Ecotoxicological Assessment of Surface Waters: A Modular Approach Integrating *In Vitro* Methods

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Summary

Today ecotoxicological evaluations of surface water quality are either based on field surveys or online biomonitoring, whereas the ecotoxicological quality of wastewater is mostly determined with standardised acute toxicity tests. In this paper we present a concept for the ecotoxicological evaluation of surface waters, where mainly in vitro tests are used for the screening of water samples, presenting the first tier of a two-tiered approach. In this first tier a battery of fast and cost-efficient test systems are used as an early warning system. Thereby, the toxic potential of water samples will be identified. This modular approach allows the exchange or addition of test systems if necessary. If a toxic potential is identified in a water sample, this sample can be investigated more thoroughly in a second tier where organisms are used. In this paper we focus mainly on the general approach and the description of the first tier.

Zusammenfassung: Ökotoxikologische Bewertung von Oberflächengewässern: Ein modularer Ansatz mit *in vitro* Testsystemen

Ökotoxikologische Bewertungen von Oberflächengewässern basieren in der Regel entweder auf Freilanduntersuchungen oder auf Online-Biomonitoring. Die Qualität von Abwasser wird hingegen meist mit standardisierten akuten Toxizitätstests untersucht. In diesem Artikel schlagen wir ein Konzept zur ökotoxikologischen Bewertung von Oberflächengewässern vor, in welchem hauptsächlich in vitro Testsysteme für das Screenen von Wasserproben verwendet werden. Dieses Screening ist die erste Stufe in dem zweistufigen Konzept. In dieser ersten Stufe werden kostengünstige und schnelle Testsysteme als Frühwarnsysteme verwendet, um das toxische Potential von Wasserproben zu identifizieren. Dieser modulare Ansatz erlaubt den Austausch und die Aufnahme von weiteren Testsystemen, falls dies notwendig wird. Ist in einer Wasserprobe ein toxisches Potential identifiziert worden, so kann diese Probe in der zweiten Stufe gründlicher untersucht werden. In dieser zweiten Stufe werden Organismen verwendet. In diesem Artikel werden einerseits das Konzept, andererseits die Auswahl der Testsysteme der ersten Stufe vorgestellt und kritisch diskutiert.

Keywords: ecotoxicological assessment, ecotoxicology, cellular test systems, surface water

1 Introduction

About 100,000 different compounds are registered on the EU-market and the number increases every year (EC, 2001). Thanks to intensive wastewater treatment, acute toxic effects caused by pollutants are nowadays rarely observed. However, chemical compounds are still inevitably released into the environment from households, agriculture, and industry. These chemicals, due to chronic exposure, still pose a potential threat to stream and riverine ecosystems. As studies on mussels and fish have shown, even low pollutant concentrations in surface waters can cause severe sublethal damages (Fent, 1996; Vos et al., 2000).

In surface waters we are confronted with a large variety of pollutants and with the problem of mixture toxicity. In an unknown mixture of pollutants chemical analyses can only detect a limited number of compounds. Thus mixture toxicity (including synergistic and antagonistic effects) is impossible to predict by chemical analysis in environmental samples only. Therefore, effect-based test systems have become essential for a hazard assessment, as it has also been shown in the second revision of the Swiss Water Protection Law (Bundesgesetz über den Schutz der Gewässer (Gewässerschutzgesetz), 1991) and the European water framework directive (EC, 2000). In these directives, the protection, preservation and restoration of streams as integral ecosystems are central issues. For a holistic assessment of streams, chemical analysis has not only to be combined with ecotoxicology, but also with various aspects of hydrology, ecomorphology and biology.

