Workshop 5.10
Ecotoxicity – applying the Three Rs

Lecture

Incorporating *in vitro* methods into aquatic environmental bioaccumulation predictions

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The bioaccumulation ("B") of a chemical in aquatic organisms can be determined by exposing fish to a chemical via water and/or food, and monitoring its uptake and loss. The standard method requires numerous animals, excessive labor, and costly analytical methods. So, a tiered approach is proposed for the evaluation of bioaccumulation in fish. *In silico* methods may be used to predict bioaccumulation potential. The current "B" models are based solely on chemical lipophilicity, and several models ignore key parameters like metabolism. Methods are being explored to incorporate absorption, distribution, metabolism, and excretion (ADME) properties in "B" predictions. ADME parameters can be quantified using *in vitro* methods, although few fish data are now available. A recent bioaccumulation workshop in Cincinnati, USA focused on ideas to optimise and validate *in vitro* fish methods because the quantitative relationship of *in vitro* to *in vivo* results is lacking. A key action step is for a subgroup to develop methods and choose environmentally significant test materials to compare results of *in vitro* and *in vivo* tests from different labs. Planned action steps and preliminary methods from this subgroup will be presented. This effort is expected to reduce time, labor, and animal usage for bioaccumulation testing needed to meet developing global regulatory demands.

Lecture

Applying the 3Rs in acute ecotoxicity

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Acute ecotoxicity tests on vertebrates are required by law for hazard characterisation and for environmental risk assessment of chemicals, plant protection products (PPP), biocides and veterinary medicines. While acute toxicity data on mammals and birds are only required for the registration of the active substances of PPP, acute fish bioassays needs to be performed in all cited substances and chemicals, and in some EU countries, for Whole Effluent Assessment.

3R approaches for mammals in environmental risk assessment are beneficing from the advantages reached in the process of hazard characterisation for humans, on the contrary little attention has been paid to acute oral toxicity data on birds. Almost no advancements have been made in applying the 3Rs in this area.

Most progress over the last 5 years has been made concerning the acute fish bioassays: Reductions up to 75% in the number of fish was proposed by Hutchinson and co-workers in 2001 with the concept of the threshold approach and is on the way to be peer reviewed by ECVAM. Refining the acute bioassay on adult fish by adopting the fish embryo test (Nagel, 2002) was already proposed and in some countries in the way to be adopted for WEA. A very interesting approach combining the 3Rs: I.e. replacement by fish cells, reducing using the threshold approach and refining by means of a fish-embryo test is in the way to be proposed for the expert group of the ECVAM taskforce on Ecotoxicology. My presentation will analyse the present situation and outline future prospects.

Lecture

Replacing vertebrate testing in regulatory ecotoxicology

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The public discussion on the Three Rs in animal testing mainly focuses on mammalian testing in human toxicology. Rarely it is realised that a considerable number of non mammalian vertebrates are used in regulatory ecotoxicology. This number may even increase when the new European chemical legislation will become effective by the end of this decade and respective toxicity data will be required for more than 10,000 substances.

By analysing the number of animals used in regulatory ecotoxicology it becomes evident that most vertebrates are used for fish acute toxicity testing. This test is a basic ecotoxicological requirement in most environmental regulations that are triggered by chemical's effects. The German Federal Environmental Agency has been given priority to the replacement of fish acute toxicity testing in regulatory ecotoxicology. The most promising and successful approach to replace fish acute testing is the fish egg test or fish embryo test. This test is using fish eggs instead of adult fish. This type of test has been standardised for waste water toxicity assessment and successfully introduced in the German legislation on waste water fees as an alternative test resulting in an overall reduction in the number of fish used for testing in Germany by more than 30 percent.

For the testing of chemicals the fish embryo test has been included into the current work plan of the OECD Testguidelines Programme under the lead of Germany. This method is designed to make the traditional acute fish toxicity testing obsolete and will probably be one key element of the ecotoxicological hazard and risk assessment within the new European chemical legislation.

