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Ecotoxicological Biomonitoring at Different Levels of Biological Organization and Its Application in *Chironomus* spp.

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다단계 생체지표를 이용한 생태독성 모니터링과 *Chironomus* spp.에의 적용

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요 약

환경오염의 조기 경보 시스템으로 생체지표를 이용한 생태독성 모니터링이 최근 널리 연구되고 있다. 환경내의 생물종에서 측정한 생체지표를 이용한 환경 모니터링은 생태계 수준의 영향에 대한 예측 정보를 제공해 줄 수도 있다. 이를 위해서는 생체지표와 개체군 수준에서의 반응과의 인과관계가 밝혀져야 한다. 오염물질에 대한 생체의 반응은 분자, 세포, 생화학, 생리적, 개체, 개체군, 군집 수준에서 나타나게 되며, 이러한 각 단계별 반응은 반응 시간의 규모와 독성학적, 생태적 관련성에 따라 구분 지어 볼 수 있다. 각 개별 수준에서의 반응을 종합하면 오염물질에 노출된 생체의 전제적인 영향을 이해할 수 있으며, 이러한 이해를 바탕으로 개체군에서 나타나는 영향에 대한 인과관계를 추론할 수 있다. 생체지표와 개체군 수준의 반응과의 인과관계 정립은 효율적인 환경오염 예방기능 수행에 필수적인 과정이며, 다단계 생체지표는 각 단계별 반응의 인과관계를 밝히기 위해 가장 적절한 접근 방법이다. 수서 무척추 생물인 *Chironomus*의 유충은 이러한 다단계 바이오마커 연구에 매우 적절한 생물학적 모델이다. 이 논문의 첫 번째 부분은 생체지표를 이용한 환경 모니터링을, 두 번째 부분은 *Chironomus*의 유충에서 생체지표의 적용에 대해서 다룬다.

Key words : biomarker, environmental monitoring, ecological risk assessment, early warning system, multi-level biomarker, invertebrate, *Chironomus* spp.

INTRODUCTION

In many developed countries, the enforcement of specific regulations had a significant positive effect on the level of environmental pollution in the last decades, especially through a reduction in point source pollution (e.g. building of sewage treatment plants) and the ban of some persistent chemicals (e.g. DDT, toxaphene). However point source pollution is still a matter of concern in numerous countries and non-point source pollution by organic (e.g. pestici-

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des, dioxins) and inorganic (e.g. heavy metals) compounds is still a matter of concern worldwide.

The assessment of environmental quality implies that the biological effects of pollutants could be monitored using adapted tools. Ecotoxicology is a multi disciplinary science which focus on the adverse effects of toxicants at various levels of biological organization and which may provide such tools. Ecotoxicological researches have first been devoted to the study the effects of environmental contaminants at the population, community or ecosystem levels (Forbes and Forbes, 1994). However, these traditional approaches are sometimes inefficient, especially to adequately assess the effects of chronic exposure of organisms to low levels of xenobiotics and to detect early biological responses. Therefore, there has been a shift in emphasis towards understanding the sublethal effects of long−term exposure to contaminants at the individual level where exposure can be adequately described and assessed (Newman and Jagoe, 1996). It has been necessary to perform studies on individuals at the biochemical and molecular levels where toxicant−induced responses are initiated.

The effects of toxicants usually begin through an interaction between toxicants and biomolecules (e.g. enzymes, receptors, DNA). Effects then cascade through the molecular, biochemical, subcellular, cellular, tissue, organ, individual, population, community and ecosystem levels of organization. Therefore, the understanding of the effects of toxicants at the molecular or biochemical levels may provide some insights into the cause of effects identified at higher levels (Newman and Unger, 2003). The biomarker approach can be an extremely useful tool for this kind of investigation and it has been increasingly used for environmental hazard assessment during the last ten years (Delpedge and Fossi, 1994; Fossi *et al.*, 2000).

