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The role of biomarkers in environmental assessment (2). Invertebrates

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The potential use of biomarkers in ecological risk assessment is explored. The biomarker concept, initially developed to form a basis for studies at the individual/population level, is extended to include community and ecosystem level studies. A strategy is outlined in which biomarkers might be used to assess chemical exposure *and* the cumulative, adverse effects of toxicants on biota *in situ*. Protocols for identifying communities, species and populations most at risk are described. The role of biomarkers in the evaluation of the effectiveness of remedial action to alleviate pollution is discussed. It is proposed that, in addition to biomarker measurements on samples obtained from organisms at field sites, biomarker screening tests should be initiated in the laboratory with a range of new chemicals and organisms relevant to the field sites thought to be at risk. This will help to establish links between laboratory-based testing and adverse effects *in situ*.

Keywords: biomarkers; environmental assessment; community level; ecosystem level.

Introduction

It has been estimated that more than 100 000 chemicals are now in regular industrial use and are therefore potential contaminants and pollutants of the global ecosystem (Maugh 1978). In attempting to create an effective management strategy, ecotoxicologists are faced with the following problems.

(1) Ranking the relative toxicities of chemicals to natural biota.
(2) Predicting the pathways, fate and final concentrations of specific chemicals in different ecosystems.
(3) Predicting the ecological damage that might ensue as a result of the accumulation of particular concentrations of chemicals in biota.
(4) Setting safe, achievable and verifiable limits for chemicals in different ecosystems.

The package of procedures used to evaluate the relative risks of chemical toxicity from raw material acquisition to ultimate disposal is referred to collectively by the terms 'life cycle assessment' (LCA); (Fava *et al.* 1993). Interestingly, whilst LCA is currently attracting a great deal of attention, it is a procedure whose value is severely limited by its most critical component – namely ecological risk assessment. For the purposes of the following account ecological risk assessment is defined as: 'the procedure by which the likely or actual adverse effects of pollutants and other anthropogenic activities on ecosystems and their components are estimated with a known degree of certainty using scientific methodologies.'

Specific limitations of current environmental management procedures have been highlighted by numerous authors. In particular, the extent to which laboratory tests alone are or ever will be capable of predicting either the likely exposure or the effects of chemical pollutants on ecosystems and their components has been questioned (Cairns 1983, 1986; Kimball and Levins 1985; Ryder and Edwards 1985; Cairns and McCormick 1992; Depledge 1992). Other deficiencies of current procedures include the failure to investigate interactions among pollutants, the influence of environmental factors on pollutant toxicity and gradual, pollution-induced changes in ecological relationships over extended time periods (several years). Also, little attention has been paid to genotypically or phenotypically determined interpopulation differences in susceptibility to pollutants or to biogeographical differences in pollutant toxicity related to differences in climate and the inherent sensitivity of species in various climatic regions. Many of the pollutants that have already accumulated in the environment are not considered at all by current management procedures.

Ecological status and prospective risk assessment

All ecological risk assessments assume that for each community or ecosystem, there is a range of conditions associated with normal, sustainable functioning (analogous to homeostasis in an individual organism) and that there is a quantifiable risk that the community or ecosystem will depart from the normal range of conditions when pollutant chemicals are added. There is also an assumption that certain qualities should be protected and that deviation outside the normal range of conditions is a threat to ecosystem structure and/or function (Cairns and McCormick 1992). Cairns (1977) refers to the normal structural and functional characteristics of ecosystems as their 'ecological integrity'. Whilst Suter (1990) criticized this concept on the grounds that it is too vague to be subject to formal quantitative analysis, Cairns and McCormick (1992) successfully refuted the accusation by pointing out that most of the components that are encompassed by the term (such as species diversity, population dynamics, nutrient cycling rates, etc.) are indeed quantifiable and can be used to measure ecosystem well-being. This latter assumption underlies much of what follows.

New approaches: the use of biomarkers

The role of environmental management is to fulfil one or more of the following objectives.

(1) To minimize threats to human health (from contaminated food or water or from the loss of vital resources such as agricultural land, fish stocks, etc.).

(2) To ensure that ecosystems are self-sustaining (or that the rate of change from one set of ecosystem characteristics to another proceeds at a natural rate).

(3) To ensure that particular species do not decline (the reasons for protecting the well-being of certain species may be commercial, recreational, aesthetic or biological).