Poster

ECVAM Key Area Ecotoxicology: Summary of ongoing activities

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ECVAM's activities in ecotoxicology started in 2001 with its workshop on "The use of fish cells in ecotoxicology" (Castaño et al., 2003; *ATLA 31*, 317-351) and was followed up with the establishment of the ECVAM Task Force Ecotoxicology (chaired by Peter Pärt, JRC, Ispra, I) in 2003.

Current activities are focused on reduction and eventual replacement of the acute LC_{50} test in fish. Thus, a new testing strategy based on the threshold (step-down) approach published by Hutchinson et al. (2003, *Environ. Toxicol. Chem. 22*, 3031-3036) for pharmaceuticals was used to retrospectively evaluate ecotoxicological data of new and existing chemical substances extracted from databases maintained at the European Chemicals Bureau and ecotoxicological data of active substances extracted from published reports for the plant protection products. The evaluation revealed that a reduction of at least 50% might be feasible (see also poster/oral presentation Jeram et al.). This new testing strategy is now reviewed by ECVAM's Scientific Advisory Committee and the Competent Authorities of the EU Member States.

Regarding the complete replacement of the acute fish test, ECVAM and its Task Force are developing a testing strategy, which is based on the use of fish cells, fish embryos and QSARs. However, all of these possible replacement methods still need to be evaluated and/or validated.

Linked to ECVAM's activities on high-throughput screening for human health effects, it is planned to establish cytotoxicity tests using various fish cell lines and determine in comparison the cytotoxic effects of the chemicals tested on mammalian cell lines.

Poster

Herring and sprat migration predictive model in the Scheldt estuary

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In this study, we developed statistical models to describe the distribution of herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) in the Scheldt estuary based on long-term using data. The influence of water quality and the characteristics of the fish assemblage were analysed using a range of regression techniques, including stepwise and linear regression. We considered fish density in response to 15 water quality factors during a 10-year period. The distribution of herring and sprat showed statistically significant correlations with the difference of water temperature in the river and at sea (DT).This illustrates the importance of temperature to distribution of herring. A connection between altering water thermal conditions and fish migratory patterns proves to be the best predictor of herring and sprat abundance in Scheldt estuary.

Poster

Variation in the energy density of herring and sprat during estuarine residency in the Scheldt

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Changes in whole body energy content of herring (*Clupea harengus*) and sprat (*Sprattus sprattus*) during estuarine residency were measured and compared in the marine part and the brackish water part of Scheldt estuary. Fish of between 4 and 10 cm were used for analysis.The average amount of energy of wet weight (EWWT) in herring and sprat in Borssele station was higher than those in Doel station in both species. Water content varied from 78 percent to 82 percent in herring, and from 77 percent to 81 percent in sprat. Energy density of wet weight (EWWT) herring ranged between 2754.1-6487.13 J/g and between 2473-6958.4 J/g in sprat during the estuarine residency. We found that the average EWWT was higher in sprat (4998.4 J/g) than in herring (4438.6 J/g). Dry mass energy densities varied between 22.5-33.7 KJ/g in herring and 23.7-33.1 KJ/g in sprat.

Poster

Biological and technological advances for ecotoxicity and human health risk assessment using zebrafish embryos

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Animal use for chemical testing and pollution assessment is an issue of serious social concern and represents an important cost-rising factor. These drawbacks are further enhanced with the application of the new chemicals testing policy (REACH) and its requirement of intensive testing and animal use reduction.

Assessment of toxicity in non-mammalian embryos is one of the most promising approaches for ecotoxicity testing, given that they provide the opportunity of testing chemicals in a real and complete developing organism in a short time, with reduced costs and avoiding many ethical constraints.

Experimentation in non-mammalian embryos is currently limited by the lack of knowledge of the biology of these species and the poor technological developments in this area. These facts result in poor embryo yields, bad embryo quality and technological handicaps that ultimately lead to difficult scalability and scarce reproducibility of the results.

ZF Biolabs (Tres Cantos, Madrid) has deepened the knowledge in the zebrafish biology to allow a high, constant and high quality embryo production. This milestone has been achieved improving key aspects of the zebrafish biology such as diet and embryo production. Besides, ZF Biolabs has developed and patented the first sorter specifically designed for zebrafish embryos.