Biomarker−based environmental monitoring

concept of biomarker

The historical development of the biomarker approach is closely linked to advances in medicine and vertebrate biology (NRC, 1987). Biomarker measurements are now equally feasible in many plants and animal species (Livingstone, 1991; Depledge and Fossi, 1994; Fossi *et al*., 2000; Lagadic *et al*., 2000). Biomarkers were originally defined as xenobiotically −induced variations in cellular or biochemical components or processes, structures or functions that are measurable in a biological system or sample (NRC, 1987). They were first classified as markers of exposure to a toxicants, markers of effects of exposure and markers of susceptibility to the effects of exposure (NAS/NRC, 1989). This definition has been challenged by several authors (Adams, 1990; Engel and Vaughan, 1996; McCarty and Munkittrick, 1996) and the term biomarker is now more commonly used in a more restrictive sense, namely sublethal biochemical changes resulting from individual exposure to xenobiotics (Hyne and Maher, 2003).

The biomarker approach has received considerable attention in ecotoxicology as a new and potentially powerful and informative tool for detecting and documenting exposure to, and effects of, environmental contamination (Newman and Jagoe, 1996). The primary use of biomarker in environmental monitoring is to assess the health of organisms in order to detect and identify potential problems so that unacceptable and irreversible effects at higher levels of biological organization can be avoided. It is important, however, to keep in mind that our current understanding of biomarker responses in wild species is limited. To achieve the full potential of this tool for the protection of the environment, a great deal of research is still needed to develop, validate and interpret biomarker based monitoring.

Potentials and limitations of biomarker in environmental monitoring

Chemical pollution is often caused by a complex mixture of compounds, which makes the exhaustive analysis of the contaminants present in polluted environment impossible (Risso−de−Faverney *et al*., 2001; Meregalli *et al*., 2002). Moreover, the mere presence of a pollutant does not indicate an impact on organisms, as its bioavailability may be influenced by many

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factors (see e.g. Landrum and Robbins, 1990). The use of biomarkers to assess the biological and ecological significance of environmental contaminants is a complementary approach to chemical analysis and is becoming an important component of many environmental monitoring programs. Organisms can provide more complete information on the impacts of the toxicants than chemical analysis alone because some of them can integrate the exposure to contaminants and respond in some measurable and predictable ways (Vermeulen, 1995). Responses can be observed at several levels of biological organization from the biomolecules level, where pollutants can cause damage to critical cellular targets and elicit cellular mechanisms of defense such as detoxication (e.g. cytochrome P450 associated enzymatic activities, glutathione S-transferases) and repair process (e.g. DNA repair enzymes), to the organismal level, where severe disturbances such as impairment in growth, reproduction, developmental abnormalities, or decreased survival may be observed (Newman and Jagoe, 1996). Biomarkers can provide not only evidence of exposure to a broad spectrum of anthropogenic chemicals, but also a temporally integrated measure of bioavailable contaminants. A suite of biomarkers should preferably be used to determine the magnitude of the problem at the individual level and evaluate possible consequences at the population or community levels (Cormier and Daniel, 1994).

Recently, the growing awareness of the possibility of using wildlife animals as sentinels for human environmentally-induced diseases has created a demand for biomarkers that are nonlethal and correlate with adverse effects in humans (Kendall *et al*., 2001). Links between wildlife and human health can serve as a premise for extrapolation in risk assessment. Indeed, humans share many cellular and subcellular mechanisms with wildlife species. Humans and wildlife also overlap in their environments and may therefore be exposed to the same contaminants. There is evidence to suggest that when highly conserved systems are targeted by environmental toxicants, both ecosystem and human health suffer (Kendall *et al*., 2001).

As biochemical changes are usually detectable before adverse effects may be seen at higher level of biological organization, the biochemical marker approach is often considered as an early warning or proactive tool. This is a great advantage because responses at higher levels are usually measurable only after a significant or permanent damage has occurred. The early detection of sublethal effects may also be used to identify the need for remedial action at a contaminated location and to monitor the recovery period after cleanup of the site (Peakall and Shugart, 1993; Depledge and Fossi, 1994; Lagadic *et al*., 2000). Regardless of their proactive or retroactive utility, the ecological realism of biomarkers is lower than for indicators based on higher-level of biological organization such as species richness or reproductive failure (Newman and Unger, 2003).

