The Use of Fish Cells in Ecotoxicology

The Report and Recommendations of ECVAM Workshop 47[1,2]

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Preface

This is the report of the forty-seventh of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). ECVAM's main goal, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures which would enable it to become well informed about the state-of-the-art of non-animal test development and validation, and the potential for the possible incorporation of alternative tests into regulatory procedures. It was decided that this would be best achieved by the organisation of ECVAM workshops on specific topics, at which small groups of invited experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward (1).

The workshop on the use of fish cells in ecotoxicology was held in Angera, Italy, on 22–24 October 2001, under the co-chairmanship of Argelia Castaño (Animal Health Research Centre, Spanish National Institute for Food and Agrarian Research and Technology, Valdeolmos, Madrid, Spain) and Helmut Segner (Centre for Fish and Wildlife Health, University of Bern, Switzerland). The participants came from international regulatory or governmental organisations, academia and industry.

The objectives of the workshop were: a) to critically review the potential use of fish cells to replace, reduce or refine existing regulatory tests which involve the use of fish for ecotoxicological purposes; and b) to discuss the advantages, limitations, and possible future applications of fish cell systems in hazard assessment, ecotoxicological research and testing, and in environmental surveillance and monitoring.

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[1]ECVAM — The European Centre for the Validation of Alternative Methods. [2]This document represents the agreed report of the participants as individual scientists.

Introduction

Man-made contamination of the aquatic environment has necessitated the development of methods and concepts to assess the effects of environmental pollution on aquatic organisms, including fish. Currently, toxicological research on fish is largely based on *in vivo* studies. This applies to all the fields under consideration, i.e. basic toxicological research and regulatory toxicity testing, as well as environmental surveillance and monitoring. The justification for *in vivo* studies is derived from the objective of ecotoxicology to evaluate the effects of chemicals on populations and ecosystems. The situation is clearly different from that in human toxicology, where the focus is on the individual and on one single species. Thus, *in vitro* systems in ecotoxicology are expected not only to allow for extrapolation from *in vitro* effects to toxic effects *in vivo*, but also to provide information on biological responses at the supraindividual and ecological levels. Therefore, scientists and regulatory authorities are reluctant to use cell-based *in vitro* tests in the context of ecotoxicology. However, a number of ethical, technical, scientific and economical reasons support the development of *in vitro* methods for use in ecotoxicology.

1. Cells, as the basic building blocks of all life forms, represent a key level of organisation for detecting and understanding common and unique mechanisms of toxicity. Knowledge of common mechanisms facilitates the interspecies extrapolation of the impact of environmental contaminants, which is one of the major challenges to ecotoxicologists. Identifying unique toxic mechanisms or susceptibilities for a species can also be of ecotoxicological value. If a unique toxic mechanism occurs in species of special ecological importance, this information can be used to assess the impact of toxicants on both the species and the ecosystem. In addition to improving the risk assessment, the understanding of toxicity mechanisms can lead to the development of biomarkers, which can be used to measure *in vivo* responses and can permit the evaluation of the effects of toxicants on animals.

2. Cell cultures provide the best experimental system for studying toxic mechanisms at the molecular and cellular levels, by allowing cells to be studied in a controlled environment and in isolation from the multiple physiological systems which regulate their activities *in vivo*.

3. Animal cell cultures permit studies on a species that might not otherwise be studied, because that species cannot be maintained in a laboratory setting and/or is not routinely available (for example, pelagic marine fish, which travel in large schools).

4. Animal cell cultures permit the comparison of species at the cellular level under equivalent conditions of toxicant exposure. This is important for understanding the relative potencies of toxicants in different species.

5. Animal cell cultures can be used as a rapid, inexpensive screening tool to evaluate the toxicities of large numbers of individual compounds and of samples from the environment. This will probably become even more important in the future, as the new concepts of genomics and proteomics become incorporated into screening tests. Cellular test systems will provide the basis for the application of automated and high-throughput technologies in ecotoxicological hazard assessment.

6. The application of animal cell cultures for the above purposes reduces the use of whole animals in toxicity testing, which is a goal supported by popular opinion in many countries around the world. As in human toxicology, the objective is the replacement, reduction or refinement of *in vivo* tests. The existing regulatory tests in ecotoxicology are *in vivo* tests for measuring organismic — not ecological — responses, such as death, growth, and reproduction. The special challenge for *in vitro* studies in ecotoxicology is the development of cellular test systems that not only can replace whole animal tests, but also have meaning at the ecological level.

Despite the fact that inherent differences exist between homoeothermic and poikilothermic organisms, basic fish cell research has received relatively little attention. Most fish cell work has been driven by commercial aquaculture and fisheries, mainly with the purpose of identifying and growing fish viruses for fish health studies (2, 3). However, the scattered literature on fish cells has also touched on various aspects of cellular physiology, molecular biology, genetics, immunology, endocrinology, nutrition, comparative biology and biotechnology (4–9). One of the first studies that used fish cells *in vitro* for toxicological studies was that of Rachlin & Perlmutter (10), who measured the cytotoxic action of zinc on an established cell line from the fathead minnow (FHM). However, it was only in the 1980s that the use of fish cell systems for toxicological purposes became more widely recognised. Since then, an increasing number of studies have been undertaken to explore the scope and the limits of the use of fish cells for ecotoxicological purposes (11–24).

Currently, the use of fish cells in toxicological experiments and tests is focused on measurements

of: a) cytotoxicity, both basal and selective (cell-specific); b) genotoxicity; and c) effects on cell-specific functions and parameters, including studies on biotransformation, the induction of specific markers, and mechanisms of toxicity. Increased knowledge about fish cells has shown that there are many fundamental similarities between fish and mammalian cells with respect to cellular mechanisms, but that fish cells also reflect a number of fish-specific traits that cannot be assessed with mammalian cells. Fish cells have many practical advantages over mammalian cells: they can be incubated at room temperature (20°C) and in the ambient atmosphere, which means that specialised incubators are not needed; and they can be stored for long periods at 4°C, circumventing the need for freezing/thawing the cultures. Fish cells can be exposed to various aquatic environmental samples at varying osmolarities, something that has been done in mammals only with renal cells. Because of commonality of endpoints and because of simpler handling, fish cells could even replace the use of mammalian cells in some specific tests (for example, for the testing of non-sterile environmental matrices). Therefore, the potential use of fish cells in general *in vitro* testing and screening is not limited only to ecotoxicological assessments.

The Use of Fish in Ecotoxicology

Fish are used as experimental animals in biomedical and basic biological research and, in particular, for mechanistic studies. Examples are the use of fish as model organisms in cancer research and in developmental biology. Another area of increasing importance is the toxicity testing of chemicals and water samples, for regulatory purposes, for the development and safety evaluation of new substances and products, or for environmental monitoring and surveillance. Fish are also used in education and for training purposes.

Numbers of fish used

The available data on the number of fish used in Europe for research, environmental surveillance and ecotoxicity testing are rather poor. The European Commission's third report (25) on the numbers of experimental animals used in the 14 Member States of the European Union (EU) indicated that a total of 614,234 fish were used in 1999, which is significantly fewer than for 1996 (1,112,791 fish in 13 Member States; 26). According to the figures for 1999, most of the animals (71%) were used "in experiments for selected purposes" and 29% for toxicological and safety evaluation purposes, including the testing of chemicals and products for or by the aquaculture industry in feed and vaccine development or in studies on diseases. The number of fish used may have increased recently, since the data from the United Kingdom show a 98% increase from 122,438 fish in 1999 to 243,019 fish in 2000 (27). However, the latest United Kingdom data from 2001 reveal a decrease of 30%, to 171,092 (28). A conservative estimate is that about 1 million individual fish are used for research and regulatory purposes per year in the EU Member States.

Regulatory tests — chemicals

A large number of fish tests are performed for regulatory purposes. New and existing chemical substances are tested worldwide to evaluate potential adverse effects on human health and the environment. Regulatory testing is established at the national level for wastewater, and at a supranational level (for example, the EU [29, 30] and the Organisation for Economic Cooperation and Development [OECD; 31]) for newly developed and/or existing chemicals. In the EU, the tests for notification of new chemicals have to be carried out according to Annex V of *Council Directive 67/548/EEC*, which involves acute toxicity for fish (method C.1), acute toxicity for *Daphnia* (C.2), and the algal growth inhibition test (C.3; 29, 30). These European testing methods are equivalent to the OECD guidelines, a collection of methods used to assess the potential hazard of chemicals and of chemical preparations. They cover tests for physical and chemical properties, effects on human health and wildlife, and accumulation and degradation in the environment (see http://www.oecd.org/ehs/). For the fish test, the use of eight species from eight genera is allowed, with the rainbow trout (*Oncorhynchus mykiss*) and the zebrafish (*Danio rerio*) being the preferred species (32). The *Strategy for a Future Chemicals Policy* (33) foresees the disappearance of the distinction between new and existing substances, and all chemicals produced at above 1 tonne per year will be subjected to a registration procedure. While the testing of substances produced or imported at 1–10 tonnes per year should be limited to *in vitro* tests, base-set testing may be requested for substances marketed at above 10 tonnes per year. It is expected that this new policy will increase the number of fish needed for ecotoxicity testing to at least 4.4 million in the proposed testing period (33).