These biological and technological advances contribute to reduce the variability associated with the use of model systems based on non-mammalian embryos, and thus support the development of reliable assays using the zebrafish embryos for ecotoxicity, and human health risk assessment of chemicals.

Lecture

A strategy to reduce the number of fish for acute aquatic toxicity testing of chemical substances and plant protection products

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ECVAM and ECB applied the threshold (step-down) approach, recently described by Hutchinson et al. (2003, *Environ. Toxicol. Chem. 22*, 3031-3036) for pharmaceuticals, to chemical substances and plant protection products.

According to the current EU regulatory requirements, acute aquatic toxicity is determined by three endpoints, namely algae EC_{50} 72h, daphnia EC_{50} 48h, and fish LC_{50} 96h. The proposed testing strategy takes into consideration that only the lowest value of the three endpoints is considered for hazard and risk assessment, and that fish is often not the most sensitive species. Therefore, algae and daphnia tests are carried out first and then, with the lowest of the two EC_{50} concentrations, a test using five test and five control fish is performed. When fish toxicity occurs at this concentration, further testing on fish at lower concentrations would be needed to determine the LC_{50}.

Ecotoxicity data of chemicals and plant production products from various data bases were retrospectively evaluated by selecting the lowest value from the reported daphnia and algae data and by subsequently calculating the step-down tests needed to reach the reported fish toxicity. Comparison of the numbers of fish needed in both testing strategies revealed a possible reduction of 55% to 70% when applying the threshold (step-down) approach. The evaluation report is now peer-reviewed by the ECVAM Scientific Advisory Committee.

The new testing strategy is a promising approach to reduce number of animals and costs for ecotoxicity testing not only in the current regulatory framework but also with regard to REACH.

Lecture

Ecotoxicological tests in non-ecotoxicological research: Contribution to 3Rs

Anne Kahru

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Due to the increase of population and industrial development the role of chemicals in everyday life is constantly increasing. Some unforeseen properties of pollutants have already been discovered, e.g., endocrine-disrupting side effects.

The framework of "chemicals – human – environment" contains two opposite scientific tasks: 1) to discover new (toxic) chemicals/mixtures to cure diseases, fight pests and microbes with minimum unwanted side-effects and 2) how to live safely in this world of chemicals, i.e. to minimise the unwanted effects of xenobiotics already accumulated in the environment (e.g., PCBs) in hazardous amounts or going to be produced in high-production-volumes in the nearest future (e.g., nanoparticles). The growing awareness of hazard of chemicals is clearly shown by introduction of REACH project. The increasing need for toxicity testing creates a big challenge for scientists to work out relevant alternative methods to laboratory animals or fish. One not yet widely explored area is the use of simple prokaryotic and eukaryotic models. Bacteria, fungi, protozoa, insects, plants and invertebrate animals – normal test organisms for ecotoxicological studies – are well suited for implementation of 3Rs in scientific and regulatory research.

This talk will focus on use of invertebrate organisms (e.g., bacteria, crustaceans, protozoa) in toxicity screening as well as for mechanistic studies.

For the illustration, the toxicity data for 50 MEIC programme chemicals (MEIC – Multicenter Evaluation of In vitro Cytotoxicity) involving pharmaceuticals, solvents, pesticides etc as well as cationic polymers (nanoparticles) – potential targeted drug delivery agents – will be presented. The potential of genetically modified bacteria as models for mechanism-targeted toxicity will be discussed.

Poster

Alternative to fish testing for acute ecotoxicity screening of cosmetic ingredients

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The EU notification process requires toxicological and ecotoxicological evaluation of new chemicals. Tests for environmental hazard assessment are carried out according to Council Directive 67/548/EEC Annex V, which involves fish acute toxicity (guideline OECD N°203), Daphnia acute toxicity, and Algal growth inhibition test. Fish, as any living vertebrate other than man, fall into the scope of European regulations for the protection of laboratory animals. This protection extends to immature forms including larval stages of development, but excluding embryonic stages before they become capable of independent feeding.