2 The Swiss Modular Concept

The Swiss Modular Concept has been developed in order to evaluate the quali-

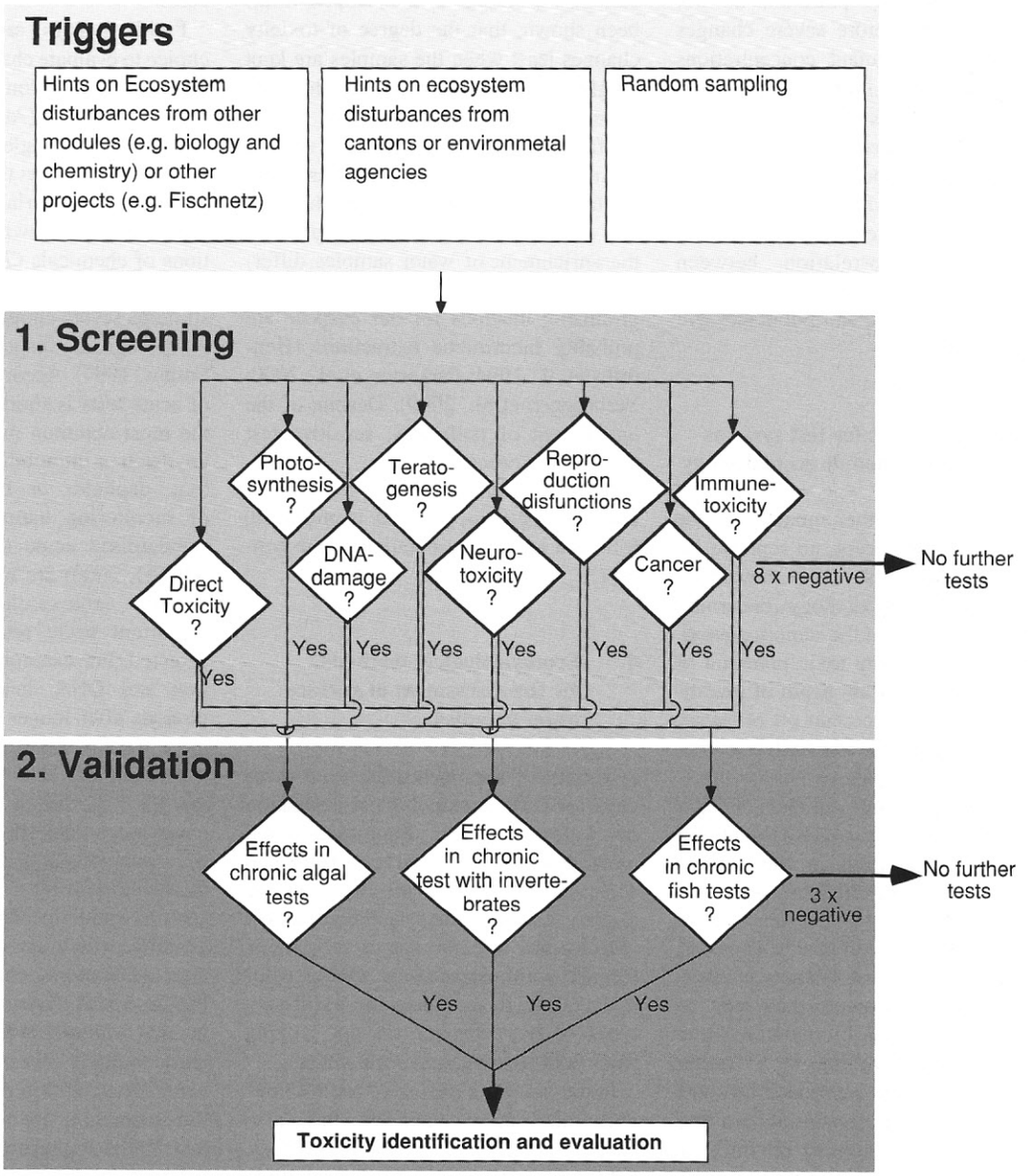

Fig. 1: A two-tiered approach to the module ecotoxicology for the assessment of surface waters.