Table 1 shows the existing and draft EU/OECD Guidelines on tests on fish (29–31). The number of fish used per toxicity test is higher than in tests involving mammalian species: for the acute toxicity of chemicals, at least seven but preferably ten fish per concentration of the test compound, and at least five concentrations plus a control, are required. In the classical LD50 method (B.1/TG 401) with mammals

Table 1: Existing/draft fish tests in the EU/OECD Test Guidelines (29–31)

TG number OECD	TM number Annex V	Title	Endpoint
203	C.1	Fish, acute toxicity test	Mortality
204	—	Fish, prolonged toxicity test: 14-day study	Mortality
210	—	Fish, early-life stage toxicity test	Lethal and sub lethal effects Fertilised eggs are used
212	C.15	Fish, short-term toxicity test on embryo and sac-fry stages	Lethal and sublethal effects on embryo and sac-fry stages
215	C.14	Fish, juvenile growth test	Effects on growth rates Juvenile fish are used
305	C.13	Bioconcentration, flow-through fish test	Bioconcentration factor
—	—	Fish screening test for endocrine disruptors	Gross morphology, vitellogenin production and histology data on gonads (not yet harmonised)
—	—	Fish full life cycle test for endocrine disruptors	In addition to the three points above, growth rate, sex reverse rate, reproduction and secondary sexual characteristics (not yet harmonised)

EU = European Union; OECD = Organisation for Economic Cooperation and Development; TG = Test Guideline; TM = Test Method.

(both recently deleted), a maximum number of five rodents per concentration (if higher vertebrates are used, the number can be further reduced) and a minimum number of only three concentrations (oral toxicity), are required. Recently, the LD50 method (B.1/TG 401) has been replaced by alternatives that permit the use of even fewer animals and include the use of endpoints other than mortality (Fixed Dose Procedure [B.1bis/TG 420], Acute Toxic Class Method [B.1tris/TG 423] and the Up-and-Down Procedure [TG 425]).

As shown in Table 1, the current fish tests are designed to evaluate endpoints on mortality, lethal and sublethal effects, reproduction, and growth rate. Among these tests, the Fish Acute Toxicity Test (TG 203) can be considered as the most widely conducted test for the various purposes. In the OECD High Production Volume programme, for example, the acute fish test is required as one of the essential data for the Screening Information Data Set (SIDS) report, as well as the acute *Daphnia* test (TG 202) and the algal test (TG 201). Furthermore, in the context of the Globally Harmonised System (GHS) for Hazard Classification and Labelling, the 96-hour LC50 for fish is applied to designate both acute and chronic toxicity classes by applying an acute to chronic ratio (ACR) or safety factor. The bioconcentration factor (BCF) is determined by using the TG 305 (Annex V, C.13) bioconcentration, flow-through fish test. Other fish tests are used to evaluate chronic effects and effects on specific stages of development.

With regard to the assessment of potential endocrine disruptors, two new fish test methods (a short-term test and a long-term test for evaluation of reproduction) are currently under consideration at the OECD level. The objectives of these tests are to evaluate endocrine effects in fish by measuring endocrine-related biomarkers, such as vitellogenin induction, gonad histology, sex ratio, secondary sexual characteristics and reproductive parameters. If these new regulations come into force, the number of fish used for regulatory testing will increase even more.

Regulatory tests — effluents

In Canada, effluents from pulp and paper mills must be tested periodically in an acute lethality test involving rainbow trout (34, 35). A similar approach will soon be applied to mine effluents. Ten fish are exposed for 96 hours to a whole-water sample of effluent. If after 96 hours six or more of the fish have died, the effluent has failed the test. If after 96 hours five or fewer fish have died, the effluent has passed. Recently, the possibility of replacing this test with a fish cell test has been explored, with promising results (36).

In Germany, the *Waste Water Act* requires the testing of wastewater effluents by means of a 48-

hour acute fish lethality test according to DIN 38412-L31, with the golden ide, *Leuciscus idus melanotus*, as the test species. The test determines the dilution of wastewater that is not lethal to fish within a 48-hour exposure period. However, this test has recently been replaced, and wastewater toxicity is now evaluated with a test involving fish eggs, according to DIN 38415-T6 (37).

The European Commission White Paper on a *Strategy for a Future Chemicals Policy* and its impact on the use of fish

As shown in Table 1, the current chemicals regulations in the EU rely on *in vivo* data from fish tests. For instance, for base-set (acute lethality) testing (for chemicals with production volumes between 1 and 100 tonnes), the number of animals required is 210, 20% of them being fish, and for level 1 (prolonged toxicity) testing (production volume 100–1000 tonnes), 602 animals are required, 59% of them being fish (33). For the large majority of existing chemicals, no or only fragmentary toxicological and ecotoxicological data are available; if toxicity data needed to be generated for those compounds, this would lead to a tremendous increase in animal use. For instance, if base-set information were required for 30,000 chemicals (1–100 tonnes production), the number of fish required for testing would be 1.260 million, and for level 1 (100–1000 tonnes production), 2.024 million.

In the White Paper on a *Strategy for a Future Chemicals Policy* of the European Commission (33), the number of animals that would be required for chemical toxicity testing to achieve the goals of the proposed strategy was not discussed. Calculations made by the Medical Research Council Institute for Environment and Health (38) suggested that 12.8 million vertebrates (8.4 million mammals and 4.4 million fish) would be required for the testing of 30,000 substances. These numbers would be substantially increased, if the offspring produced in reproductive studies and the animals used in some higher tier tests were taken into account. In addition, the inclusion of novel tests for mammalian neurotoxicity and endocrine disruption would further increase the number of animals required. Moreover, the testing of these chemicals in *in vivo* experiments would be extremely costly and time-demanding. In view of these problems, the White Paper on a *Strategy for a Future Chemicals Policy* suggests a change in testing strategy, that is, to use *in vitro* methods for the initial hazard evaluation of chemicals. An enhanced emphasis on *in vitro* methods, however, would require established and validated *in vitro* test protocols. Since fish represent a major test organism in current test strategies, and since fish-specific toxic effects and endpoints are not reflected in mammalian cell systems, fish cell bioassays would have to be incorporated in future *in vitro* test batteries. The development and validation of fish cell-based *in vitro* test methods will therefore be of primary importance in future research.

The Use of Fish Cells in Ecotoxicology

The fish cell systems which are currently used for toxicological studies, are based on either primary fish cells or established fish cell lines. They differ in their functional properties, and have therefore been used for different purposes.

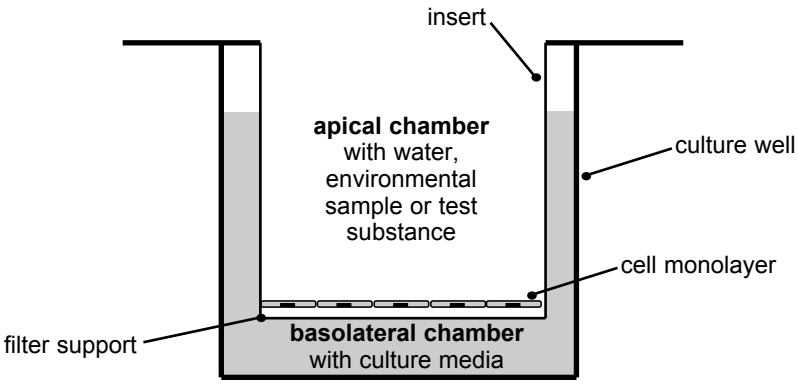
Primary fish cells

The isolation and culture of primary cells have been achieved from a wide range of fish tissues, including the liver (39–42), gill epithelia (43), gonads (44, 45), kidney macrophages (A. Castaño, unpublished data), skin epithelia (46–48), endocrine tissues (49), muscle cells (50), and white blood cells (51). Primary cells express many of the differentiated cellular structures and functions of their source tissues (for example, 44, 52, 53), and as such may be particularly suitable for mechanistically oriented studies on cell-specific toxicant fate and action. For example, gill epithelial cells grown on permeable filters have many features in common with cells in the intact branchial epithelium, of which the most important is that they tolerate exposure to water on the apical (mucosal) epithelial side (54). Due to its physiological realism, this system could be an attractive model for studying toxic effects on gill barrier function, toxicant uptake and metabolism. This preparation is particularly well suited for testing water samples without previous treatment (Figure 1).