These are very demanding objectives and it is unlikely that any one scientific approach will be able to fulfil all of them. Nonetheless, the identification of specific molecular, biochemical, physiological and behavioural changes in populations of animals and plants following pollutant exposure does appear to offer considerable promise (McCarthy and Shugart 1990; Cairns and McCormick 1992; Depledge 1992; Peakall and Shugart 1992;

Fossi and Leonzio 1993). The use of these so-called 'biomarkers' to evaluate pollution hazards has noticeably increased in the past few years. Indeed, the biomarker approach has now attracted the attention of international regulatory agencies as a new and potentially powerful tool for detecting and documenting exposure to and the effects of environmental contamination (Giesy *et al.* 1988; McCarthy and Shugart 1990).

Various definitions of biomarkers have been proposed, but here we use the following broad definition 'a biological response to a chemical or chemicals that gives a measure of exposure and sometimes, also, of toxic effect'.

A working terminology has been etablished to facilitate biomarker discussions. For convenience, ecotoxicological biomarkers may be assigned to one of four classes: class 1, exposure biomarkers, class 2, effect biomarkers, class 3, exposure/effect biomarkers and class 4, latent effect biomarkers. Examples of each class were provided by Depledge (1993).

The biomarker approach was developed initially to chart the responses of individual organisms to increasing pollutant exposure and stress (Depledge 1989, 1993; Depledge *et al.* 1992). It is assumed that a healthy individual exposed to increasing pollutant loads will suffer a progressive deterioration in health which is eventually fatal. Early departures from health are not apparent as overt disease, but are associated with the initiation of biochemical and physiological responses. When these compensatory responses are activated, the survival potential of the organism may already have begun to decline because the ability of the organism to mount compensatory responses to new environmental challenges may have been compromised. If an organism has acquired a pollutant load that cannot be tolerated, detoxified or excreted, then pathological processes will result in the development of overt disease and, finally, death. Nonetheless, even in individuals that are no longer able to compensate for pollutant toxicity, if conditions improve sufficiently, they may still be able to recover as repair mechanisms continue working to restore compensatory responses. In other words, an organism can return from a diseased to a healthy state (Depledge 1989). Biomarkers raise the possibility of determining where an organism is located on this continuum and so potentially offer a warning of early, reversible pollution-induced departures from health (see Depledge 1992, 1993 for further details).

So far, biomarkers can be identified that indicate that organisms have been exposed to stress (general biomarkers; Mayer *et al.* 1992) and in some cases, even specific stressors (metallothionein responses following metal exposure (Benson *et al.* 1990), acetyl cholinesterase inhibition following exposure to certain organohalogen pesticides (Mayer *et al.* 1992), etc.). Whilst signalling that an exposure has taken place, such biomarkers (class 1 in the classification given above) contribute little to the prediction of the direct consequences for the organism or population in question. For this to be possible, a particular biomarker response should, if possible, be related to a given degree of impairment of growth or reproductive output or energy utlization which directly affects the survivorship of the organism and which can be attributed to exposure to a known amount of the specific pollutant (class 1 type).

Linking the responses of individuals to higher level effects

The analogy that can be drawn between the sequence of responses of individual organisms to increasing pollutant loads and the responses of populations, communities and whole ecosystems to pollutants is obvious. Risks to the ecosystem and its compo-