ty of Swiss streams bringing together all these before-mentioned aspects (Bundi et al., 2000). The biological part includes modules examining fish, macro invertebrates, higher plants and algae. In each of these modules the distribution of species and population densities are analysed. The intensity of each investigation depends on the size of the investigated area, which can vary between a stream section (e.g. 0.1 or 1 km), entire stream systems and regional/cantonal scale. While intensive analyses can be performed for the former, only rapid and cost-efficient methods can be applied in the latter case. Evaluation protocols are elaborated for each module for the different investigation procedures. The result of the biological surveys are compared with historical data, whenever possible. Statistical evaluations of this comparison lead to the status of health of the investigated community. It is, however, difficult to trace back the reasons for observed

changes in ecosystem communities. Due to natural fluctuations in ecosystems, damages caused by pollution do not become obvious before severe changes occur. At low pollutant concentrations this can signify an impact during years before the damage is obvious.

The module ecotoxicology will help to determine if toxic compounds are present in concentrations, which have the potential to cause damages in the ecosystem. Moreover the correlation between changes in the ecosystem, determined in the biology modules, and pollutants will be ascertained.

3 Requirements for test systems used in the module ecotoxicology

In contrast to the other modules of the Swiss Modular Concept, no separate investigation protocols will be developed for the module ecotoxicology concerning the different scales of the sampling areas. In order to detect the toxic potential of water samples a certain depth of investigation – meaning a certain set of tests – is necessary and these tests will therefore be used for all samples.

The analysis of large numbers of water samples causes further requirements for the test systems used in the module ecotoxicology. Cost-efficiency and rapid responses of the test systems were important criteria. Additionally, a possible automation of the test systems is desirable. As the experiments shall later be routinely performed by environmental agencies, easy handling is a further requirement. A high reproducibility and the relevance of the test results are also conditions, which have to be fulfilled. Last but not least, the aim was to avoid the use of experimental animals whenever possible.

A goal of the module ecotoxicology was to be able to discover most of the to date known relevant toxic responses with test systems fulfilling the requirements mentioned above. Endocrine disruption and DNA damage are examples for these toxic responses (Fig. 1).

Additional conditions precedent to an ecotoxicological hazard assessment are the storage of the environmental samples and the enrichment of the pollutants from the samples, due to the low pollutant concentrations in surface waters. Concerning the storage of the samples it has been shown, that the degree of toxicity changes least when the samples are kept in glass containers and when they are stored at 4°C (de Maagd et al., 2001; Geffard et al., 2001). The time of storage should however be kept as short as possible to minimise changes of the chemical and toxic properties of the sample. For the enrichment of water samples different methods are available, the most promising methods for our purpose are probably biomimetic extractions (Hendriks et al., 1994; Parkerton et al., 2000; Verbruggen et al., 2000). Despite of the enrichment of pollutants, sensitive test systems are necessary.

In the following chapter a selection of ecotoxicological approaches is presented, followed by the presentation of the concept.

4 Ecotoxicological methods for the assessment of surface water samples

A variety of approaches are used these days for the ecotoxicological evaluation of water samples. Ecotoxicological methods include field surveys, simulated ecosystem studies, acute and chronic toxicity tests (laboratory and field) and *in vitro* studies (e.g. the use of cells transformed with recombinant DNA). Biomarkers can be measured in all different types of experimental set up, ranging from field surveys to *in vitro* studies.

In the last years the use of test batteries with alternative methods has also become popular in the field of ecotoxicology (den Besten, 1998; Girling et al., 2000; Janssen, 1998; Juvonen et al., 2000; Toussaint et al., 1995). In these test batteries usually a range of organism groups and/or a variety of physiological, biochemical and/or immunological endpoints is investigated (Janssen, 1998; Juvonen et al., 2000; Toussaint et al., 1995; Triebskorn et al., 2001). The test battery presented here is of advantage since it systematically covers the relevant toxic responses.

The choice of the approach to use strongly depends on the objectives of the assessment. Some classic and new approaches will briefly be discussed below.

