Session I-6: Advances in alternative methods for ecotoxicology

Session I-6: Oral presentations

I-6-172

Fish cell lines as alternatives to fish toxicity tests

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Fish are the dominant vertebrate species for the regulatory evaluation of ecotoxicity and are generally afforded the same legal protection as mammals. It is for this reason that the establishment and validation of cell culture assays as alternatives to fish tests, as initially suggested almost 40 years ago, is a desired and urgent societal goal. On this background, we establish novel fish cell cultures models with a particular focus on cell lines and develop strategies to overcome common limitations in the application of cell culture assays as substitutes for fish. The most recent development is a cell line from rainbow trout intestine; we are currently working to establish this cell line as an intestinal barrier model. To elucidate active transport mechanisms for chemical and toxicant distribution, we identified mRNA expression and activity of nine selected ABC transporters belonging to the ABCB, ABCC and ABCG families in seven rainbow trout cell lines. Finally, improved procedures for exposure of a rainbow trout gill cell line led to toxicity results that are well comparable to those obtained in the acute fish toxicity test. Thus, fish cell lines hold great potential for deciphering the molecular mode of action of chemicals and, provided the right choice of *in vitro* model and exposure conditions, may supplement or even substitute fish toxicity tests. Our vision is to build an *in vitro* fish test which not only provides knowledge on cell type interactions in physiology and toxicology but also supports the development of computational models toward a virtual fish.

I-6-340

Quantitative and comparative analysis of alternatives to *in vivo* tests for endocrine disrupting chemicals (EDCs) in fish and amphibians

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Endocrine disruption plays an important role in environmental safety assessment of chemicals. Therefore, appropriate screening assays have been developed, such as the US EPA Tier 1 screening assay in fish and frog species (adopted in 2009). Both assays are closely aligned with the OECD test guidelines 229 and 231. However, these assays use a large number of animals. Furthermore, they are costly and quite long in duration relative to an ideal screening assay. A shorter-term and alternative to an-

imal tests would be advantageous in order to reduce the number of animals used. A literature search was conducted to identify potential alternatives and a database with alternatives to fish and frog testing methodologies was assembled. Data from 1995 to the present were collected related to the detection/testing of estrogen-, androgen-, and thyroid-active chemicals in the following test systems: cell lines, primary cells, fish/frog embryos, yeast, bacteria, cell free systems, and "omics" technologies. A critical analysis was performed to (1) determine the strengths and limitations of each assay and (2) present conclusions regarding chemical specificity, sensitivity, and correlation with *in vivo* data. Due to the relatively large amount of data available for estrogenic compounds comparative analyses were performed specifically for this group of EDCs. For example, a high correlation was observed between ligand binding and reporter cell assays and between fish and frog estrogenic data. Furthermore, alternative assays appear to be able to detect specific hormone receptor binding. A summary of these and other data on alternative assays for EDCs will be presented.

I-6-423

Adverse outcome pathways and extrapolation tools to advance the Three Rs in ecotoxicology

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Adverse outcome pathways (AOPs) are conceptual frameworks for identifying and organizing predictive and causal linkages between cellular-level responses and endpoints conventionally considered in ecological risk assessment (e.g., effects on survival, growth/development, and reproduction). The proposed paradigm for "Toxicity Testing in the 21st Century" advocates the use of mechanistically-based, high-throughput *in vitro* assays as a potential cost effective and scientifically-sound alternative to some whole animal hazard testing. To support the development of this approach, there is a recognized need to (1) identify and catalog common adverse outcome pathways (AOPs) and (2) based on these pathways, strategically develop appropriate batteries of alternative assays. Furthermore, there is a need to develop a variety of extrapolation tools which can translate *in vitro* assay data into credible predictions of *in vivo* outcomes, preferably for a wide range of organisms. This presentation will highlight the utility of the AOP concept and discuss extrapolation tools needed to define and expand the applicability domains of mechanistic high throughput *in vitro* assay data, with specific emphasis on how these approaches can support the reduction, refinement, and/or replacement of animal use in ecotoxicology and ecological risk assessment.

I-6-536

What reductions in fish use can be made employing alternatives for wastewater effluent assessment?