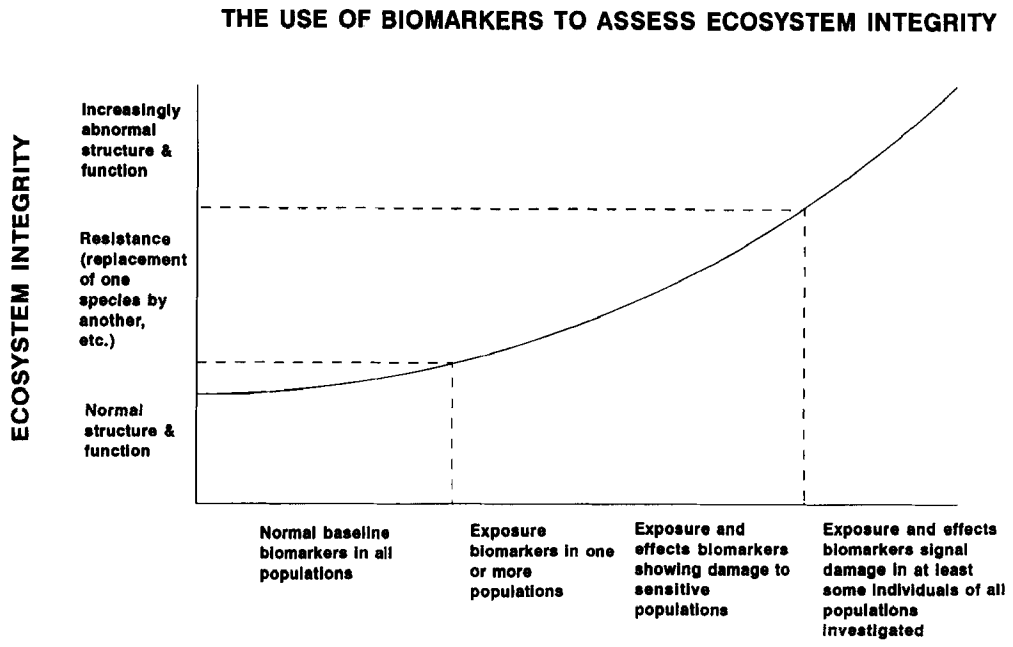

Fig. 1. The use of biomarkers to assess ecosystem integrity.

nents are expected to increase as the amount of pollutant entering the system increases. Furthermore, there will be a degree of self-compensation in each ecosystem which will tend to preserve structure and function to some extent. This is embodied in the concept of ecosystem 'resistance' as expounded by Webster *et al.* (1975) and Harwell *et al.* (1978), which is analogous to the 'compensatory responses' exhibited by individual organisms exposed to pollutants (Depledge 1989). A resistant ecosystem may show little change in structure or function if, for example, loss of one or more populations from the ecosystem following pollutant exposure is associated with replacement by alternative species that serve the same role (Kelly and Harwell 1989). However, should key species be lost or their well-being sufficiently impaired such that ecosystem structure and/or function are affected, then the ensuing ecosystem change shows that ecosystem resistance has been overcome (Fig. 1). Interestingly, the replacement of sensitive species in ecosystems by more tolerant species without significant changes in ecosystem structure and function could in itself be interpreted as an early warning of a pollutant impact if loss of the species can be directly attributed to exposure to a particular chemical. If biomarkers were to be used to measure toxicity in a sensitive species, population decline might well be detected at an even earlier stage. This illustrates an important principle, namely that monitoring changes in populations of sensitive species might provide a valuable insight into the status of the whole ecosystem.

There are a number of caveats worth mentioning with regard to Fig. 1. For example, it is not known at present whether a linear model is suitable for describing ecosystem decline. Also, the situation may be further complicated in ecosystems passing through

successional changes (i.e. they are never structurally or functionally 'stable'). These matters deserve attention but as yet, other better models have not been proposed.

A protocol for the use of biomarkers in ecological risk assessment

Ecological risk assessment consists of two components – assessment of current ecosystem status and prediction of future status. With regard to the former, any measurement that is made must clearly indicate how far the ecosystem under investigation has departed from normal conditions and for what reason. With regard to the predictive component, this necessitates knowledge of how populations comprising the ecosystem will respond to a given pollutant load. This can perhaps be estimated to some extent from laboratory biomarker studies and from databases amassed from investigations in other ecosystems with similar characteristics. Such a database is currently being assembled (D.B. Peakall, personal communication).

A protocol for the use of biomarkers in ecological risk assessment was formulated by Fossi and Leonzio (1993). The approach focused on establishing the risk to key components in the ecosystem, the inference being that if these components are adversely affected, the ecosystem structure and/or function (ecological integrity) will be at risk. Their strategy is developed and reformulated below.

Three phases of investigation are envisaged which constitute a sequence moving towards the acquisition of increasingly precise data.

Phase 1. Identification of ecosystems at risk

This involves the identification of potential pollutants, pathways and fate together with recognition of critical populations and communities in the ecosystem under study.

Complex interactions among polluting molecules and the ecosystem should be investigated in an interdisciplinary study using a database evaluation of regional features, analysis of diffusion models, chemical analysis and studies of biotic communities (Fossi and Leonzio 1993). A selected suite of general exposure biomarkers would also be utilized in an initial screening of a broad range of invertebrates to detect pollutant exposures (the justification for focusing on invertebrate species is discussed below). An estimate of how much biomarker values differ among species for a given concentration of the pollutant in the environment may aid the identification of the species at risk.