Field surveys are the appropriate choice to evaluate changes in populations or communities from sites suspected to be contaminated (Attrill and Depledge, 1997). Acute single species tests are being used to detect the toxic potential of wastewater, industrial effluents and other compartments showing high concentrations of chemicals (Tonkes et al., 1998). Acute refers to a condition involving a stimulus severe enough to induce rapidly a biological response (Forbes and Forbes, 1994). Accordingly, the duration of acute tests is short (up to 4 days) and the most common measured parameters are death or immobilisation of organisms (e.g. daphnids or fish). For purposes of monitoring natural water samples, standardised acute single species tests (OECD, 2000) are not adequate for the module ecotoxicology since several important toxic responses cannot be detected. For example endocrine disruption and DNA damage only become obvious after longer time periods or by more sensitive test methods.

The general rule for chronic tests is that the exposure to the chemical has to cover more than 10% of the organism's life span (Rand and Petrocelli, 1985). However, with regard to toxic endpoints such as endocrine disruption and mutagenicity, which are often only detected after generations, one has to reconsider this time span. A recommendation would be that chronic experiments should at least include investigations over two generations. Within this time of exposure the chemical or its effects may accumulate in the organism, leading to effects not seen in acute toxicity tests. Chronic toxicity tests are extremely expensive and work intensive, therefore, they cannot be considered as a screening tool for the module ecotoxicology.

In vitro toxicity tests are no substitutes for *in vivo* toxicity tests, but they are useful to provide a first screening in the process of an assessment of environmental quality. Few comparative studies on estrogenic effects, hepatic biotransformation and genotoxicity have been performed in fish. Studies concerning the estrogenic effects and the hepatic bio-

transformation show a good qualitative agreement between *in vitro* and *in vivo* results, but *in vivo* assessments have mostly proven to be more sensitive (Le Gac et al., 2001; Legler et al., 2000; Legler et al., 1999; Matthews et al., 2001; Sturm et al., 2001). Investigations on the genotoxicity of fish with the comet assay show that *in vitro* assays can also be more sensitive than *in vivo* assays. Differences in the metabolisation of the pollutants and in the DNA-repair are probably responsible for these results. Regrettably *in vivo* investigations on the mutagenicity of the substances and the environmental samples are missing (Schnurstein et al., 1999).

Until now *in vitro* tests as well as molecular biological methods have mainly been used in research projects (e.g. Triebskorn et al., 2001) and not in routine assessments. Considering most of the requirements mentioned above, *in vitro* test systems are the most desirable for the task defined in this paper. Additional advantages are: no use of animals, rapid responses, relatively low costs, ease of experimental manipulation, modest requirements of space, ready availability of test material, higher reproducibility of results, independence of systemic problems of test animals (such as parasitism) and the fact that they can be automated in many cases. In combination with modern, biochemical or molecular biological tools the sensitivity of *in vitro* test systems can be strongly enhanced. For example, the use of these tools allows to assess water samples for their potential to cause endocrine disruption within a few days by measuring the vitellogenin concentration in hepatocytes (Smeets et al., 1999) or by the use of recombinant fish cell lines or recombinant microorganisms (Ackermann et al., accepted; Routledge and Sumpter, 1996; Zacharewski, 1998).

In vitro methods have also several potential shortcomings due to the simplification of the *in vivo* situation. *In vitro* assays cannot completely reflect complex *in vivo* events, such as bioavailability and toxicokinetics of a compound and cell-cell communication and interaction are partly missing. *In vitro* assays may predict the potential toxicity of a pollutant once it has reached the target cell, but they cannot predict the final bioavailability of the substance to the cell. These aspects have always to be kept in mind when *in vitro* test systems are used. They are mainly responsible for the problems of transferability of test results from *in vitro* to *in vivo*. It should, however, be noticed that the aim of the first tier in our concept is just to predict a potential risk for organisms not to predict the effects to the organisms.