The choice of the appropriate biomarker requires an accurate knowledge of a variety of factors (Mayer *et al*., 1992; Peakall and Shugart, 1993). Thus, it is critical to use well-defined biological material, for which the changes in biochemical activity with development, age and tissue is known, in order to predict toxicity from changes in biochemical biomarker response following the exposure to a chemical (Hyne and Maher, 2003). The selection of biomarkers applicable in many species is frequently limited by a lack of knowledge on their intrinsic characteristics (e.g. basal level, feedback control, role of repair mechanisms). The reliability of use of biomarkers depends on knowledge of the mechanism involved in the particular response. Once suitable biomarkers are selected, it is important to conduct field studies to establish how environmental and biotic factors will modify the biomarker responses to toxicants relative to those seen in laboratory conditions where those factors are controlled (Hyne and Maher, 2003).

Multilevel biomarker based approach

As mentioned above, biomarker responses could be used as an early warning system for environmental monitoring (Peakall and Shugart, 1993; Depledge and

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Fossi, 1994 Lagadic *et al*., 2000). Nevertheless, biochemical endpoints alone do not seem to be sufficient to assess environmental quality. Pollutant-induced biochemical effects may potentially have consequences at higher levels of biological organization, such as changes in population dynamics or in biological diversity at both the intra- and interspecific levels (Depledge *et al*., 1993; Caquet and Lagadic, 2000). Such changes may have adverse ecological consequences (Caquet and Lagadic, 2000). Therefore, multilevel biomarker approach, evaluating different biological responses ranging from molecular to physiological level, would be more conservative for useful environmental monitoring (Depledge and Fossi, 1994; Lagadic *et al*., 1994, 2000; Dickerson *et al*., 1994; Choi *et al*., 2002).

The multilevel biomarker concept is originally based on the fact that biological responses of an organism in natural environment progresses through homeostasis, compensatory and repair phases, as the exposure level or duration increases (Depledge, 1994). While an organism is exposed to contaminants, physiological compensatory mechanisms become active and changes in physiological processes or functions occur, which indicate that exposure has occurred. If the exposure persists or the level of exposure increases, these compensatory mechanisms become overwhelmed, damages occur, and physiological repair mechanisms become active. Under natural environmental conditions, as an organism progresses through these phases, the energy allocated for natural maintenance is reduced as more energy is needed for compensatory response and repair. The organism weakens and may be quickly eliminated from the population. Therefore, in situ survey of populations may not allow to detect diseased organisms even though exposure and effects have occurred (Newman and Jagoe, 1996). In the context of the multiple-response paradigm, the objective is not to quantitatively measure the amounts of different toxicants, but to determine where an organism is located on the continuum between homeostasis and disease. Responses indicate whether the organism is challenged but readily coping with toxicant stress (compensatory phase) or is deeply stressed and needs to use its energy resources to repair damages. This approach is essential to determine the general health status of the organism; moreover, it makes possible to extrapolate the relationship between responses at different levels of biological organization (Fossi *et al*., 2000).

Some biochemical biomarkers do not appear to have a direct relationship to a defined mechanism of toxicity. In this case, the use of such biomarker will not give a reliable prediction of toxic effects and is, therefore, only ever likely to indicate exposure to chemicals. These biomarkers of exposure cannot be used to predict effects at the population level from biomarker changes measured in a sample of individuals (Hyne and Maher, 2003). To relate the effects measured at the individual level to higher levels of biological organization, the biomarker response should be related to an impairment of growth, reproduction, or metabolic function which directly affects the survival of the organism and which can be attributed to exposure to a known amount of specific contaminants (Delpedge and Fossi, 1994).

Environmental monitoring using *Chironomus* spp.