It is important to add here that the biomarker approach is not a replacement for conventional assessment techniques, but is an important supplementary approach of great ecological relevance. Ecological research methods based on the evaluation of the general state of the population (birth rate, mortality, fertility index, relationship between ages) are indispensable for interpreting links between biochemical and cell changes (biomarkers) and adverse effects on populations and communities.

Phase 2. Identification of critical species and target populations

Obviously, it is not feasible to perform comprehensive biomarker studies on all the components of an ecosystem. Identification of the most important populations is therefore necessary. Once the general extent of pollutant exposure has been assessed more specific effects of pollutant toxicity can be examined using extended suites of exposure and effects biomarkers (classes 1–4) in a limited range of species occupying different trophic levels and ecological niches; in this way *in situ* verification of the adverse effects

of a chemical or mixtures of chemicals can be obtained. The biomarkers may also illuminate previously unsuspected chemical and/or natural stressors in the study area or reveal that damage has been caused by a pollutant that has since degraded and is no longer detectable by residue analysis. Unless serial baseline studies have been carried out, the assessment of impact in the study area will have to be based on a comparison with biomarker responses determined in other control areas supplemented with data obtained in the laboratory where responses to particular chemicals can be correlated with physiological status and fitness parameters. It is important that at least some of the samples collected for biomarker analysis are also subject to residue analysis and, if possible, genetic analyses aimed at detecting alterations in gene frequencies within populations. This will provide links between biomarker responses, chemical exposures and long-term effects such as the evolution of tolerant populations at chronically-polluted sites.

Species of invertebrates occupying critical trophic positions (e.g. top predators) should be investigated next, the rationale being that interspecific differences in susceptibility to chemical toxicity may be pronounced (see Mance 1987). For example, it is well known that different species exhibit different levels of tolerance to liposoluble xenobiotics. In some cases, this may be due to differences in the handling capacities of mixed-function oxidase systems which are then reflected in varying abilities to survive in highly polluted environments (Fossi and Leonzio 1993).

Phase 3. Predicting the likely impact of chemical pollutants

Prediction of the potential of known amounts of specific pollutants to perturb (or further damage) ecosystems can also be aided by the use of biomarkers. It is proposed here that combined laboratory and field biomarker screening tests be evaluated as a means of etablishing a firmer scientific basis for extrapolating from laboratory data to real environments. This might comprise of the following.

(1) Selection of a range of invertebrate species from diverse phyla that exhibit different feeding strategies and that are present in the ecosystem in question. Sample populations of these organisms should then be exposed to a range of concentrations of the test chemical in the laboratory.

(2) Measurement of a suite of biomarkers (biochemical, physiological and behavioural) to assess responses to and toxicity of the test chemical should then be performed. Biochemical biomarkers should reveal the type of detoxification mechanisms induced by the chemical whilst physiological and behavioural biomarkers will signal exposures resulting in adverse effects at the level of the whole organism (such as altered scope for growth, loss of endogenous behavioural rhythmicity, etc.). They will also permit time relationships between chemical exposure and biomarker responses to be established.

(3) Residue analysis of the test organisms should be carried out to relate biochemical biomarker responses in specific tissues to tissue concentrations of the test chemical or its derivatives.

(4) If the test chemical has been released into other similar ecosystems and biomarker responses have been measured *in situ*, then results obtained in the laboratory test can be compared with the database compiled from field tests.

(5) Once the test chemical has been evaluated and safe concentrations determined, the biomarker approach offers the possibility of genuine validation of the test procedure. For

example, if the test chemical is allowed to be released into an ecosystem, the same biomarker analyses that were carried out in the laboratory can be performed on animals taken from the ecosystem under study. Moreover, tissue residue analyses can be carried out on field samples. In this way, it is possible to verify that the chemical in question, at a given concentration, induces the same biomarker responses in the field as it does in the laboratory. If not, then there is a good basis for establishing whether animals *in situ* are more or less sensitive to the pollutant in the field than they were in the laboratory and by how much.

Based on the database of biomarker responses that will accumulate for a diverse range of organisms subjected to varying degrees of pollutant exposure both in the laboratory and *in situ* it may be possible to relate the extent of biomarker responses to the degree of ecological deterioration at any given site. If pollutant concentrations in the field are then held below concentrations that do not produce adverse biomarker responses in a significant proportion of the test population, then protection of the ecosystem should be ensured.