Fairly new techniques, which could also be very useful for the field of ecotoxicology, are gene arrays and proteomics (Afshari and Hamadeh, 2000; Bandara and Kennedy, 2002; Hamadeh et al., 2001; Lobenhofer et al., 2001; Templin et al., 2002). They are, however, in their infancy and still have to prove their applicability.

In vitro tests can be performed on different levels of organisation. They can be performed either with isolated biomolecules (e.g. DNA) (molecular level), or with subcellular fractions (e.g. membrane vesicles or mitochondria). Another possibility is the use of cellular systems; here either microorganisms (e.g. yeast) or cells freshly isolated from the organisms or stable cell lines derived from higher organisms (e.g. fish hepatocytes) can be used. Stem cells are already an alternative for some mammalian toxicity tests (Rohwedel et al., 2001), however, no such cells exist for aquatic organisms up to now.

The main differences between the various levels of organisation are the integration of the toxic responses, which is increasing from the molecular to the cellular level and the probability to obtain false positive or false negative responses. False negative responses are defined as no responses in the investigated test system, but toxicity at a higher level of organisation, these may for instance occur when a toxic compound is metabolically activated. False positive responses are defined as positive responses in the investigated test, but no toxicity at higher level of organisation. False positive results may be obtained if a repair system is active or if a compound is detoxified.

For the first tier of our ecotoxicological hazard assessment concept, false positive results are less of a problem than false negative ones, because the error will be detected in the next tier. In contrast, false negative results will not be investigated further and will only be detected when damages have caused severe changes in the ecosystems or the toxicants are discovered in the module chemistry. Therefore it is most important to avoid false negative results on the screening level.

Nearly all toxicity mechanisms have a molecular basis. (Exceptions are behavioural changes due to impregnation during early life stages, which will certainly be difficult to trace with ecotoxicological methods.) The molecular basis of damages is very similar between organisms; therefore one should theoretically be able to detect nearly all damages using molecular test systems (with the exception of the behavioural changes). Of course hundreds or even thousands of different test systems would be necessary to detect all damages with molecular test systems.

A step towards lower numbers of necessary tests and therefore a more realistic approach is the use of subcellular test systems, where cellular subunits are used. Using this more integrated approach several molecular toxicity mechanisms can be detected in one test system (e.g. several mechanisms of membrane damage can be detected with membrane vesicles (Escher et al., 1997). The possibility to obtain false negatives is relatively low as no cellular repair systems are active and these cellular components are well conserved in all organisms, but the number of tests that has to be performed in order to cover all possible toxic responses is still huge, a test battery on this level would therefore be rather cost-intensive and it would still lead to many false-positives.

A further integration of toxicity mechanisms is thus necessary and can be reached by the use of cellular test systems. Here, different cell functions integrate a series of molecular defects (e.g. inhibition of photosynthesis). However, several questions and problems arise: a) the choice of the cell to be analysed; which organism and which tissue shall be used. b) Cells have repair mechanisms, which repair parts of the damaged biomolecules and, therefore,

not all damage will be detected, but on the other hand this clearly decreases false positives. c) Isolated cells loose some of their typical features and the loss of tissue specific properties (e.g. cell-to-cell signaling) is even more elevated when permanent cell lines are used (Ashby, 2000; Segner et al., 2001). d) The interaction between different cell types and tissues cannot be examined, which can be important for studies of hormonal and immunological responses. e) The metabolism of compounds is only active in some tissues, as in the liver. In other tissue cultures liver extract has to be added in order to simulate metabolism. However, in recent years fish cell lines with a high metabolic activity have been cultivated (Leguen et al., 2000).

In order to study toxic responses, which depend on tissue interactions a further integration is necessary. The use of embryos is in this case an alternative. The observation of the embryo development is the only possibility to detect teratogenicity. An additional advantage is that no artificial metabolic activation is needed and that the effects on all tissues can be examined at once. Test systems using embryos share several characteristics as relatively low costs and modest space requirements with *in vitro* test systems. If embryos are used, the duration of the test should, however, be kept to a minimum, in order to complete the experiment before the development of the nervous system is too advanced and the organisms are able to sense the pain caused by the exposure and handling. Schulte and Nagel have developed a protocol for a 48 hours test with zebrafish eggs (Schulte and Nagel, 1994). Tests with zebrafish eggs are already used as *in vitro* test systems for the assessment of water, wastewater and sludge (DIN, 2000).