A disadvantage of primary cell cultures is that they can show considerable fluctuations in their responses, depending on the physiological status of the donor fish and/or the quality of the isolation procedure (41).

Primary cells, being more differentiated, may deviate from cell lines in their cytotoxic responses. Segner & Schüürmann (55) showed that isolated trout hepatocytes were much more sensitive to iron(II) sulphate than the continuous trout liver cell line, R1; they speculated that this difference might be due to a higher level of unsaturated fatty acids in the hepatocyte membranes, leading to a particular sensitivity to iron-catalysed oxidative stress. When the cytotoxicities of the 50 Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) chemicals to fish cell lines, RTG-2 and R1 (56–58), were compared with their cytotoxicities in primary gill and liver cells from rainbow trout (56–60), the correlation coefficients (r^2) were 0.58 and 0.69, respectively, i.e. the correlations between primary

Figure 1: Method for growing gill epithelial cells on permeable filters



cells and cell lines were slightly worse than those observed between different cell lines. A major factor contributing to this difference between primary cells and cell lines might be the higher metabolic capacity of the primary fish cell cultures. However, reports supporting this assumption are rare. One example might be the case of carbon tetrachloride. In isolated fish cells, this compound showed the highest cytotoxicity toward metabolically active liver cells (EC50 between 3.5mM and 5mM; 61), while the sensitivity of metabolically less active or inactive fish cell lines was clearly lower (RTG-2: 18.6mM, R1: 21.6mM; 57, 58).

Further examples of cell type-specific toxic responses of fish cells come from genotoxicity studies. T. Braunbeck and co-workers (unpublished data) compared the genotoxicities of five chemicals to zebra fish primary liver and gill epithelial cells with their genotoxic activity in the two fish cell lines, RTG-2 and RTL-W1. In both cell systems, genotoxicity was assessed by means of the comet assay. The responses of the primary cells and the cell lines differed, depending on the test compound. Also, pronounced differences in their sensitivities to individual genotoxicants were observed among cell lines (62).

Fish cell lines

More than 150 continuous cell lines have been established from fish. Most of them are either fibroblast-like or epithelial-like, and originate mainly from the tissues of salmonids or cyprinids (2). In contrast to other areas in biomedical research, where a lot of recombinant cell lines are widely used, only two recombinant fish cell lines have been constructed to date: RTG-2 cells, stably transfected with the rainbow trout oestrogen receptor (23), and BF-2 cells, stably transfected with active luc gene, BF-2/luc1 cells (63).

The cytotoxicity data from various fish cell lines agree reasonably well. The inter-cell line r^2 values are mostly higher than 0.8, while the slopes of the regression lines show more variation. For instance, Babich & Borenfreund (13), when comparing the cytotoxicities of several classes of organic compounds in two fish cell lines, FHM and BF-2, observed an almost identical cytotoxicity ranking in the two cell systems, although the FHM cells were consistently more sensitive than the BF-2 cells. Similar findings were reported with respect to metal cytotoxicity, where the cell lines RTG-2 and BF-2 showed identical relative ranking, but different absolute sensitivity (11). Good agreement of cytotoxicity data was also reported for the cell lines, CHSE-214 and RTG-2 (64). However, exceptions to this generally good agreement between cell lines must not be overlooked. For instance, cytotoxicity data for a series of chlorophenols were very similar between six fish cell lines, but one cell line — PLHC-1 — showed markedly different responses (22).

The good correlations between cytotoxicity data among various fish cell lines are consistent with the basal cytotoxicity concept proposed by Ekwall (65, 66). According to Ekwall, a majority of chemicals cause acute toxicity by interference with fundamental structures and functions common to all cells, irrespective of their origin. Thus, as long as a chemical does not show a specific mode of action and/or does not attack a specific cellular function, the cytotoxic responses of various cell types to a compound should be similar.

Although cell lines are often thought of as being less differentiated than primary cultures, they represent a standardisable and easy-to-handle system with relatively low variability. Their application is more convenient and less laborious than that of primary cells, which have to be freshly isolated each time they are needed. Thus, fish cell lines have been preferred for cytotoxicity tests to date and might be expected to become more important in the future (19, 22, 23).

Culture conditions

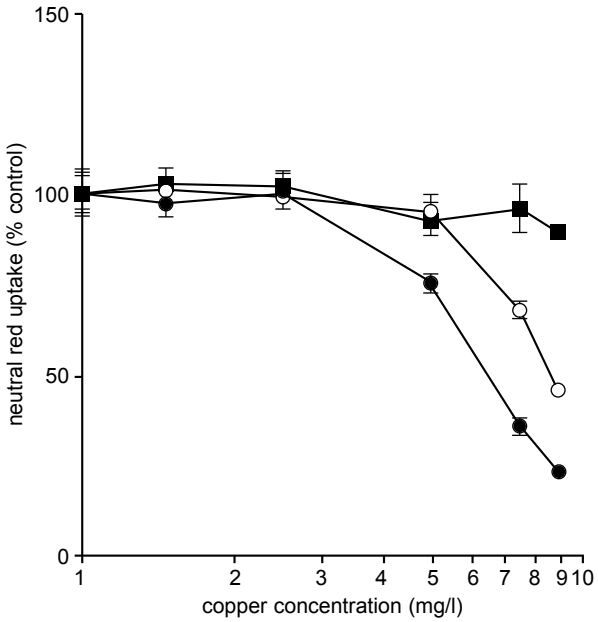
Both primary fish cells and continuous fish cell lines are usually incubated in mammalian culture media. While primary cells are often maintained in serum-free media, media for continuous fish cell lines are usually supplemented with mammalian sera, although, with some cell lines, the addition of more-defined supplements such as albumin may be adequate (67). However, the presence of mammalian serum may influence the physiology of the fish cells (68) and their toxicological responses (22, 69).

Incubation temperature is an experimental variable in fish cell culture. Primary cell cultures clearly reflect the temperature dependency of *in vivo* processes in fish, such as the temperature effect on xenobiotic metabolism or endocrine-mediated responses (70–72). With respect to fish cell lines, growth and cellular functions are strongly temperature dependent (9, 73). As a consequence, temperature affects the cytotoxic responses of fish cells (Figure 2; 74, 75).

At low incubation temperatures, fish cell lines are able to retain their viability over prolonged periods, and grow normally after returning to the optimum temperature. For example, the RTG-2 cell line derived from rainbow trout gonad can remain viable for 2 years at 4°C, without any medium change (3).

A major limitation in the more intensive application of fish cell systems is an insufficient characterisation, both with respect to their cellular and functional properties and to their culture requirements. Substantial work is still needed to support a future, more extensive use of isolated fish cells.

Figure 2: Effects of exposure temperature on copper cytotoxicity in the rainbow trout established cell line RTG-2



RTG-2 cells were exposed for 96 hours at 20°C (●), 14°C (○) and 4°C (■) (A. Castaño, unpublished data).

Values are given as means ± SD.

The Use of Fish Cells for Predicting Acute Fish Lethality

As discussed above, a number of regulatory guidelines require acute lethality tests in fish. These tests are of ethical concern, since they can inflict severe suffering on the animals. In addition, they have technical limitations; for example, extensive laboratory facilities, trained personnel for the maintenance of various fish species, and large volumes of testing samples and residues are needed. Furthermore, the scientific value of an LC50 value for ecotoxicological hazard evaluation might be questioned. Therefore, ethical, technical, scientific and economical reasons demand the development of alternatives to acute fish lethality tests.

Over the last 30 years, various authors have proposed the use of cytotoxicity tests based on fish cells as an alternative to *in vivo* tests (19, 76–80). Various *in vitro* systems have been used to test a variety of chemicals, as well as environmental samples. In most of the studies, good correlations in the toxicity rankings were obtained when comparing *in vitro* EC50 values with *in vivo* LC50 values. An example is given in Table 2, which shows that the correlation coefficients were usually higher than 0.8. Exceptions were reported for lipophilic solvents (81), certain metal complexes (82) and carbamate and organophosphate pesticides (83), where the correlations were poor. In the latter case, this can be explained by the toxic mechanism of organophosphate pesticides — inhibition of acetylcholinesterase, which would not be expected to be reflected in cell lines. Similarly, acute metal lethality to fish results from the impairment of branchial ion regulation and osmoregulation — another physiological mechanism which cannot be seen in cell lines. The data set from the international MEIC study (56–58) allows the predictive powers of various *in vitro* fish cell systems for *in vivo* toxicity in fish to be compared (Table 3). For the R1 cell line, r^2 values of 0.86–0.89 and a slope of greater than 1.2 were found for the *in vitro/in vivo* comparison of the effects of 12 MEIC chemicals (H. Segner, unpublished data). In the case of the RTG-2 cell line, r^2 values were 0.90–0.93 and the slope was 1.3 (A. Castaño, unpublished data).