Other important considerations in the measurement of invertebrate biomarkers in ecological risk assessment

Having dealt with the major conceptual considerations regarding the use of biomarkers in ecological risk assessment it is important to consider several other practical matters. The three greatest challenges posed by the approach outlined above are that it is as yet impossible to determine what proportion of a population and which individuals must be adversely affected by pollutants before decline in the population ensues (Depledge 1990). Secondly, it is not clear how environmental and biotic factors will modify biomarker responses to pollutants relative to those seen in controlled laboratory conditions. Thirdly, the establishment of dose–response relationships between pollutant exposure and biomarker responses may be very complex indeed. These issues are currently being addressed by our research groups.

Why perform biomarker studies on invertebrates?

The historical development of the biomarker approach can be seen to have had a strong link with medicine and vertebrate biology (NRC 1987). However, biomarker measurements are equally feasible in invertebrate samples. There are several reasons why in ecological risk assessment, studies on invertebrates are preferable. For example, invertebrates represent 95% of all animal species (Barnes 1968). They are major components therefore of all ecosystems and are found occuyping all trophic levels. Invertebrate populations are often numerous, so that samples can readily be taken for analysis without significantly impacting upon population dynamics. There are also ethical and legal considerations which favour their use (for example, licences are not required for invertebrate work and dilemmas associated with destructive analysis of large vertebrates are reduced).

Increasing knowledge of the biochemistry and physiology of invertebrates (see, for example, Livingstone 1991; Stegeman *et al.* 1992) now permits reasonable interpretation of biomarker responses in terms of the threat to the well-being of individual organisms.

Analytical procedures and databases

To be confident in assessments based on biomarker measurements it is absolutely vital that analytical procedures should be reliable and reproducible and that the range of inherent variability in the biomarker measuremetns in healthy organisms is well known. Precise quantification of biochemical biomarkers in easily accessible body fluids, tissue samples or of physiological and behavioural biomarkers and establishment of 'normal' reference values are integral parts of the biomarker approach. Adding this information to a database can help to provide the basis for detection and monitoring of exposure to pollutants and when screening for toxicity and disease.

Persistence of biomarker responses

Many biomarker responses are transient. An exposure to a pollutant may elicit a response that lasts for a matter of hours. However, some biomarker responses persist for weeks or months with continued exposure and others may be detectable throughout the lifetime of the organism. As alluded to earlier, it is vital to establish in advance what duration of response can be expected. This is important because confidence must be gained that the absence of a biomarker response among the individuals of a population indicates that the population is not exposed to or affected by a pollutant. If a biomarker response occurred but then receded despite continued exposure or adverse effects, it may be misinterpreted and lead to the conclusion that the population and indeed the ecosystem, were unaffected.

Interindividual variability in biomarkers responses

One of the most difficult factors to deal with in making ecological predictions based on biomarker measurements is to take account of interindividual differences in response within each population of organisms studied (Depledge 1990). Among the individuals tested, some will exhibit a weak biomarker response whilst others will exhibit a very marked response. This could arise because the population was not homogeneously exposed to the chemical stressor. In natural ecosystems, this is very likely as changes in physicochemical and biological characteristics often vary over very small distances. Such heterogeneity will then be reflected in small-scale differences of the bioavailability of pollutants. However, an equally important source of variability may be inherent differences in the morphology and biochemical/physiological status of exposed organisms. Size, age, genotype, ontogenetic stage, environmental conditioning, etc., are all important sources of interindividual differences in response (Depledge 1993).

If biomarker responses can be monitored in a number of key components of ecosystems and confirm that the physiological condition (and perhaps Darwinian fitness) of a significant proportion of those organisms sampled is impaired due to exposure to specific pollutants (either singly or in combination), this should be viewed as a clear signal that remedial action is required.

The special case of non-destructive biomarkers

Biomarker test procedures are often limited by the size of the tissue sample available for analysis or, if physiological measurements are being considered, by the site of the organism to which transducers can be attached. With regard to invertebrates, their small size often renders measurements on individuals difficult and usually requires destruction

of the whole organism (and perhaps even pooling of several organisms) when biochemical analyses are required. Fortunately, such organisms are often readily obtained and are so plentiful that destructive techniques, although not desirable, offer a pragmatic solution without affecting *in situ* population numbers significantly. With many of the larger invertebrates it is possible to obtain small tissue or body fluid samples without significantly damaging or affecting the well-being of the whole animal. The advantages of this non-destructive approach are as follows:

(1) Non-destructive sampling allows repeated measurements to be made on the same individual (providing that sampling frequency is not so high as to affect the biomarker response itself). Such repeated measurements may allow an individual to be used as its own control.