Ecotoxicological significance of invertebrate biomarkers

To link the measurement of a biomarker in individuals to changes at the population level, it is necessary to understand the mechanisms, which link the effects at the subcellular level to the response of individuals. Quantitative dose-response relationships for the biomarker may then link the molecular effect of the toxicant to the toxic response of the individual organism. Linkage of whole organism responses to changes in populations can then be obtained by statistical or numerical inferences (Hyne and Maher, 2003). Invertebrates are good biological models for such studies. They are major components of all animal communities and they represent 95% of all animal species on Earth (Barnes, 1968). Their populations are often abundant and their life cycles are frequently short, so samples

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can be taken for analysis without significantly affecting population dynamics and population level effects can be examined concomitantly with the response of biomarkers. Increasing knowledge of the biochemistry of invertebrates (James, 1989; Livingstone, 1991), now permits reasonable interpretation of biomarker responses in terms of ecological risk assessment (Depledge, 1994; Depledge and Fossi, 1994). Numerous biomarkers are extensively studied in various invertebrates species to evaluate their potential for predicting population level changes. This is for example the case of DNA damage (Deplege, 1998; Wilson *et al*., 1998; Atienzar *et al*., 1999; Fossi *et al*., 2000; Guecheva *et al*., 2001), heat shock proteins induction (Snyder and Mulder, 2001; Wheelock *et al*., 2002; Guecheva *et al*., in press), energy reserves (Baturo and Lagadic, 1996) or of the alteration of the activity of various enzymes (Abele-Oeschger, 1996; Baturo and Lagadic, 1996; Fossi *et al*., 2000; Hyne and Maher, 2003; Guecheva *et al*., in press).

Particularities of *Chironomus* as a sentinel invertebrate species

Various factors need to be considered when selecting a species for ecotoxicological monitoring, including knowledge of its physiology and of its demoecology, the availability of laboratory rearing protocols. The aquatic larvae of non-biting midges (Chironomidae, Diptera), which are widely used in freshwater environmental monitoring and laboratory toxicity testing, fulfill those criteria. They are ubiquitously distributed, sensitive to many pollutants, easy to culture and have a short life cycle (Ingersoll and Nelson, 1990), which make them suitable for ecotoxicological monitoring.

The midges frequently represent the most abundant group of macroinvertebrates and up to more than 50 % of the total number of macroinvertebrate species in freshwater ecosystems, especially in the profundal and sublittoral zones of lakes. The Chironomidae, and especially the sub-family Chironominae, are the most widely distributed group of insects, having adapted to nearly every type of aquatic or semiaquatic environment. The larvae are diverse in form and size, but they are easily recognized because they usually have anterior and posterior pairs of prolegs and a distinct head capsule. Larvae are an extremely important part of aquatic food chains as detritivores and serving as preys for many invertebartes and for several species of fish (see review in Cranston, 1995).

The chironomid life cycle includes egg, larva, pupa and adult stages (Fig. 1). The females lay their eggs in a group as an eggmass (Fig. 1A) with up to 800 eggs (in *Chironomus duplex*), either directly into water or attached to plants or stones at the water's edge. After a couple of days the eggs hatch as larvae, which, in the sub-family Chironominae, are usually red-colored due to the presence of hemoglobin in the hemolymph (Fig. 1B). The larvae go through four instars. In the fourth instar larvae, polytene chromosomes develop in some tissues (e.g. salivary glands), reaching their greatest size just before the larva pupates in a silk-lined tube. The larvae are 2 to 30 mm long, depending on species and larval instar and often exhibit a slightly curved shape, particularly when preserved in alcohol or formalin. The duration of the larval period may range from two weeks to several years and depends mostly on temperature. The pupal stage lasts no more than a few days. After 2 or 3 days the pupa swims to the surface and the adult midge emerges. Usually, the adults live only a day or two, mating in swarms, laying their eggs and then dying. Adult chironomids are minute- (e.g. wing length 0.8 mm in Orthosmittia reyei) to medium-sized (e.g. wing length 7.5 mm in *Chironomus* alternans) insects (Fig. 1C, 1D). In temperate regions, many chironomid species are uni- or bivoltine, but up to four generations in a year are not uncommon. Species living in the cold, profundal zones of deep lakes may take more than one year to complete their life cycles, and circumpolar species require at least two years, and occasionally as many as seven (see review in Cranston, 1995).