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Fish testing has many applications which may include new chemicals screening, product safety assessment, and water supply safety. The majority of fish testing use can be attributed to compliance and investigational testing of treated wastewaters throughout the world. Permitted wastewater discharges to surface waters are routinely tested in North America for effluent toxicity to determine compliance to state and national water quality standards. Similar mechanisms or goals exist in certain parts of Europe and Asia. Larval, juvenile and adult fish are employed for acute and/or chronic toxicity determinations. A major advantage of effluent toxicity testing is the integration of mixture toxicity and bioavailability. This is unlike chemical-specific analysis which identifies only individual constituents and can make it difficult to determine if receiving waters are protected. We suggest that a 3Rs approach for effluent testing will lead to adequate or new alternative solutions that are better designed

for their purpose and enable fast, cost-effective equivalents.

Alternatives for fish effluent testing include streamlining existing tests to use fewer fish, *in vitro* methodologies and fish embryo testing. We will review several options to reduce numbers of fish using techniques such as: combining control treatments, test designs using fewer effluent concentrations and possible development of fish embryo tests yielding equivalent results to standard assays using swim-up/larval forms and chronic toxicity endpoints. A program has been initiated to test assumptions and assay conditions using fathead minnow and zebrafish. Comparisons of embryo tests and classical effluent survival and growth assays for both species are planned using chemicals and representative effluents. Successful results in the pilot experiment would prompt a more extensive program. Lastly, these assays may have additional utility to develop fish toxicity perspectives on complex mixtures and metabolites using bench top wastewater treatment plant models (e.g., CAS units) during the development of new or existing chemical assessments.

I-6-447

Product stewardship "incorporating the 3Rs while improving bioaccumulation assessments"

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Product stewardship is an integral, on-going aspect of environmental programs at major corporations. Animal welfare concerns, as exemplified by the 3Rs, are an important consideration in product stewardship testing and the evolution of regulatory testing guidelines. Intelligent testing strategies that incorporate tiered testing and evaluation schemes help guide efficient use of limited resources, including test organisms, and facilitate scientifically-based chemical evaluations to ensure achievement of product stewardship and environmental protection goals. An example of a tiered assessment framework for bioaccumulation assessment is presented that incorporates data mining, modeling, physical-chemical property measurements, *in vitro* assays, and *in vivo* toxicity tests to provide an assessment of bioaccumulation potential in fish. Examples of the application and limitations of the framework for bioaccumulation assessments will be presented including an evaluation of the animal welfare benefits of the framework and suggestions for additional research to improve the framework.

I-6-114

Harmonizing and optimizing fish testing methods: The OECD framework project

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The Organisation for Economic Cooperation and Development (OECD) serves a key role in the international harmonization of testing of a wide variety of chemicals. An integrated fish testing framework project was initiated in mid-2009 through the OECD with the US as the lead country. The objectives of the project were to review regulatory needs and data requirements for fish testing in the context of existing OECD Test Guidelines. One goal was to support animal welfare concerns by identifying unnecessary test methods and ensure the optimal use of data derived from *in vivo* studies. A September 2010 workshop with participation from over 40 international experts was organized with the goal of producing a guidance document that provides a detailed discussion of technical issues, relevant endpoints, and specific recommendations for a harmonized testing framework for fish. In addition to detailed reviews of individual OECD fish test guidelines, topic areas included general testing issues, regulatory needs and data requirements for fish testing, statistical issues, animal welfare considerations and alternative approaches to testing. General guidance on possible strategies for approaching hazard testing with fish was developed by identifying broad principles to guide testing sequences which can then be adapted for specific circumstances and types of chemicals. This presentation will highlight the conclusions and recommendations of the workshop and discuss the resultant framework document.

Session I-6: Poster presentations

I-6-095

Statistical power of the OECD 210 chronic fish early life stage test and what this suggests for future animal alternative approaches