Table 2: Correlation coefficients between EC50 values for various chemicals obtained with established fish cell lines and LC50 data obtained with *in vivo* tests

Fish cell line	Endpoint	Fish species	n	r	r^2	Chemical class	Reference
FHM	NRU	Golden orfe	49	0.89	0.79	Heterogeneous	81
GFS	NRU	Carp	18	0.69	0.48	Organophosphate pesticides	83
GFS	NRU	Carp	34	0.85	0.72	Pesticides	83
GFS	NRU	Fathead minnow	31	0.96	0.92	Narcotics, anilines, phenols	226
GFS	NRU	Guppy	29	0.96	0.92	Aldehydes and pesticides	226
GFS	MTT	Goldfish	7	0.96	0.93	Chlorophenols	227
GFS	MTT	Medaka	15	0.92	0.84	Chlorophenols	227
GFS	MTT	Guppy	8	0.96	0.92	Chlorophenols	227
RTG-2	ATP content	Rainbow trout	26	0.97	0.94	Heterogeneous	80
RTG-2	NRU	Rainbow trout	26	0.98	0.96	Heterogeneous	80
RTG-2	Cell detachment	Rainbow trout	26	0.98	0.95	Heterogeneous	80
BG/F	NRU	Rainbow trout	4	0.98	0.96	Organo-mercurial compounds	18
RTG-2	Cell attachment	Rainbow trout	9	0.92	0.85	Phenols, benzenes, anilines	91
RTG-2	MTT	Zebra fish	5	0.95	0.90	Heterogeneous	228
RTG-2	NRU	Zebra fish	4	0.99	0.98	Heterogeneous	228
PLHC-1	MTT	Medaka	9	0.80	0.64	Organo tin compounds	229
PLHC-1	NRU	Medaka	8	0.86	0.74	Organo tin compounds	229
BG/F	NRU	Platessa	4	0.99	0.98	Organo lead compounds	230
FHM	Total protein content	Golden orfe	25	0.90	0.81	Heterogeneous	78

NRU = neutral red uptake.

Primary cell cultures, due to their specific metabolic properties, may show *in vivo*/*in vitro* correlations different from those for cell lines. A comparison between the EC50 values of MEIC substances for freshly isolated trout hepatocytes in suspension and LC50 values for fish showed a comparatively poor agreement: r^2 was 0.53 and the slope of the regression line was 0.58 (59). This low concordance between hepatocytes and fish seems not to have been due to the use of cells in suspension or to non-homogeneous fish data. Sandbacka *et al.* tested a set of surfactants (n = 12) in primary cultures and in acute toxicity fish bioassays, and the correlation coefficient was slightly better (r^2 = 0.60), but the slope of the regression line was still poor (84).

Gill epithelial cells in suspension were more sensitive to the MEIC chemicals than were hepatocytes, and they showed a clearly better correlation with *in vivo* data for acute toxicity in fish (r^2 = 0.87,

Table 3: Correlations between *in vitro* cytotoxicity (EC50) with R1, RTG-2 fish cell lines, epithelial and hepatocyte primary fish cell cultures, and fish lethality (LC50) for 12 environmentally relevant chemicals from the MEIC list (56–58)

Correlations	r	r^2	Slope	n
Gill epithelial and fish (logmM)	0.838	0.702	0.849	12
Hepatocytes and fish (logmM)	0.742	0.551	0.757	12
R1 (NRU) and fish (logmM)	0.945	0.893	1.258	12
R1 (protein, 24 hours) and fish (logmM)	0.941	0.886	1.320	12
R1 (protein, 144 hours) and fish (logmM)	0.930	0.864	1.249	12
RTG-2 (protein) and fish (logmM)	0.951	0.905	1.343	12
RTG-2 (NRU) and fish (logmM)	0.965	0.931	1.385	12

MEIC = Multicentre Evaluation of In Vitro *Cytotoxicity; NRU = neutral red uptake.*

slope 1.07; 60). A comparison of data from primary gill epithelial cells in suspension exposed to surfactants also showed a good concordance with acute toxicity data for fish ($r^2 = 0.75$, slope 0.97), as did a similar comparison with gill epithelial cells in primary culture ($r^2 = 0.75$, slope 1.00; 84). There appear to be no differences between primary gill cells in suspension and cells in primary monolayer culture in their responses to chemicals, since a comparison with surfactants showed a very high correspondence ($r^2 = 0.99$, slope 0.98; 84). Gill epithelial cells grown in primary culture and then brought into suspension behaved in a way that was very similar to that shown by freshly isolated cells in suspension (85).

It should be noted that the *in vivo* database lacks information, which makes the *in vitro*/*in vivo* comparisons rather uncertain (86). In addition, the fish acute lethality data are non-homogeneous, i.e. the data are derived from various laboratories and were generated with various fish species. Reliable *in vivo* data (under standardised test conditions, etc.) for chemicals of ecological relevance are rare. High variability (up to four orders of magnitude) in the sensitivity of the fish to some chemicals in the acute lethality tests has been reported (87, 88). This variability was mainly due to differences in sensitivity between the various fish species to chemicals and the water quality during the test, whereas differences in the test design contributed less to the variability.

Fish cell cultures can successfully be used to study how environmental variables can modify the acute lethality of chemical contaminants. Two of the most important variables are temperature and light. An example of the latter is the photocytotoxicity of many polycyclic aromatic hydrocarbons (PAHs; 89). Eleven of sixteen PAHs showed no signs of acute lethality when presented to the rainbow trout gill cell line (RTgill-W1) in the dark, but were acutely toxic when presented concurrently with ultraviolet light irradiation, which by itself was not cytotoxic.

Fish cells have been used to test complex water samples, particularly wastewater effluents. Again, a good *in vivo*/*in vitro* correlation of toxicity ranking was obtained (77, 79, 90). In Germany, an interlaboratory study involving nine laboratories was conducted to measure the toxicity of 144 wastewater samples, both with the golden ide (*L. idus melanotus*), and in the neutral red uptake assay with the RTG-2 cell line (unpublished results). The intralaboratory reproducibility of the cytotoxicity test was found to be very good, and the interlaboratory reproducibility was at least as good as, or even better than, that of the *in vivo* assay. Further, the *in vivo* and *in vitro* tests showed good correlations in toxicity ranking: r values ranged between 0.66 and 0.74. Importantly, the *in vitro* test did not indicate any false positive samples. However, a major drawback of the cell test was its lower sensitivity; on average, the cytotoxicity assay was 10 times less sensitive than the *in vivo* test.

A difference in absolute sensitivity between *in vivo* tests and *in vitro* fish cell tests has been noted by several authors (80, 91, 92). The clearly lower absolute sensitivity is the most controversial point against the use of fish cell lines as an alternative to LC50 tests, since it would limit the replacement of the *in vivo* test in all areas where absolute sensitivity is of importance, such as in wastewater testing.

The question arises as to whether low sensitivity is specific to fish cells and might be avoided by using other cell systems. The database of the MEIC study offers the opportunity to directly compare the sensitivities of the fish cell lines, R1 and RTG-2, to those of mammalian cell lines. In Table 4, EC50 values for the first 30 MEIC chemicals of R1 and RTG-2 cells are compared with the average EC50 values from human cell lines and from non-human, mammalian cell lines. From this table, it is evident that, except for compounds with a species-specific metabolism, such as digoxin, the sensitivity of fish cells is in the same range as that of mammalian and human cells. Thus, the problem of low sensitivity of cytotoxicity assays is not a problem specific to fish cells; in other words, the replacement of fish cells by mammalian cells would not solve the problem of the too-low sensitivity (compared to fish *in vivo*) of the *in vitro* methods.

The sensitivity of cytotoxicity tests with fish cells can be increased by a number of measures related to the design of the test protocol. For instance, replacement or reduction of fetal calf serum in the test medium was shown to increase sensitivity toward some classes of chemicals (75, 93, 94; A. Castaño and co-workers, unpublished data). In addition, the selection of the cytotoxic endpoint (80, 95) or alterations to the cell density used (96) can enhance sensitivity. Nonetheless, these modifications will clearly not totally abolish the current *in vitro–in vivo* difference in sensitivity, although proteomics or gene microarrays technology could circumvent this problem in the near future.

The Use of Fish Cells in Genotoxicity

An evaluation of the genotoxic potential of a particular chemical is essential for hazard assessment in the regulatory process. However, this requirement is not included in the ecotoxicological dossier of a chemical.