The Cosmetics Directive and the REACH proposal promote the use of *in vitro* methods for the hazard evaluation of chemicals.

We present an ongoing tiered approach for acute ecotoxicity assessment of cosmetic ingredients, aimed at replacing the fish acute toxicity testing required by the European legislation on chemical safety: Algae and Daphnia acute toxicity assays are realised on tier 1, then selected chemicals are submitted on tier 2, to a fish embryo test in replacement of juvenile or adult fish required by OECD guideline N°203.

We present data, obtained with two different fish species (Medaka and Zebrafish), showing similar sensitivity in adult and embryonic stages of development, to 26 reference chemicals (surfactants and quaternary ammoniums). Sensitivity differences between embryonic stages, before and after hatch, are observed. With regard to the selective permeability of the fish eggs envelops (chorion), especially to quaternary ammoniums, we recommend testing on fish embryonic stage after hatch (named Eleutheroembryo), which still relies on autotrophic vitellogenic supply.

Lecture

Evaluation of the fish embryo test as a potential replacement for the standard acute fish toxicity test using juveniles

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The 96 hrs acute fish toxicity test is the standard early tier method used in prioritising chemicals and effluents for subsequent hazard and environmental risk assessment. Current and proposed European chemical legislation and ethical stands on the use of animals for the purpose of risk assessment has resulted in an increased desire to implement the Three Rs in regards to vertebrates, including fish. Present regulatory definitions of protected and unprotected life stages of fish have led us to evaluate fish eggs (embryos) and sac fry (eleutheroembryos) as potential replacements in acute toxicity test protocols for fish. Because several species are preferred in different parts of the world and our companies operate globally we are developing an understanding of the relationship between embryo, eleutheroembryo, and juvenile sensitivities with Japanese Medaka, Zebrafish, and fathead minnow. In this paper we review: (a) the first stages of results with Zebrafish and Medaka embryonic stages side-by-side with 96 hrs juvenile tests using 26 compounds on a nominal basis, and (b) Zebrafish egg (48 hrs), eleutheroembryo (48 hrs), and juvenile (96 hrs) tests (4 compounds) with detailed confirmatory analytical to explore issues with chemical sorption and loss when dealing with small volumes. Sensitivity of Medaka and Zebrafish embryonic stages appear promising for the purpose of an early tier replacement test. We will review additional high priority research needs. These early studies are being used to develop protocols that we believe can be useful for development of future robust validation plans.

Lecture

Practical applications of the principles of the 3Rs in environmental assessment

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Experimental bioassays are currently used in ecotoxicology and environmental toxicology to provide information for risk assessment evaluation of new chemicals and to investigate their effects and mechanisms of action; in addition, ecotoxicological models are used for the detection, control and monitoring of the presence of pollutants in the environment. As a single bioassay will never provide a full picture of the quality of the environment, a representative, cost-effective and quantitative test battery should be developed. In order to study the effects of chemicals in the environment, a test battery has been applied. Such a battery should represent a wide range of organisms belonging to different trophic levels. A number of ecotoxicological model systems with more than twenty endpoints were evaluated at different exposure time periods. The systems included the immobilisation of the cladoceran *Daphnia magna* (1st consumer), bioluminescence inhibition in the marine bacterium *Vibrio fischeri* (decomposer) and growth inhibition of the alga *Chlorella vulgaris* (producer). Total protein content, neutral red uptake, lactate dehydrogenase (LDH) activity and MTT metabolisation were investigated in Vero monkey kidney cells (model of 2nd consumer). Neutral red uptake, total protein content, MTS metabolisation, LDH activity, lysosomal function, succinate dehydrogenase activity, G6PDH activity, metallothionein levels, EROD activity, apoptosis induction and changes in morphology were studied in the RTG-2 cell line, derived from rainbow trout gonad (*Oncorhynchus mykiss*), and in the hepatoma fish cell line PLHC-1, derived from *Poeciliopsis lucida* (models of 1st consumer).