The predictable responses of populations of certain species of midges to different levels of various pollutants have resulted in the use of larval chironomids as

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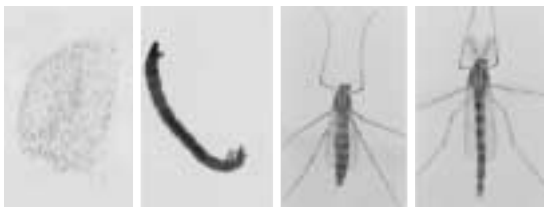

Fig. 1. Chironomid A: egg mass, B: larva, C: female adult, D: male adult.

biological indicators of water quality. Additionally, chironomid larvae are essential components in the efficient biological processes that take place in the oxidation ponds of sewage treatment plants. Water quality also determines chironomid distribution in the field, and within the family Chironomidae a wide range of tolerance is displayed. Some Tanypodinae and Chironominae are very tolerant towards low levels of dissolved oxygen. *Chironomus plumosus* larvae are able to withstand a pH value of 2.3. *Cricotopus bicinctus* is known for its tolerance for many substances, including electroplating wastes and crude oil. Other members of the family are known for their intolerance for poor water quality (see review in Pinder, 1986). Larvae of midges of the genus *Chironomus* are among the rare invertebrate species to possess hemoglobin (s) (Hbs), which give them some biochemical and physiological particularities. *Chironomus* Hbs exhibit many interesting features, such as a high degree of polymorphism, a high affinity for oxygen and an extracellular localization (Osmulski and Leyko, 1986). From an evolutionary point of view, it is generally admitted that the presence of Hbs in invertebrates reveals the adaptation of these organisms to unfavorable environmental conditions, since these pigments help to sustain aerobic metabolism under low-oxygen conditions (Weber and Vinogradov, 2001). *Chironomus* Hbs appear to fulfill clear physiological roles in transporting and storing oxygen in the larvae that burrow in polluted and hypoxic muds (Osmulski Leyko, 1986). According to Weber (1980) and Lindegaard (1995), the extracellular Hbs enhance the good exploitation of hypoxic oxygen. Moreover, a possible but still undefined role has been proposed for *Chironomus* Hbs in the metabolism of xenobiotics in frequently polluted environments, where these animals flourish (Osmulski and Leyko, 1986; Weber and Vinogradov, 2001).

Multilevel biomarkers in *Chironomus* spp.

The above-mentioned physiological and ecological particularities make chironomid larvae a suitable invertebrate model for biomarker-based environmental monitoring and ecological risk assessment. Biomarkers have frequently been studied in this insect group. Various endpoints, ranging from molecular to population level, have been employed in chironomid for environmental quality assessment, especially for species of the genus *Chironomus* (Table 1 and 2). Changes of enzyme activities or chromosome-level alterations were mainly used as molecular or biochemical biomarkers (Table 1), whereas development time, life cycle or reproduction performances were investigated as population-level descriptors (Table 2). Most studies have been performed under laboratory condition, and few studies were undertaken in natural ecosystems.

As shown in Table 1 and 2, despite the increasing use of biomarkers for assessing environmental toxicity, there have been few studies in which effects on biochemical responses have been compared with subsequent effects on individual fitness or population health. Organisms show homeostatic responses to changes in environmental conditions, and differences in biomarker measurements may be within the usual range of expression and have no long-term significance for organism fitness (Peakall, 1994; Olsen *et al*., 2001). Alternately, the responses of biomarkers may only be measurable when obvious damage to the fitness of an organism has occurred, which considerably reduces their usefulness. Therefore, it is clearly important to be able to relate changes in biomarkers to meaningful effects at higher levels of biological organization, and to determine that these

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Table 1. Examples of biomarkers studied in *Chironomus* spp.