From the ecotoxicological point of view, the evaluation of genotoxicity has a particular importance, because of the delayed manifestation of the

Table 4: *In vitro* cytotoxicities of the first 30 MEIC chemicals for human, mammalian and fish cell lines

Chemical	Human cell lines, 24-hour, average EC50 n = 8	Non-human mammalian cell lines, 24-hour, average EC50 n = 6	R1 24-hour, NRU50	RTG-2 48-hour, NRU50
Paracetamol	–1.85	–2.14	–1.97	–2.04
Acetylsalicylic acid	–2.28	–2.20	–2.60	–2.18
Iron(II) sulphate	–2.49	–2.28	–2.69	–1.96
Diazepam	–3.96	–3.72	–3.42	—
Amitriptyline hydrochloride	–4.10	–3.98	–4.07	–4.14
Digoxin	–6.28	–3.24	–3.01	–4.49
Ethylene glycol	–0.53	–0.14	0.04	0.59
Methanol	0.03	0.15	0.21	0.10
Ethanol	–0.23	–0.86	–0.01	–0.29
Isopropyl alcohol	–0.78	–1.16	–0.31	–0.43
1,1,1-Trichloroethane	–1.70	–1.91	–1.67	–1.87
Phenol	–2.13	–2.77	–1.91	–2.32
Sodium chloride	–1.00	–1.00	–0.74	–0.93
Sodium fluoride	–2.16	–2.87	–2.19	–2.32
Malathion	–2.83	–4.24	–3.48	–3.04
2,4-Dichlorophenoxyacetic acid	–2.61	–2.94	–2.77	–2.85
Xylene	–1.95	–2.27	–1.86	–2.21
Nicotine	–2.30	–2.16	–2.05	–1.51
Potassium cyanide	–2.43	–2.68	–1.91	–1.97
Lithium sulphate	–1.41	–1.83	–1.08	–1.49
Theophylline	–1.95	–2.13	–2.45	–1.72
Dextropropoxyphene hydrochloride	–3.25	–3.28	–3.54	—
Propranolol hydrochloride	–3.79	–3.58	–3.80	–3.46
Phenobarbital	–2.14	–2.36	–1.92	—
Paraquat	–2.74	–3.07	–1.73	–2.00
Arsenic trioxide	–4.33	–4.92	–3.41	–4.59
Copper(II) sulphate	–3.30	–3.41	–3.31	–3.46
Mercury(II) chloride	–4.71	–5.07	–5.00	–4.27
Thioridazine hydrochloride	–4.74	–4.91	–4.11	–4.38
Thallium sulphate	–3.28	–3.65	–3.28	–3.34

Data from (57, 58).

All values are given as logM.

MEIC = Multicentre Evaluation of In Vitro *Cytotoxicity; NRU = neutral red uptake.*

genotoxic effects that may require months or years to manifest fully, and these later events may be crucially important at the population and community levels. Chemical pollutants can have a selective effect against sensitive phenotypes, leading to a reduction in the overall genetic diversity of the affected population. Thus, predicting the effects of chemicals or complex mixtures on populations prior to their release, permits the establishment of safety levels in risk assessment protocols.

The assays currently used to measure the genotoxic potencies of water samples or environmental chemicals are mainly prokaryotic tests (Ames, Umu) and/or mammalian cell tests. However, a eukaryotic test for aquatic animal species exposed to environmental chemicals is desirable, since the genotoxic responses of eukaryotes to a given compound can be different from those of prokaryotes. In order to assess genotoxic effects in fish, fish cells offer the following advantages over assays based on prokaryotic or mammalian cells.

Low repair capacity

Fish cells appear to have a low DNA repair capacity compared to mammalian cells, and they may therefore be more sensitive to DNA damage. The activity of the nucleotide excision repair mechanism in fish is lower than that in mammalian cells (97). Trout cells exhibit only limited capacity for DNA repair, especially for the removal of bulky DNA adducts, and negligible glutathione transferase-mediated detoxication of the epoxide, which accounts for a high sensitivity to carcinogens (98). Comparative experiments with piscine and rodent cell lines showed a higher sensitivity of the piscine RTG-2 cells than of the mammalian V79 cells to the genotoxin, 4-nitroquinoline-*N*-oxide (T. Braunbeck and co-workers, unpublished data). However, more experimental data are needed before a conclusive statement on the enhanced sensitivity of fish cells to genotoxic agents can be made.

Fish-specific metabolism of xenobiotics

Significant species differences in the metabolic conversion of xenobiotics are evident between mammals and fish (see, for example, 99, 100), as well as between various fish species (101, 102), so target organism-derived cells should be the preferred test system for genotoxicity assessment in fish toxicology.

Many genotoxic chemicals exert their effects only after metabolic conversion to chemically reactive forms. Differences in metabolic efficiency have been shown for a variety of chemicals even between closely related species; for example, rainbow trout are three times more effective than are salmon in activating aflatoxin B1 (103). Therefore, the choice of fish cell system is important in the assessment of metabolism-mediated genotoxicity. Primary cultures of hepatocytes, gill cells and several fish cell lines, including RTG-2, BF-2, BB, FHM, PLHC-1 and RTL-W1 cells, have been found to retain at least rudimentary cytochrome P450-dependent mono-oxygenase activities, and therefore are able to metabolise compounds such as benzo[*a*]pyrene (BaP) to water soluble intermediates.

Masfaraud and co-workers (104) proved that the pattern of DNA adducts in cultured rainbow trout hepatocytes was identical to that obtained *in vivo*, suggesting that DNA repair mechanisms are active in primary cultures of rainbow trout hepatocytes. T. Braunbeck and co-workers (unpublished data) compared the genotoxicities of five chemicals to primary cultures of liver and gill epithelial cells of zebra fish with their genotoxicity to the fish cell lines, RTG-2 and RTL-W1. In both cell systems, genotoxicity was assessed by the same endpoint, the comet assay. The responses of the primary cells and the cell lines differed, depending on the test compound. For example, primary cells displayed no genotoxic response to BaP at concentrations of up to 11mg/l, while in RTG-2 cells, the lowest obervable effect concentration (LOEC) for BaP was 0.61mg/l. On the other hand, nitrofurantoin evoked no genotoxic response in RTG-2 cells at concentrations of up to 31mg/l, while nitrofurantoin had a LOEC value of 0.26mg/l in hepatocytes (T. Braunbeck and co-workers, unpublished data). Among cell lines, significant differences in sensitivity to individual genotoxicants can also occur (62).

Differences dependent on the endpoint used can be up to one order of magnitude. Using the same cell line, RTG-2, but with different endpoints, the genotoxic concentrations for BaP were 0.05mg/l for altered DNA fingerprints, 0.1mg/l for the induction of micronuclei, and 0.605mg/l in the comet assay (T. Braunbeck and co-workers, unpublished data; 105, 106). Furthermore, these concentrations are in the lower reported range of genotoxic concentrations when compared with those obtained in *in vitro* tests with mammalian cells (from 0.1mg/l to 1670mg/l) for the same chemical. The genotoxicities of pollutants are directly related to their effects on the structure and function of DNA molecules, which can be determined by using a number of laboratory methods. Fish cells have been used with a large variety of genotoxic endpoints (Table 5). Since most fish karyotypes consist of large numbers of small, irregular chromosomes, chromosomal analyses are complicated and time-consuming. Therefore, methods other than chromosomal analysis are recommended.

In addition, the use of fish cells simplifies the analysis of genotoxicity in fish populations. Genotoxic studies, based on alterations in DNA fingerprints, are difficult, because a marked genetic polymorphism in fish leads to variations in the genetic complement between individuals and populations. This fact makes genetic comparisons between control and exposed fish very difficult, particularly in field situations. Fish cells have the advantage of being a population of homozygous individuals. After exposure to genotoxic substances, alterations detected in the genomic fingerprint are the result of the interaction of the agent with the DNA, and not due to polymorphisms. Becerril and co-workers demonstrated a good comparability between *in vivo* and *in vitro* systems with respect to their genomic pattern, with an inter-population similarity index *in vivo*/*in vitro* of 0.931 (107). Also, we know that there is a good comparability between *in vivo* and *in vitro* systems with respect to genotoxic responses. Therefore, genotoxicity detected in a fish cell line exposed to environmental genotoxicants will most likely also be manifested *in vivo*, when fish are exposed to the same agent. It is probable that the agent could also alter the genomic complement of a given population (105, 107–109).