In vitro test systems and embryo tests (which belong according to the EU legislation to *in vitro* test systems) are of special interest for screening purposes. The application of test batteries allows examining simultaneously large sample numbers for different toxic responses with ease, rapidity and at relatively low cost.

At the organism level, the risk of false negatives cannot be excluded as the sensitivity of species towards chemicals varies (DeLorenzo et al., 2001; Slooff et al., 1983; Vittozzi and De Angelis, 1991). This is, however, also a problem with the nowadays currently used toxicity tests, which are recommended by the OECD (OECD, 2000). Ideally species, which have proven to be more resistant to a variety of chemicals than other species – as it is the case for the fathead minnow – should be avoided (Vittozzi and De Angelis, 1991). The enrichment of the samples as well as the use of sensitive methods shall help to reduce this problem.

5 A new concept for the ecotoxicological assessment of surface waters

Based on requirements and available ecotoxicological methods discussed above, the following concept has been developed (Fig. 1): Water samples will be examined in a two-tiered approach. In the first tier, mainly *in vitro* test systems are used to determine the toxic potential of the water samples. In a second tier it is investigated if the observed toxic effects also manifest themselves on the level of whole organisms. The experimental setup and organisms to be analysed are chosen based on the toxic mechanism detected in tier 1.

Trigger: Spot checks shall be made to identify locations that are exposed to a higher risk. Additionally assessments may be triggered by indications on discharges from the chemistry module or signs of biological disturbance seen in the biology modules. Hints from local environmental agencies shall also been pursued.

Tier 1: The toxic potential of a water sample is mainly assessed with single-cellular organisms. The test battery proposed in this first tier consists of various tests, which cover the majority of relevant biological responses to toxicants. This modular test battery can be adapted to changing needs and technical capabilities. Individual tests may be replaced by newer or more sensitive tests, or the number of tests may be expanded in order to include toxicity mechanisms which were previously unknown, or where no screening test methods existed.

In addition to direct toxicity, the test battery is also expected to uncover more subtle toxic effects, including effects on photosynthesis, DNA damages, disturbances in reproduction and teratogenicity. Direct toxicity is assessed using two commercially available (Lumistox® (DIN, 1998a,b) and FluoroMetPLATE™ (Jung et al., 1996) bacterial bioassays. The standardised Lumistox® test is more sensitive to organic pollutants while FluoroMetPLATE™ is particularly sensitive to heavy metals (Jung et al., 1996). Single-cellular algae serve as representatives of algae and plants and are evaluated for inhibitory effects on the photosynthetic apparatus by measuring chlorophyll fluorescence. DNA damage is assessed with a combination of a bacterial test system (e.g. umuC test) (Oda et al., 1985) and a test system using cells of higher organisms (comet assay) (Devaux et al., 1997). Estrogenic, anti-estrogenic, androgenic and anti-androgenic effects – as part of the reproduction disturbance – can be detected using recombinant yeast cells (Mak et al., 1999; Routledge and Sumpter, 1996). The metabolic activation and inactivation of chemicals occurring in higher organisms can be imitated by the use of S9 extract, as it is done since a long time for the identification of mutagenic compounds (De Flora et al., 1984; Maron and Ames, 1983). The measurement of further hormone responses (e.g. progesterone) will soon be available (García-Reyero et al., 2001). Teratogenicity will be measured in fish embryos. For the toxic responses of neurotoxicity and immunotoxicity no simple test systems, which can be used in a screening battery and can cope with the complexity of these toxic responses, are known yet. Therefore these toxic responses cannot be analysed for the moment, but they are planned to be integrated later on, for the moment we can only rely on detection in the biological modules. Research in these fields is strongly encouraged.