Species	Stressors	Endpoints	References
C. tentans	Insecticides	Acetylcholinesterase	Karnak and Collins (1974)
C. riparius	Insecticides	Cytochrome P450	Estenik and Collins (1979)
C. riparius	Parathion	Acetylcholinesterase	Detra and Collins (1991)
C. ninevah	Cooper	Balbiani ring	Aziz *et al*. (1991)
C. tentans	Benzo[a]pyrene, ActynomycinD, Dimethylnitrosamine	Chromosome puffing	Bentivegna and Cooper (1993)
C. salinarius	Contaminated sediments	Polytene chromosome	Hudson and Ciborowski (1996)
C. riparius	Heavy metals	Polytene chromosome	Michailova *et al*. (1998)
C. tentans	Heat shock	Stress protein (hsp70)	Karouna−Renier and Zehr (1999)
C. riparius	Fenitrothion, chromium	Antioxidant enzymes	Choi *et al*. (2000)
C. tentans	Cadmium	alpha−tubulin cDNA	Mattingly *et al*. (2001)
C. riparius	Fenitrothion, chromium	Energy metabolism	Choi *et al*. (2001)
C. riparius	Contaminated sediment	Mouthpart deformity / Nucleolus activity	Meregalli *et al*. (2002)
C. riparius	Fenitrothion	Acetylcholinesterase, Superoxide dismutase	Choi *et al*. (2002)
C. riparius	Pirimiphos methyl	Acetylcholinesterase, Glutathione*S*−transferase	Crane *et al*. (2002)
C. riparius	Aluminium	Polytene chromosome	Michailova *et al*. (2003)
C. riparius	Phenobarbital, permethrin	Cytochrome P450	Fisher *et al*. (2003)

Table 2. Examples of the population level effects studied in *Chironomus* spp.

Species	Stressors	Endpoints	References
C. tentans	DDE	Egg viability	Derr and Zabik (1972)
C. riparius	Cadmium	Oviposition/egg viability	William *et al*. (1987)
C. decorus	Cooper	Partial life cycle	Kosalwat and Knight (1987)
C. riparius	Cadmium	Larval development/adult emergence	Pascoe *et al*. (1989)
C. riparius	Aluminum	Life cycle	Palawski *et al*. (1989)
C. riparius	Lindane	Life cycle	Taylor *et al*. (1993)
C. riparius	Ethynyloestradiol/bisphenolA	Development/reproduction	Watts *et al*. (2001)
C. riparius	Hexachlorobiphenyl	Life cycle	Hwang *et al*. (2001)
C. riparius	Fenitrothion	Emergence	Choi *et al*. (2002)
C. riparius	Pirimiphos methyl	Emergence	Crane *et al*. (2002)
C. riparius	Ethynylestestradiol/bisphenolA	Emergence/reproduction	Segner *et al*. (2003)

changes occur earlier and may truly act as an “early warning” (Depledge and Fossi, 1994). This kind of approach has recently been conducted in *Chironomus* (Choi *et al*., 2002; Crane *et al*., 2002).

In the field, chemical pollution often occurs as a complex mixture of pollutants and this may impede the prediction of pollutant effects. In this context, the measure of multiple biological parameters presents several advantages. It is fundamental to accumulate data at different levels of biological organization in order to fully understand the effect of toxicants on organisms. Furthermore, the measure of population−level parameters may facilitate the interpretation of the data at the lower biological levels (Atienzar *et al*., 1999). In situ application of the multilevel biomarkers approach will help develop a better understanding of the ecological consequences of low−level environmental contamination in the field, and *Chironomus* system provides a promising biological model system to address these approaches.

CONCLUSION

A fundamental challenge in ecotoxicology is to

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link the presence of a chemical in the environment with a valid prediction of hazard for biota. A biomarker-based approach may help to resolve this difficulty by providing a direct measure of toxicant effects in the exposed species (Dickerson *et al*., 1994). Understanding molecular and biochemical effects enhances our ability to assign causal linkage to effects at higher levels of biological organization and to predict effects of chemicals based on similar molecular interactions with biomolecules. To better diagnose environmental quality, multilevel biomarkers-based approach, which permits better understanding of the impact of pollutants on organisms, should be implemented in environmental monitoring procedures. Moreover, the interconnections between ecologic heath and human health should not be overlooked. What is needed, in the future, are new and innovative approaches that integrate effects across different levels of biological complexity and provide a clear understanding of all the hazards posed by environmental pollution, not only to ecological systems but for human health as well.

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