Table 5: Endpoints for estimating *in vitro* genotoxicity in primary fish cell cultures and in established fish cell lines

Endpoint	Agent	Established fish cell line	Primary fish cell culture	Reference
Cellular transformation	MAMAc	BG/F		231
Induction of ouabain-resistant mutants	BaP, MNNG	BF-2		232
	MNNG	GEM 199		233
Alkaline unwinding assay	MND, PQ	BB		234
Nick translation	Creosote-treated wharf, BaP, MNNG		Rainbow trout hepatocytes	235
Alkaline precipitation assay	Creosote-treated wharf, BaP, MNNG		Rainbow trout hepatocytes	235
DNA adduct formation	BaP, DMBA	BF-2, RTG-2, BB		183, 236
	Aflatoxin B1		Rainbow trout hepatocytes	101
	BaP		Rainbow trout hepatocytes	104
Anaphase aberration	BaP, MNNG, MMC, AA, 3-MC	RTG-2		237–239
	Marine sediments	RTG-2		238, 240, 241
	Atrazine, meturxoron, 4-chloroaniline, lindane, pentachlorophenol, alachlor, carbofuran	R1		241
	N-nitroso-*N*-methylurea	ULF-23HU		242
	BP, DBA, BA, PY	Ul-h		243
Comet assay	MNNG, H_2O_2	R1, RTG-2		244
	Organic extracts of a marine sediment	EPC		245
	Cadmium		Trout hepatocytes	246
	BaP, 4NQO	RTG-2, RTL-W1		62
	Environmental samples	RTG-2, RTL-W1		Nehls & Segner, unpublished data
	Water samples		Zebrafish hepatocytes	247
	Water samples		Zebrafish gill cells	247
	H_2O_2, BaP		Rainbow trout hepatocytes	248
	H_2O_2, 3-chloro-4-dichloromethyl-5 hydroxy-5*H*-furanone, BaP, 1-nitropyrene		Hepatocytes/ blood cells from Brown trout (*Salmo trutta*)	249

*AA = 9-aminoacridine; BA = 1,2-benzanthracene; BaP = benzo[*a*]pyrene; BP = 3,4-benzopyrene; DBA = 1,2,5,6-dibenzanthracene; DMBA = 7,12-dimethylbenz[a]anthracene; EMS = ethyl methanesulphonate; MAMAc = methylazoxymethanol acetate; 3-MC = 3-methyl cholanthrene; MMC = mitomycin C; MND = menadione; MNNG = N-methyl-N´nitro-N-nitrosoguanidine; MMS = methyl methane-sulphonate; 4NQO = 4-nitroquinoline N-oxide; PQ = 9,10-phenanthrenequinone; PY = pyrene; VS = vincristine sulphate.*

Table 5: continued

Endpoint	Agent	Established fish cell line	Primary fish cell culture	Reference
Comet assay	H_2O_2		Red blood cells (*Pleuronectes americanus*)	250
	Methyl mercury		Lymphocytes (Bottle-nosed dolphin; *Tursiops truncatus*)	251
	Cyclophosphamide		Erythrocytes (*Cyprinus carpio* and *Ameirus nebulosus*)	102
Micronucleus induction	Organo-mercurials (phenyl, ethyl, methyl)	BG/F		18
	MNNG, 4NQO	Ul-h		100
	BaP, potassium dichromate, 1,4-butane sultone, EMS	RTG-2		170
	MMC, BaP, VS	RTG-2		106
	Complex mixtures	RTG-2		171
Sister-chromatid exchange	MMC, MNNG, methyl methane sulphonate	*Ameca splendens*		252
	MNNG	ULF-23		253
	N-nitroso-*N*-methylurea	ULF-23		242
			Blood leucocytes in culture	254
			Blood leucocytes in culture	255
	EMS, MMS		Fish leucocytes	256
Unscheduled DNA repair synthesis (UDS)	MNNG, 4NQO, aflatoxin B1	RTG-2		257
	MNNG, 4NQO	Ul-h		100
	BaP, aflatoxin B1	RTG-2 + S9		149
	Sediments	BB		258
			Fish hepatocytes	259
			Fish hepatocytes	260
			Fish hepatocytes	261
	UV irradiation	CAF-MM1		262
	Gamma irradiation	CAF-MM1		263, 264
Alterations in DNA fingerprint (RAPD)	MMC, BaP	RTG-2		105, 109

*AA = 9-aminoacridine; BA = 1,2-benzanthracene; BaP = benzo[*a*]pyrene; BP = 3,4-benzopyrene; DBA = 1,2,5,6-dibenzanthracene; DMBA = 7,12-dimethylbenz[a]anthracene; EMS = ethyl methanesulphonate; MAMAc = methylazoxymethanol acetate; 3-MC = 3-methyl cholanthrene; MMC = mitomycin C; MND = menadione; MNNG = N-methyl-N´nitro-N-nitrosoguanidine; MMS = methyl methane-sulphonate; 4NQO = 4-nitroquinoline N-oxide; PQ = 9,10-phenanthrenequinone; PY = pyrene; VS = vincristine sulphate.*

The Use of Fish Cells in Endocrine Toxicity

The potential hazard represented by endocrine disrupting chemicals in the environment has been widely discussed for several years, among both scientists and the general public (110–112). There is evidence that compounds with oestrogenic activity have the potential to affect the development of the reproductive and central nervous systems, as well as behaviour and the immune response in vertebrates (113, 114). For fish *in vivo*, several biomarker endpoints have been developed for detecting and/or assessing the oestrogenic activity of xenobiotics or water samples. These endpoints include plasma steroid concentrations (115), alterations in sex differentiation (116, 117), and binding to and activation of the oestrogen receptor (118, 119), as well as induction of the production of vitellogenin, the major egg yolk precursor protein (120–125). Some oestrogenic endpoints, particularly vitellogenin production and oestrogen receptor activation, can be assessed by using piscine *in vitro* systems. Emphasis has been given to primary cultures of fish hepatocytes (for references, see 126), and routine testing with primary hepatocytes has already been initiated. In contrast, the first test systems with permanent fish cell lines and fish hepatoma cells have only recently become available (127, 128).

Cultured fish hepatocytes have been found to be particularly well-suited for studies on the induction of vitellogenin (129). The most evident function of fish liver in relation to reproduction is the synthesis and secretion of vitellogenin. Vitellogenin is specifically synthesised in the liver of oviparous female vertebrates under the control of 17β-oestradiol, secreted into the blood, and finally taken up by oocytes via receptor-mediated endocytosis.

The induction of vitellogenin protein and/or mRNA production in fish hepatocytes *in vitro* has been used to quantify the oestrogenic effects of xenobiotics (for references, see 72 and 126). The use of primary cultures of fish hepatocytes to detect vitellogenin induction by oestrogen-active compounds illustrates two important aspects of the use of primary cell isolates from fish: the pronounced temperature dependency of the cellular responses, and occasional fluctuations of cellular responsiveness. In a study with rainbow trout hepatocytes, vitellogenin induction proved to be quite temperature sensitive, with higher induction rates at 18°C, when compared to 14°C (72). Cells isolated from different donor fish and at different periods of the year showed considerable variability in vitellogenin induction, due to seasonal and inter-individual variation (130; Figure 3).