If all tests in tier 1 are negative, the water samples can be considered harmless, and no further tests are performed.

If one of the toxic responses is positive, a confirmation of the result in a second tier has to be considered, especially for newly developed test systems, where the validation is still poor. Results of well-established test systems by contrast might not need further testing.

Tier 2: Only the group of organisms (algae, invertebrates, or fish), which is likely to be affected by the positively responding toxicity mechanism in tier 1, is subjected to further tests in tier 2. Thereby unnecessary testing on animals can be reduced to a minimum. For example, an increase in photosynthesis inhibition calls only for a chronic toxicity test with algae. Test organisms for a particular group should be selected such that they are typical representatives of the stream from which the water sample was obtained. It should, however, be a species, which is relatively sensitive and can be maintained in the laboratory. These organisms are then used in long-term tests focusing on the effects observed in tier 1. During a first period experts will decide the choice of the species as well as the specific test set up, as the case arises. This experimental phase shall lead to the development of a decision-support tool, which can be useful for the experimental design later on.

No toxic response in tier 2 indicates that the water sample exhibits a toxic potential that is not manifested at the organism level. In this case, no further investigations will be performed for the moment; however, the site will be investigated later again (e.g. after 1 year). If the toxic effects of tier 1 are confirmed, the compounds responsible for the damage need to be identified by a toxicity identification evaluation (TIE) procedure in order to devise appropriate mitigation measures. Extended protocols for TIE procedures have been published by the U.S. EPA. (EPA, 1991; 1993a, b). This toxicity identification is not part of the module ecotoxicology. If the observed toxic effects are the result of an interaction between two or more compounds, identification can become difficult or even impossible. In such cases, one must resort to a more pragmatic solution by reducing contaminant levels across the board.

6 Problems to solve

The module ecotoxicology is still under development and several problems will have to be solved before this approach can be used in a routine assessment of surface water samples: For several toxic responses (e.g. neurotoxicity, immunotoxicity) test systems for fish, which could be used in a screening assay, are still lacking. For invertebrates the case is even more problematic. For some of the test systems suggested for tier 1, it has yet to be shown that the toxic responses observed in the test system correspond to toxic responses in organisms. Unfortunately this link has often been neglected during the development of *in vitro* test systems.

It has also to be decided how strong the toxic responses in tier 1 and also in tier 2 have to be in order to discriminate between positive and negative responses. In the literature the general tendency goes in the direction of EC_5 (concentration with 5% of observed effect) values instead of LOEC (lowest observed effect concentration) or NOEC (no observed effect concentration) (Chapman et al., 1996; Laskowski, 1995; Murell et al., 1998). Additionally test systems for endogenous species for the second tier have to be developed.

It is not the aim of this project to develop new test methods or to validate methods, in this project we want to establish a screening test battery for the ecotoxicological evaluation of surface water samples using available methods. However, we strongly want to remind ecotoxicologists that the validation and standardisation of existing tests is as necessary as the development of new test systems especially for the detection of immuno- and neurotoxicity.

7 Conclusion

The proposed test battery of tier 1 involves a general screening for different toxic responses. The main objective is to identify water samples possessing an ecotoxic potential that may damage aquatic organisms. The idea is thus not to take the results from the screening tests and to project them up with extrapolation procedures to higher levels of biological organisation and to determine the health of the ecosystem, but to use the screening data as a warning tools in order to direct more effect specific testing. One of our main goals during the establishment of tier 1 was to avoid the use of test animals in this screening step, which was successfully done. We are aware that still many questions need to be answered and problems need to be solved. We are, however, convinced that this concept is an alternative screening method for environmental samples and that it will contribute to a reduction of test animals in other screening tests. This is especially important considering the increasing demand of screening tests for surface water, wastewater and chemicals. In the long run animal experiments in the second tier might be further decreased due to more experience with the test systems used in the first tier.

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