Poster

In vitro biotransformation and bioconcentration of surfactants – development of assays and strategies for improving the prediction of the bioconcentration potential

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Bioconcentration is the process of accumulation of chemicals from water into an organism. The occurrence of significant bioconcentration is a prerequisite for further accumulation in the food-chain and possible secondary poisoning effects. Hence, the bioconcentration potential is highly relevant for the environmental risk evaluation of chemicals. It is also one of the criteria triggering whether or not a chemical is assigned to the "persistent, bioaccumulative and toxic, PBT" or the "very persistent, very bioaccumulative, vPvB" type chemicals.

As a first approximation, the bioconcentration potential (BCF) is estimated on the basis of the octanol-water partitioning coefficient. This approach yields high values for the BCF of surfactants which are in contrast to experimental determination. The discrepancy between measured and estimated BCF is often caused by biotransformation reactions in the organisms, which counteract the buildup of elevated internal surfactant concentrations.

Refined non-animal methods for the assessment of bioconcentration of surfactants therefore require information about the rates of biotransformation. Such information might be obtained through *in vitro* biotransformation experiments or by comparison of the bioconcentration behaviour with that of a metabolically stable reference compound. Possible strategies for utilising this information are discussed with regard to replacing whole animal studies, reducing the use of test animals, and with regard to refining computational estimates of the bioconcentration potential.

Lecture

Application of *in vitro* alternative methods to ecotoxicology

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Detection of environmental pollutants is major problems in ecotoxicology. Usually chemical analyses have been applied for the testing of contaminated samples. Although chemical analyses are becoming accurate by advanced analytical technology, they detect only targeted chemicals among test sample mixed with various chemicals. Bioassay systems are also used to detect pollutants in the waters using green algae and fish etc. We applied mammalian cell culture systems using several *in vitro* methods to detect their toxicities because of high sensitivity and comparable with the results of chemical analyses. Liquid samples, river waters and cooling tower waters were used after filtration for cytotoxicity test with two cell lines. Solid samples, ashes from incinerators, river sediments or airborne particulates, were extracted with organic solvent. Extracts were examined with *in vitro* phototoxicity test which was recently adopted as OECD Test guideline 432. Some samples were also examined in the mutation assay with mouse lymphoma TK assay.

The results are as follows: The data from river waters obtained in the colony formation assay were highly correlated with that of chemical analyses, although samples showed different response between two cell lines. The results of phototoxicity and mutagenesity with extracts of airborne particulates collected from in the last 20 years showed that the air in the area has become cleaner recently which was supported by chemical analysis. Our results suggested that *in vitro* tests are useful to assess and screen environmental pollutants as well as chemical analysis and other bioassays using animals.

Poster

The use of *in vitro* estrogen-reporter assays to predict potential endocrine disrupting effects in fish

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Numerous chemicals of industrial, agricultural or domestic origin, which show estrogen-like properties and which can interfere with wildlife reproduction, do occur in the aquatic environment. It is not evident to identify and quantify all these target compounds and biological active metabolites by analytical techniques. Therefore a screening approach, based on two *in vitro* bioassays, was first applied to assess the overall exposure to pollutants with estrogenic activity. The estrogen-inducible screen with a recombinant yeast strain (YES-assay) and human breast cancer cells, stably transfected with pVit-tk-Luc (MVLN-assay), both based on estrogen receptor binding, were used. Both assays were previously compared with an *in vivo* test with Zebrafish for their performance characteristics (sensitivity, reproducibility) and response spectrum for known estrogenic compounds. As part of two environmental monitoring projects many samples from Flemish waters (rivers, effluents of municipal wastewater treatment plants and industry, drinking water resources) were analysed. Next, sites with different estrogenic potential were selected in order to evaluate the likelihood of *in vivo* adverse effects on reproduction success in fish. The *in vivo* model used was the Zebrafish, *Danio rerio*, which was exposed to environmental water samples for 3 weeks and biomarkers for estrogenic exposure (vitellogenine in blood plasma and the gonado-somatic index) were measured. It was investigated whether measured levels of estrogenic activity, as determined by *in vitro* assays could indicate potential hazardous effects in fish. This step-wise approach using *in vitro* screening has been proposed for future studies on natural fish populations in Flemish rivers.