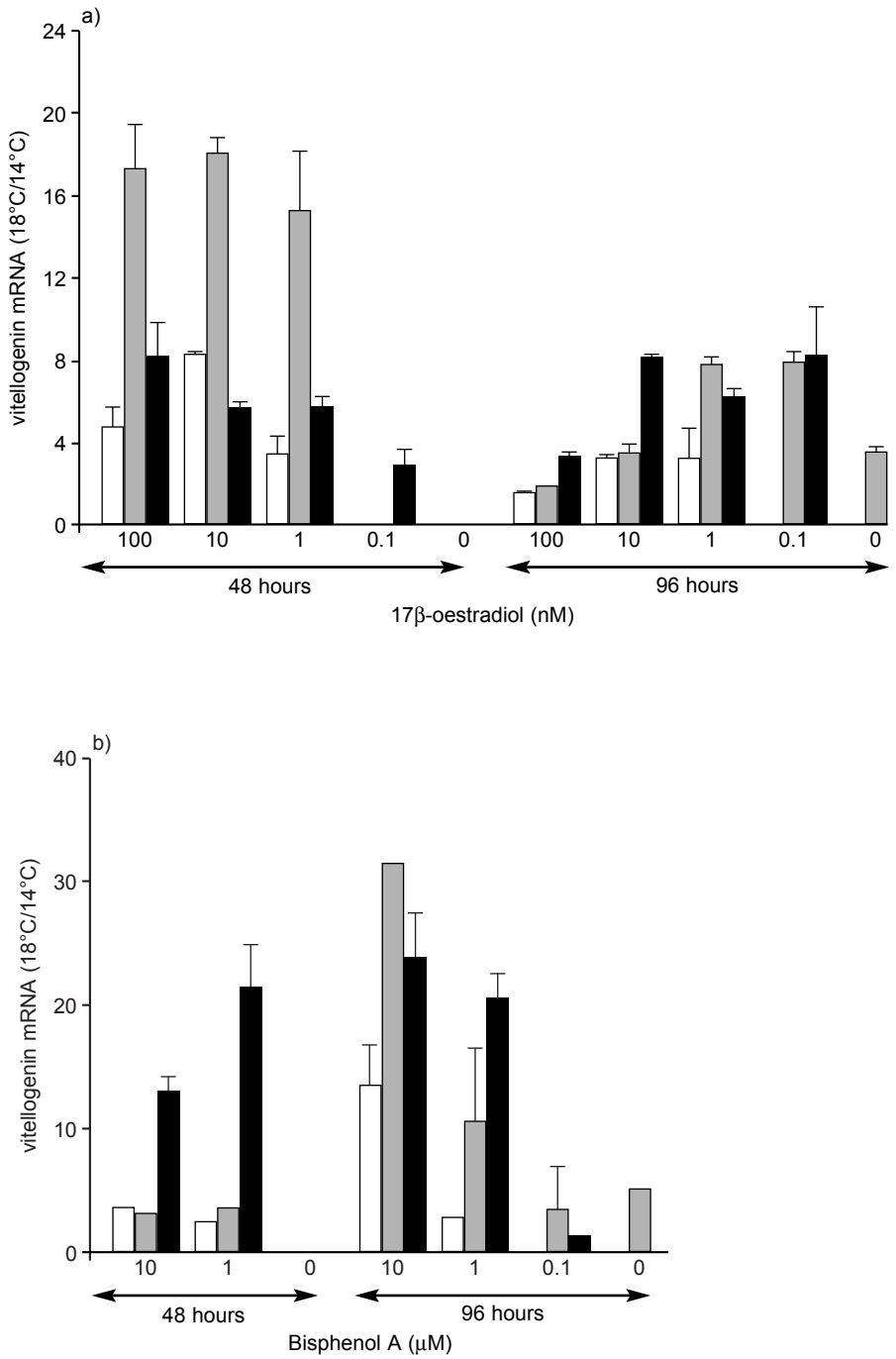
A closer analysis of the relative sensitivities of *in vitro* and *in vivo* techniques for the assessment of vitellogenin induction by oestrogens revealed that hepatocytes are less sensitive than small aquarium fish, such as zebra fish (*D. rerio*), medaka (*Oryzias latipes*) or fathead minnow (*Pimephales promelas*) in life cycle experiments (Table 6). This overt disadvantage, however, implies that false positive results with *in vitro* systems can be excluded. Nevertheless, *in vitro* systems, such as the non-radioactive dot-blot assay with primary rainbow trout hepatocytes (126), proved sensitive enough for the identification of oestrogen-positive environmental samples, including effluents from wastewater treatment plants (Figure 4). In all fish hepatocyte systems, the relative sensitivity for various endocrine disruptors was similar (Table 6).

Permanent fish cell lines have rarely been used as *in vitro* screens for oestrogen-active compounds or with environmental samples. This is due to the apparent lack of oestrogen-sensitive fish cell lines, or to an insufficient knowledge of the oestrogen-dependent cellular responses of fish cell lines. In fact, the first two studies only became available in 2000, and reported the successful detection of oestrogen-dependent responses in fish hepatoma cell lines (128) and in a transiently or permanently transfected permanent fish cell line (127). In the first case, a fish hepatoma cell line available from the American Type Culture Collection was used to visualise the expression of vitellogenin mRNA, as well as the secretion of vitellogenin into the medium under the influence of 17β-oestradiol, by means of the reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridisation techniques (128). In the second study (127), the rainbow trout gonad cell line (RTG-2) was transfected with the gene of the rainbow trout oestrogen receptor, in order to establish an oestrogen-responsive bioassay. Comparison of the EC50 values from the RTG-2 system with EC50 values reported for non-fish reporter gene assays (yeast cells, mammalian cells) revealed that, despite the fact that mammalian oestradiol receptors generally have a higher sensitivity for 17β-oestradiol, in the presence of environmental oestrogens, the rainbow trout oestradiol receptor reached half-maximal activation at similar concentrations or at somewhat lower concentrations than the non-fish systems. In contrast to the results with the RT-PCR, attempts to perform Northern blotting or ELISAs proved negative (127). Oestrogen-sensitive endpoints other than vitellogenin induction and oestrogen receptor activation that may be of use for oestrogen screening by fish cell lines, remain to be identified.

The Use of Fish Cells to Detect Dioxin-like Activity

Chemicals that elicit toxic effects similar to that of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are of great toxicological concern, due to their ability to cause hepatotoxicity, embryotoxicity, teratogenicity,

Figure 3: Temperature dependence of the oestrogenic potential of 17β-oestradiol, bisphenol A and 4-nonylphenol in isolated hepatocytes of the rainbow trout (*Oncorhynchus mykiss*)



a) 17β-oestradiol and b) bisphenol A.

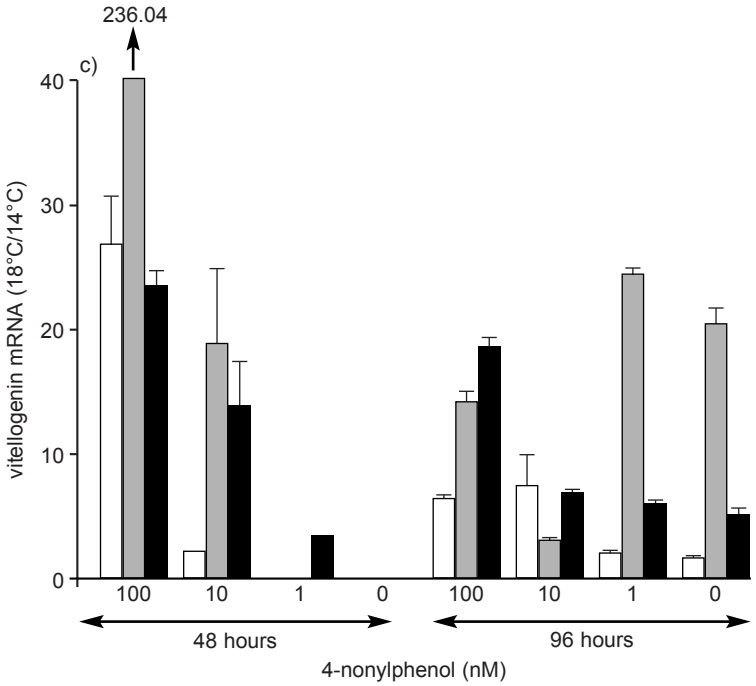
□ = *fish 1,* ▒ = *fish 2,* ■ = *fish 3.*

Data from the non-radioactive dot blot/RNAse protection assay, according to Islinger et al. *(126).*

Note the considerable variability between hepatocyte preparations from different donor fish.

Data are presented as ratios (means + SD) between 18°C and 14°C from Pawlowski et al. *(72).*

Figure 3: continued



c) 4-nonylphenol.

□ = *fish 1,* ▒ = *fish 2,* ■ = *fish 3.*

Data from the non-radioactive dot blot/RNAse protection assay, according to Islinger et al. *(126).*

Note the considerable variability between hepatocyte preparations from different donor fish.

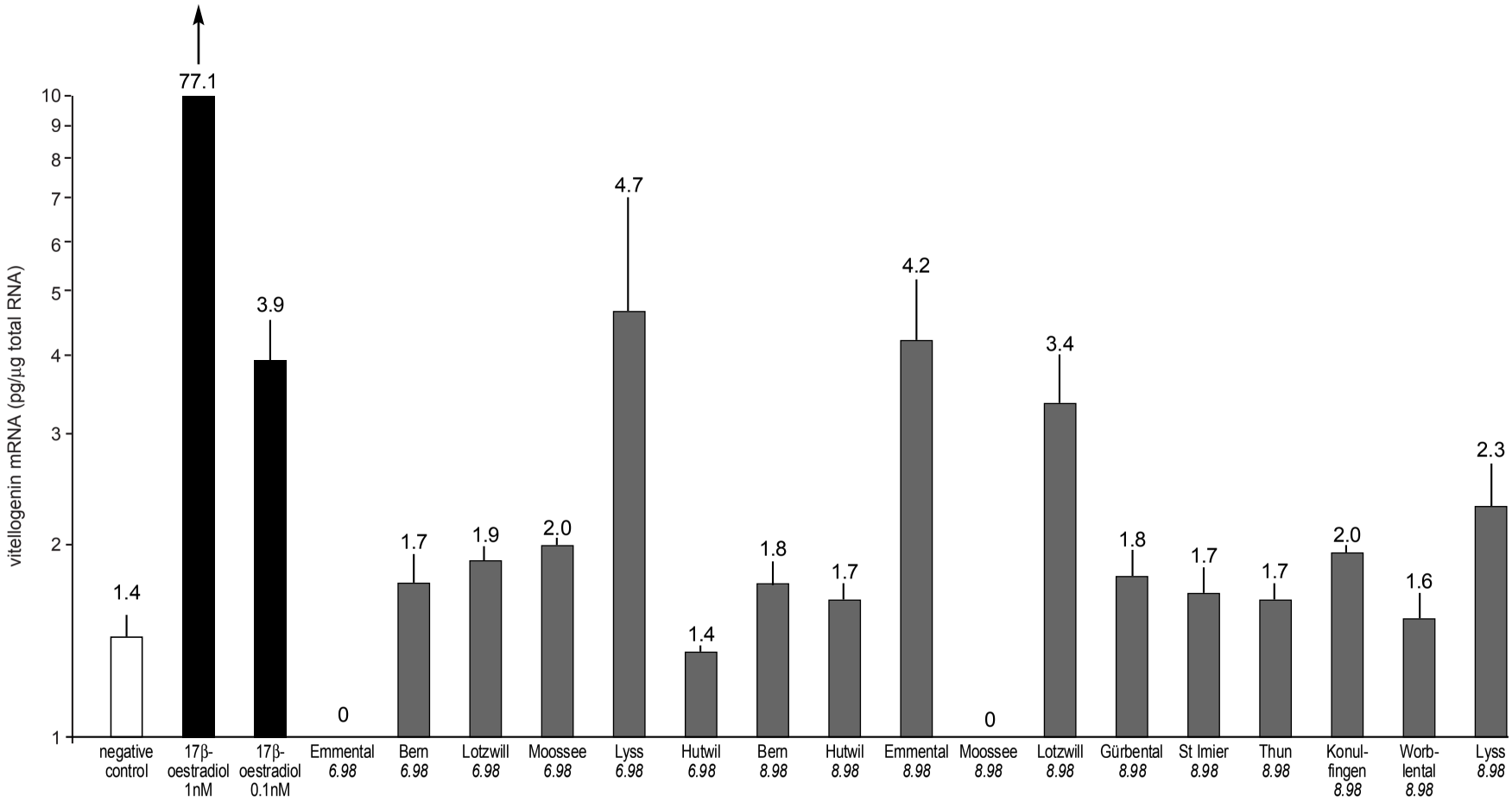
Data are presented as ratios (means + SD) between 18°C and 14°C from Pawlowski et al. *(72).*