(2) The persistence of biomarker responses in relation to pollutant exposure can be more accurately assessed.

(3) Non-destructive sampling does not involve loss of animals from the population. This is especially important if a rare or threatened species is being investigated.

(4) Long-term studies may be carried out on individuals as they pass through different ontogenetic stages.

(5) Non-destructive studies facilitate the detection of interindividual variability in response to pollutant exposure.

Blood, urine, faeces, exoskeleton, shells, tissue and organ biopsies, may be suitable for a broad range of biochemical biomarker measurements. Non-destructive physiological biomarkers include oxygen consumption, ventilation, cardiac activity (and the relationships among them), urine output, faeces output, body temperature, protein turnover, growth and reproductive output.

The use of biomarkers in improvement assessments and in bioremediation

Biomarkers may in future serve a role in bioremediation – the active restoration of damaged ecosystems. The success of clean-up procedures and the effectiveness of recolonization by various organisms should be evident in the return of biomarker values to within predetermined normal ranges. The use of biomarkers in this way is analogous to their use in medicine to chart the effectiveness of treatments for various diseases and the return of the patient to health. As pointed out above, the advent of techniques permitting non-destructive sampling of body fluids and tissues has the advantage that it permits the health status of individual organisms to be followed through time. In the context of bioremediation, such serial sampling offers the possibility of following the recovery of organisms. In turn, this permits the effectiveness of bioremediation measures to be assessed. It may also allow a mechanistic understanding of population recovery to be gained. For example, it is not always clear whether the recovery of a population in a polluted area is due to the recovery of organisms that had previously been adversely affected or to the success of individuals colonizing the formerly polluted area from elsewhere or some combination of the two processes. Biomarkers might be used to differentiate between organisms that have suffered pollutant exposure but survived and those which have never been exposed to the pollutant. Changes in the relative proportions of these individuals could provide a valuable means of establishing how recovery takes place.

Numerous studies have dealt with the depuration of pollutants following return of organisms to clean conditions (Phillips 1980; Livingstone 1991). However, it should be noted that there is often a poor correlation between tissue residue loads and the extent to which organisms recover from pollutant exposure. For example, when the shore crab, *Carcinus maenas* is acutely exposed to copper (0.75 mg l^{-1}), the heart rate declines and becomes increasingly variable and metallothionein concentrations in the gills and midgut gland increase as tissue concentrations of copper rise (M.H. Depledge, unpublished). Within minutes of transfer to clean conditions, the heart rate rapidly returns to normal so even a little depuration of copper occurs in the short-term. However, by the time that copper concentrations have returned to almost normal values, the heart rate, oxygen consumption and other physiological processes sometimes become highly abnormal, often as a prelude to death (Depledge 1984). Apparently, in some cases damage associated with pollutant exposure cannot be repaired, even though the bulk of the pollutant has been excreted. This greatly complicates the interpretation of dose–response relationships.

Conclusions

To summarize, the biomarker approach permits acquisition of information that cannot be obtained from the measurement of chemical residues in environmental and biological media. Biomarkers enable integration of pharmacokinetic and toxicological interactions in organisms resulting from exposure to complex mixtures of chemicals; they indicate the cumulative effects of toxicant interactions in molecular or cellular targets and they integrate different episodes of exposure in time and space. Different biomarkers are capable of indicating rapid responses to toxicant exposure or of providing an early warning of long-term effects or of changes in Darwinian fitness due to pollutant toxicity. The significance of chemical residues in tissues can only be assessed if detailed toxicological studies have been carried out on the target organism, relating residue levels to biomarker responses and adverse effects. Even if this information is available for individual pollutants, data is certainly not available for the complex mixtures of pollutants that occur in the real world. Thus, chemical and biomarker monitoring are complementary approaches. Another attractive feature of the biomarker approach is that it may be useful for assessing the effects of repeated exposures to single pollutants or mixtures (Depledge 1993). At present, this is seldom attempted in toxicity tests which focus primarily on lethality.

With regard to ecological risk assessment, biomarkers allow exposure to pollutants to be detected and populations especially at risk to be identified. It is proposed here that laboratory-based biomarker screening tests used with new chemicals should be developed. The data obtained can be used with a biomarker database compiled from field studies, as well as conventional approaches, in prospective risk analysis. Finally, biomarkers may permit progress associated with bioremediation efforts to be assessed.

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