro-crustacean can be used to predict acute toxicity levels. A second example will demonstrate the quantitative relationships between a recently developed biomarker, Cellular Energy Allocation, and chronic population level effects.

In vitro digestive enzyme inhibition assays with *Daphnia magna*

Previous studies have indicated the importance of the digestive physiology of the waterflea under toxic stress (Flickinger *et al.*, 1982; De Coen and Janssen, 1997a, 1998b). Since the organism's gut is considered as one of the first organs to be exposed to the toxicant, we quantified the inhibiting potential of several chemical compounds of various digestive enzymes.

Enzyme extracts were obtained by homogenising adult daphnids (>14 days old) using a motor-driven potter in 0.1M Tris-HCl buffer pH 7.0. After centrifugation (10,000g, 15 min, 4°C) the toxicants were added to the supernatant. After a 4h exposure period on ice, 5 different digestive enzymes (β-galactosidase, amylase, cellulase, esterase and trypsin) were measured using specific fluorescent and chromogenic substrates as described in De Coen and Janssen (1997a). Eight different model chemicals were used: 3 heavy metals: cadmium chloride, potassium dichromate, mercury chloride, one organo-metal (tributyl-tin: TBT-Cl), one pesticide (lindane: gamma-hexachlorocyclohexane), one fungicide (sodium pentachlorophenolate: NaPCP), one detergent (linear alkyl sulfonic acid: LAS) and one herbide (2,4-D: 2,4-dichlorophenoxy acetic acid). For 4 of the enzymes tested a significant ($p<0.05$) and linear relationship was obtained between the enzymatic EC_x values and the conventional 24h EC50 values based on immobility (Table 1).

Table 1. Results of the linear regression analysis between the short-term enzymatic endpoints and the conventional 24h EC50 values based (immobility).

Enzyme	Equation linear regression	R
β-galactosidase	Log EC5 = 0.19 + 0.69 . log 24h EC50	0.86
Esterase	Log EC10 = 0.19 + 1.02 . log 24h EC50	0.98
Trypsin	Log EC10 = 0.72 + 1.05 . log 24h EC50	0.93
Cellulase	Log EC10 = -0.44 + 0.66 . log 24h EC50	0.91

These data demonstrate that effects such as the direct enzymatic inhibition of important enzymatic pathways could be linked to effects at the organismal level.

Relationship between changes in the Cellular Energy Allocation parameter and the subsequent population level effects

Since its first publication in 1995 (De Coen *et al.* 1995), the CEA methodology has been succesfully applied in daphnids (De Coen and Janssen, 1997b) and fish larvae (Nguyen, 1997). The concept of the technique is based on the "metabolic cost" hypothesis (Calow, 1974) and resembles much to the principle of Scope for Growth (Warren and Davies, 1967). Based on a biochemical approach both the energy reserves available (Ea) and the energy consumption (Ec) are quantified over a certain exposure period. Energy consumption is estimated by measuring the electron transport activity (ETS) at the mitochondrial level, while the energy reserves available for metabolism are assessed by measuring the total lipid, protein and sugar content of the test organism. The combination of these 2 factors, Ea - Ec, represents the net energy budget of the test organism. For a complete description of the method we refer to De Coen and Janssen (1997b) which demonstrates the application of the technique in daphnids exposed for a short-term (96h) to mercury and lindane. Recently, a more extended study – using the same 8 toxicants as mentioned higher - was finished which aimed at "validating" the short-term (96h) biomarker criterion towards effects at the population level determined through life-table analysis (De Coen, 1999). The ecological relevance of the CEA assay was assessed by comparing the sub-organismal response with population level parameters such as the intrinsic rate of natural increase (r_m), net reproductive rate (R), the mean brood size (MBS), the mean total young per female (TMYF) and the adult body size (L). Two different methodologies were used to assess the effect levels: the lowest observed effect level (LOAECs) approach and the regression-based approach. All toxicants caused a significant concentration-dependent decrease in the net energy budget of *D. magna*. The observed reductions in CEA values were both the result of a decrease in energy reserves (Ea) and an increase in energy consumption (Ec). From all individual CEA components analysed, the lipid reserve criterion was the most sensitive endpoint studied, illustrating the importance of this reserve fraction for the energy metabolism of the waterflea. Both the CEA-based LOAEC values and the EC10 values were significantly ($p<0.05$) and linearly correlated with the chronic LOAEC and EC10 values based on growth and reproduction. An example of this linear relationship between the LOAEC based on CEA and r_m is given in Figure 6.

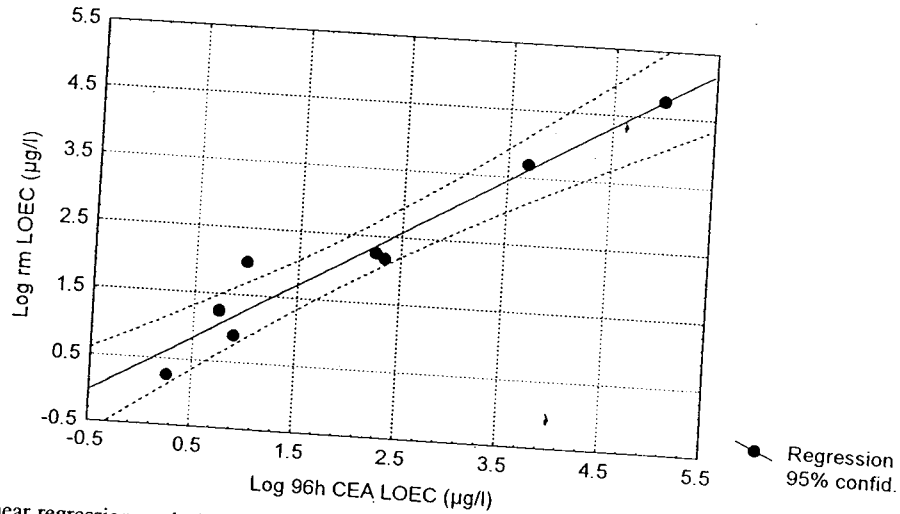

Figure 6. Linear regression analysis between the log-transformed 96h CEA-based LOAEC values and the log-transformed LOAEC thresholds based on intrinsic rate of natural increase. Log r_m LOEC = 0.40 + 0.86 * Log CEA LOEC; $r^2=0.94$; $p<0.05$.