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The Fish Early Life Stage Test (OECD 210) is the most common chronic fish ecotoxicity assay in use for assessing industrial chemicals, biocides, pharmaceuticals, and agrochemicals. The assay encompasses exposure from egg through juvenile life stages accompanied by determinations of hatchability, survival, growth (length and weight) and developmental abnormalities. The guideline is flexible with respect to species and key aspects of experimental design. With cooperation from several industry partners, we gathered over 100 OECD 210 studies (82 compounds, 16 laboratories). Fathead minnow, zebrafish, and rainbow trout comprised 71%, 16%, and 13% of the studies, respectively. Experimental design, water quality, and test endpoints were summarized. Information was collected at the level of individual chambers to allow determination of the statistical power of the reviewed studies to detect biologically meaningful effects. Statistical overdispersion in tests indicated that extra bionomial variation was frequently present, consistent with the presence of significant chamber-to-chamber variability. In order to increase the sensitivity of the assay, we recommend maximizing numbers of test chambers rather than the number of fish per chamber. Power analysis indicated that most endpoints could detect a 20% change relative to controls when using at least four replicate chambers, although to detect a 10% change would often require as many as 20 replicates for some species and endpoints. The complexity of chronic tests with respect to organism development and execution make replacements and refinements difficult; however, future alternatives could consider this as a "bar" against which they can be measured.

I-6-161

Development of a mechanistically informative, genome-wide, *in vitro* chemicals screening technology

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The testing of chemicals is an important regulatory activity which, in many cases, requires the use of animals. Reducing the number of animals for testing depends on the development of alternative test procedures that do not use living animals but yield information relevant to the health and environmental impacts of the chemicals in questions. Two methods coming into use are permanent fish cell lines and early stage fish embryos. We want to increase the information content of these alternative test methods by applying refined microarray technologies, alongside powerful statistical technique, and utilising the experimental power of well-controlled experimental designs to define a large number of genes responding to toxic exposure. These transcriptomic signatures will describe the distributed nature of gene responses, which can be modelled as affected processes and pathways, and as a network of regulatory effects. We want to know whether this system-wide view of toxic effects, which relates to the full complexity of the system, can be used as a classifier to predict the toxic behaviour of novel materials, and which alternative test method offers the best prospects for discrimination between toxic compounds. Moreover, we are interested in whether ecologically relevant doses or those that relate to the dose response curve are most appropriate for testing purposes and how the results are affected by dose. We will compare and contrast the performance of fish cells and zebrafish embryos, and demonstrate how this system-wide view of toxicological effects generates more understanding and predictive power than a candidate gene-centered view.

I-6-197

Predicting acute toxicity in fish using the rainbow trout gill cell line RTgill-W1

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The OECD test guideline 203 for the determination of fish acute toxicity requires a substantial number of fish and uses death as an integrative but crude endpoint. Therefore, the development of appropriate alternative methods is timely. One promising approach is the use of fish cell lines; however, several studies indicated that fish cell lines appear less sensitive than fish. We optimized the fish cell line approach using several steps to increase the sensitivity of a fish gill cell-based assay. These steps included the modification of the exposure medium and the determination of the chemical bioavailability. We further showed that chemical toxicity is dependent on the solvent and dosing procedure. Based on these findings, we designed dosing and exposure protocols that account for factors otherwise compromising the *in vivo-in vitro* correlation. The optimized cell line approach was now used to determine the toxic potential of 34 organic chemicals towards the RTgill-W1 cell line. The selected chemicals have a wide range of mode of action and physico-chemical properties. Results reveal a good agreement of *in vivo* and *in vitro* values. Outliers from the correlation can be explained by certain modes of action. Compounds that give the greatest deviation are either neurotoxicants or chemicals that need to be metabolized into more toxic compounds, target sites or processes that are not mimicked by the gill cells. Based on this knowledge we are developing a strategy to use fish cell lines as surrogates for acute fish toxicity studies.

I-6-382

Transepithelial electrical resistance to monitor epithelial cell integrity for *in vitro* toxicity testing of water samples

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Current methods for testing water pollution are expensive and do no test for a wide variety of toxicants. In terms of testing new toxicants the OECD has set guidelines which involve exposing live fish to test toxicants for up to 96 hours and testing how many fish have died at intervals of 24, 48, 72 and 96 hours.

This study aims to develop a cell-based autonomous biosensing microsystem for water quality. Transepithelial electrical resistance measurements (TEER) will be used to monitor the integrity of a monolayer of epithelial cells in an automated fluidics system. The system will be installed at a river or stream and will sample the water. A drop in TEER will be taken as indicative of the presence of toxicants in the water sample and will be automatically relayed to an operator, notifying him or her of a problem.

A simple fluidics system for epithelial cell culture has been developed. It includes a double flow PDMS chamber, with upper and lower compartments separated by an ultrathin microporous silicon nitride membrane for cell growth. Integrated electrodes allow regular measurements of the TEER of the epithelial cell layer.

The chosen cell line for use in the model is the caco-2 (C2BbE1) cell line. This is a polarized colon cell line forming functional tight junctions after 21 days in culture. First results have shown that those cells grow well in the double flow PDMS chamber and that TEER can be monitored.

Future work is ongoing to optimize the system and to compare TEER measurements with classical toxicity endpoints.

I-6-478

Assessment of mitochondrial toxicity of environmental chemicals using a quantitative high-throughput screening approach

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As part of the U.S. Tox21 initiative, the NCGC is developing and optimizing cell-based and biochemical assays suitable for quantitative high throughput screening (qHTS) in a 1536-well format. This effort will generate pathway profiles for environmental compounds that will facilitate the evaluation of mechanisms of toxicity and prioritization for more extensive testing, as well as the development of predictive models for *in vivo* toxicity. In this study, we optimized a mitochondrial membrane potential (MMP) assay using a water-soluble fluorescent MMP sensor to detect mitochondrial depolarization in HepG2 cells and we used this method to evaluate the mitochondrial toxicity of 1353 environmental compounds provided by the NTP. In response to mitochondrial depolarization, the ratio of the mitochondrial red fluorescent aggregates to the cytosolic green fluorescent monomeric form of the dye decreases. Of the 1353 compounds screened over a 14-point concentration curve (0.59 nM to 92 μM), 107 (8.9%) compounds disrupted the mitochondrial potential in HepG2 cells after treatment for one hour and 104 (7.6%) did so after five hours, with 88 (6.5%) compounds active at both exposure durations. To evaluate the structure-activity relationship of these potential mitochondrial disruptors, we clustered these 88 compounds by structural similarity. This analysis resulted in seventeen structural clusters and 26 singletons. We selected 39 compounds for further studies, including high-content imaging and sensitivity to different energy sources. Our results confirm the robustness of this assay for identifying MMP disruptors in qHTS format.

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I-6-537

EUROECOTOX – European network for alternative testing strategies in ecotoxicology

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EUROECOTOX is a project funded by the Seventh Framework Programme (FP7) of the European Union. It aims at mapping and reviewing the current status of alternative testing approaches for environmental risk assessment. The main objectives of the project are

– to contribute to the advancement of alternative methods of eco-
– toxicity testing in Europe;
– to promote the validation and regulatory acceptance of new alternative ecotoxicity methods;
– to facilitate the networking of research groups working in the field of alternative approaches in ecotoxicology;
– to interact with stakeholders involved in the development, validation, regulatory acceptance and final use of alternative ecotoxicity testing strategies;
– to identify bottlenecks for the implementation of alternative testing approaches and
– to propose research needs for the promotion of alternative testing.

EUROECOTOX is organising a couple of events to address these objectives, such as an expert meeting, a conference and a report. Furthermore, a website has been launched (www.euroecotox.eu) which will serve as a resource centre and a database on primarily European activities on development and validation of alternative methods for ecotoxicological testing. At the 8th World Congress on Animal Alternatives and Animal Use in the Life Sciences, a first overview on the mapping results and database implementation will be given.

I-6-585

An educational program for the use of alternative methods to animal experimentation and testing

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Alternative methodology is one of the scientific fields that have been growing exponentially during the last years. Because of the robust quality, validity and human relevance of the data collected with alternatives these methods are increasingly adopted for safety testing purposes in the field of regulatory toxicology.

The continuing progress in the field of *in-vitro* methods, e.g. high throughput and high content imaging methods and advanced knowledge in the field of systems biology, protein interactions and gene expression patterns (-omics) opened up new prospects, especially in the field of toxicology, to investigate pathway related and human relevant targets with methods alternative to animal testing. Thus, today the field of alternatives is multidisciplinary, encompassing moral philosophy as well as genomics, transcriptomics, proteomics, systems biology and also regulatory toxicology. Because of the resulting complexity of this diverse field it is inevitable that the segmental knowledge has to be shared between many experts.