This relationship clearly illustrates the usefulness of the CEA methodology to predict long-term effect thresholds for pure chemicals. Furthermore, significant ($p<0.0001$) sigmoid relationships between the 96h CEA value (expressed as percentage relative to the control) and population level effects were observed. The coefficients of determination (r^2) of the

relationships between the CEA values and the MBS and MTYF parameters equal 0.75 ($p<0.001$), while for the relationship with the intrinsic rate of natural increase and net reproductive rate the r^2 values were 0.69 and 0.78. An example of this quantitative relationship between net-energy budget changes and the population's net reproductive rate is given in Figure 7. The overall observed reductions in the energy budget of the daphnids were the result of an increased energy consumption, a reduced biomass production, or a combination of both, as was predicted by Baird *et al.* (1990). In the present study, the effect on the energy consumption (E_c) of the daphnids was clearly toxicant - and exposure time – specific: both inhibition (e.g. cadmium) and stimulation (e.g. lindane) were measured. The observed reduction in E_a values over the 96h period was either the result of (1) depletion of the energy reserves caused by increased metabolic activity or (2) decreased food (energy) uptake. Consequently, the CEA methodology not only provided an integrative quantification of the organism's energy budget, but could also help to elucidate the different modes of action of toxicants. Indeed, this technique could provide insights in some of the primary mechanisms of toxicity. For example, mechanisms such as non-specific narcosis or neurotoxicity are reflected in feeding inhibition, while uncoupling of oxidative phosphorylation is leading to increased metabolic rate and inhibition of metabolism is resulting in reduced respiratory activity. On the other hand, this CEA concept might not be applicable to compounds with a very rapid mode of toxic action (e.g. organophosphate pesticides). Chemicals which interact with certain molecules/receptors in the organism's body and cause lethality by "saturating" their specific sites of interaction, generally will cause their adverse effect within a relatively short time period before the net energy budget might even become affected. However, as the CEA methodology is aimed at predicting chronic toxicity levels using sub-chronic exposure periods, acutely toxic effects might be detected during the initial course (i.e. during the first days) of the experiment.

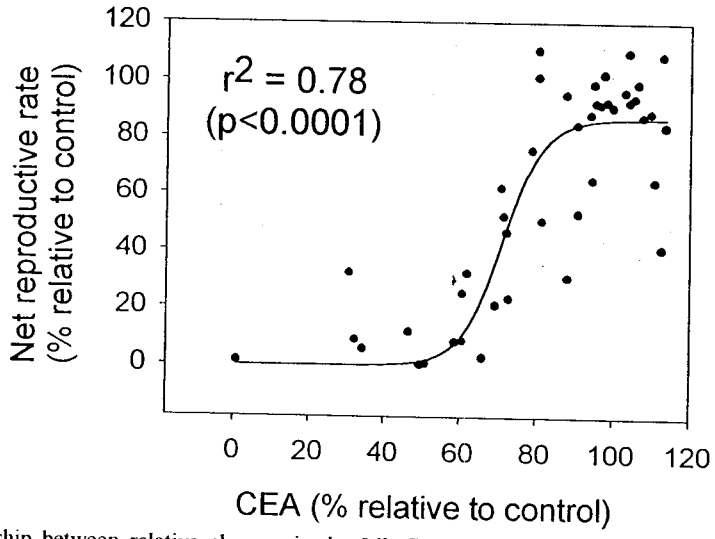

Figure 7. Relationship between relative changes in the 96h CEA parameter and relative changes in the net reproductive rate of *D. magna* exposed for 21 days.

CONCLUSIONS

Based on the results presented in this study, we conclude that the short-term biomarker assays may be useful for predicting long-term effects at higher levels of biological organization. Although the obtained relationships have no causal nature but are rather descriptive, the physiological implications of the toxicant-induced inhibition of these toxicant perturbations can be interpreted in a more broader theoretical concept. As inhibitory concentrations of crucial physiological processes such as digestive enzyme activity are similar to the lethal effect concentrations, the importance of these enzymatic pathways as well as the contribution of the specific gut-exposure route for the overall health of the organism is clearly

demonstrated. Furthermore, the relevance of measuring energy metabolism – related biomarkers to assess the impact on exposed organisms has been quantitatively illustrated. Most remarkably, the concentration that causes a 10% decrease in the net energy budget after a short-term exposure period, induces a similar decrease in the population growth and reproduction parameters. Such a quantitative relationship is the first in its kind and could give rise to several other applications in effects assessments such as Quantitative Structure Activity Relationships (QSARs) and Chronic Toxicity Identification Evalution (TIE) evalutions. Most interestingly, all of the presented methods are relatively simple to perform and the use of multi-well plates together with automated spectrophotometric readings allows the routine application of these assays.

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