However there are very few relevant educational programs in the field of alternatives, i.e. programs which are comprehensive and embedded within the framework of scientific curricula. CAAT-Europe brought together key teachers in the field of alternative methods and engaged them to complement their expertise. The synergistic outcome with regard to specified target groups resulted in an advanced and comprehensive educational setup including different modules for different audiences with different demands.

I-6-586

Center for Alternatives to Animal Testing – Europe (CAAT-Europe)

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Two universities renowned for their experience in the area of alternative methods in Europe and the US have joined forces to complement the 30-year-old Center for Alternatives to Animal Testing (CAAT) at Johns Hopkins University with a corresponding Center for Europe (CAAT-Europe) at the University of Konstanz. The "Excellence University" of Konstanz has twenty years of experience in alternatives to animal experiments, employing five professors in pharmacology and toxicology along with numerous coworkers researching human-relevant alternative methods. CAAT-Europe brings together industry and academics to address the needs for human-relevant methods, to use strategic funds to fill gaps in the development and implementation of alternative methods, to coordinate information days and workshops in Europe on relevant developments in the area of alternatives, to develop strategic projects with sponsors for the promotion of humane science and "new toxicology" and to set up a joint education program between Johns Hopkins and the University of Konstanz.

I-6-591

Application of the 3Rs in the field of ecotoxicology

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Despite constituting 11% of total UK animal usage in 2009, fish are often overlooked with respect to the 3Rs. Here we describe several initiatives to address this issue.

Reduction: For evaluating endocrine disruptors, fish full life-cycle tests are considered the "gold standard," but use many animals. By utilizing all available information (e.g. preclinical data), however, it is possible to focus testing on sensitive life-stages and endpoints. We recently employed this strategy to design a "targeted" chronic study that used fewer fish than the standard test.

Refinement: Although there is pressure to improve environmental enrichment for fish, most current approaches are based on mammalian research. Consequently, we aimed to identify enrichments that are suitable for fish and compatible with prescriptive regulatory protocols. In addition, little information is available on pain alleviation in fish, so we are developing alternative procedures that are both scientifically and ethically preferable to existing approaches to anaesthesia and analgesia.

Replacement: Cell culture is a promising alternative to fish use, although questions remain around *in vivo* translation. We assessed a fish hepatocyte culture for endocrine disruptor (ED) testing that proved sensitive for certain biomarkers, and exhibited good metabolic capability. We have also evaluated algae and crustaceans as surrogates for fish genotoxicity assessment, the data generated suggesting effective metabolic activation and measurable induction of DNA damage.

In summary, various initiatives have been undertaken, some of which have the potential to improve the 3Rs in fish, provided appropriate levels of comparability with *in vivo* studies can be demonstrated.

I-6-599

OECD validation study on the transferability, intra- and inter-laboratory reproducibility of the zebrafish embryo toxicity test

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The OECD Fish Acute Toxicity Test Guideline (TG 203) is an integral component in the environmental safety assessment of industrial chemicals, agrochemicals, pharmaceuticals, feed stuffs, and biocides. One of the most promising alternative approaches to the fish acute toxicity test is based on the use of zebrafish embryos. In 2005, the German Federal Environment Agency submitted the draft TG "Fish embryo toxicity (FET) test" to the OECD Test Guideline Program and a supportive Background Paper. Subsequently, OECD established the *Ad hoc Expert Group on the Fish Embryo Toxicity Test*. Based on the outcome of expert meetings, OECD decided to perform a validation study (coordinated by ECVAM and steered by a validation management group). The validation study aims to evaluate the transferability and the intra/interlaboratory reproducibility of the Zebrafish FET (ZFET) test for different chemicals newly fertilised zebrafish embryos are exposed to for up to 96 h. Four apical endpoints are recorded daily as indicators of acute lethality in fish: coagulation of the egg, lack of somite formation, non-detachment of the tail bud from the yolk sac and lack of heart-beat. LC_{50} values are calculated for 48 h and 96 h exposure. A total of 20 chemicals will be tested at five different concentrations in three independent runs in at least three laboratories with appropriate controls. Stock solutions and test concentrations of at least one laboratory are analytically confirmed. This presentation will give an overview on the study design and discuss the preliminary results.

Disclaimer: The opinions expressed and the arguments employed herein are those of the authors and do not necessarily reflect the official views of the OECD or of the governments of its member countries.