immunotoxicity, carcinogenesis and lethality, in many different species and at low concentrations (131). Several biochemical and toxic effects of dioxin-like chemicals are mediated through the aryl hydrocarbon receptor (AhR), a ligand-dependent transcription factor located in the cytoplasm. The ligands for the AhR have been described as hydrophobic aromatic compounds with a planar structure of a particular size that fits the binding site of the AhR. These dioxin-like or AhR-active compounds include planar congeners of polychlorinated dibenzo-*p*-dioxins, polychlorinated biphenyls (PCBs), several PAHs and polychlorinated naphthalenes (132). They have various abilities to bind and activate the AhR, leading to changes in gene expression. One process regulated by the ligand-activated AhR is the induction of the gene for cytochrome P450-1A (CYP1A). In fish toxicology, the detection of CYP1A induction at the mRNA, protein or catalytic levels (usually as 7-ethoxyresorufin-*O*-deethylase [EROD] activity) has found wide application as a biomarker of exposure to compounds with a dioxin-like mode of action (133).

In most environmental samples, dioxin-like compounds occur as complex mixtures, and in order to evaluate the risk of such samples, the toxic equivalency approach has been developed (134). For this, specific dioxin-like compounds are assigned a potency or toxic equivalency factor (TEF) relative to TCDD, which has usually been found to be the most toxic dioxin-like compound and has been assigned a value of 1.0. TEFs are decided upon by an international committee, after consideration of the results of a variety of *in vivo* and *in vitro* toxicity tests (135). The concentration of a specific compound in a sample can then be expressed as a toxic equivalent concentration or quotient (TEQ) by multiplying the concentration of the compound, as determined by analytical chemistry techniques, by its TEF. Next, the dioxin-like compounds in a sample are assumed to act in an additive manner. Therefore, the TEQ for the sample can be determined by adding together the TEQs for each dioxin-like compound in the sample, and the final TEQ can be used in risk assessment.

Fish cell cultures are invaluable for providing information that can be used to derive TEFs. In whole-animal studies, some dioxin-like compounds were shown to have toxic potencies in fish that were different from those found in mammals (136, 137).

Figure 4: Induction of vitellogenin mRNA expression in isolated hepatocytes of the rainbow trout (*Oncorhynchus mykiss*), after exposure to selected effluents from sewage water treatment plants



Data from the non-radioactive dot blot/RNAse protection assay, according to Islinger et al. *(126).*

The sites (date) from which the samples were taken are indicated below each bar.

Values are given as means + SD.

Table 6: LOEC data for endocrine disrupting activity of selected chemicals in different assays

	Oestradiol	Ethynyl-oestradiol	DHEA	4-Nonyl-phenol	Octyl-phenol	Bisphe-nol A	Genistein	Methoxy-chlor
Oncorhynchus mykiss in vitro[a]	20ng/l	20ng/l		200µg/l			130µg/l	170µg/l
RTG-2, rtER[b]	90ng/l			26µg/l	27µg/l	14µg/l		
Danio rerio in vivo[a]	20ng/l	1ng/l[c]	20µg/l	20µg/l	20µg/l			
Oryzias latipes in vivo[a]	20ng/l	20ng/l	100ng/l	2µg/l	2µg/l	685µg/l		
Xenopus laevis in vivo[d]		2µg/l		22µg/l	2µg/l	22µg/l		
Pimephales promelas in vivo[a]			1–3ng/l					
Surface water[c]								
Median	1.9ng/l	3.4ng/l		0.04µg/l	0.03µg/l	0.25µg/l		
Average	6.2ng/l	9.0ng/l		0.23µg/l				
Maximum	29.1ng/l	42.9ng/l		2.72µg/l	0.27µg/l	0.41µg/l		
Sewage water effluent[c]								
Median	21.1ng/l	10.6ng/l						
Average	22.8ng/l	14.7ng/l						
Maximum	50.3ng/l	35.1ng/l						

Data summarised by T. Braunbeck according to personal communications from: [a]T. Braunbeck; [b]G. Ackermann (ETH Zurich); [c]A. Wenzel (FHG, Schmallenberg), analytically determined concentrations of oestrogenic substances; [d]W. Kloas (University of Karlsruhe/IGB).

DHEA = dehydroepiandrosterone; LOEC = lowest observable effect concentration.

Thus, TEFs derived specifically in fish systems appeared to be necessary for risk assessment. The number of compounds that potentially have dioxin-like activity is too large for them all to be tested *in vivo*. Two chemical classes of environmental importance can be used to illustrate this problem: the PCBs consist of 209 congeners, and the PAHs contain hundreds of members. CYP1A (EROD) induction in cell cultures is an inexpensive, rapid and convenient approach for obtaining relative induction potencies (REPs), which can be used as TEFs or used together with other toxicity information to decide upon TEFs. As a result, several different fish cell culture systems have been used to evaluate the ability of substances to activate the AhR pathway and to induce CYP1A (24, 138–142), including a stably transfected cell line (143).

The widest range of dioxin-like compounds have been tested with the rainbow trout liver cell line, RTL-W1 (144), and the topminnow hepatoma cell line, PLHC-1 (5, 139). In the case of RTL-W1, EROD-induction potencies have been compared with potencies in whole rainbow trout (145) and in primary rainbow trout hepatocyte cultures (69). The relative rankings for several classes of compounds were similar in these various rainbow trout systems. Also, the potencies for several dioxins, furans, non-ortho PCBs, mono-ortho PCBs, PAHs and polybrominated diphenyl ethers have been compared in RTL-W1 cells and in the rat hepatoma cell line, H4IIE (139, 146–148). Although many compounds showed similar potencies in the mammalian and piscine cells, some significant differences were found. The mono-ortho PCBs had little or no potency in RTL-W1 cells (146). Two non-ortho PCBs (118 and 149) were less potent in RTL-W1 cells, whereas two furans were 4–5 times more potent in the RTL-W1 cell line (146). These kinds of results suggest that cultures of piscine cells, rather than mammalian cells, should be used for aquatic toxicology studies on dioxin-like compounds.

The influence of species-specific TEFs on the TEQs for environmental samples has been illustrated with extracts of livers from lake trout (150, 151). Generally, the TEQs were higher when using TEFs derived with H4IIE cells than with those derived with RTL-W1 cells. However, this depended on the kinds of dioxin-like compounds in the sam-

ple. For samples in which PCBs predominated, the TEQs calculated with mammalian TEFs were higher, whereas for samples in which dioxins and furans were most abundant, the situation was reversed. The outcome of this study should dictate the set of TEFs to be used. If the purpose were to assess the risk to humans of eating the trout, the TEQs derived from mammalian TEFs would be most meaningful. If the purpose were to evaluate the risk of the dioxin burden to the fish, the piscine TEFs would appear to be best. To date, the importance of species-specific TEFs is hard to judge, as only a few out of thousands of fish species, and only 50–60 out of hundreds of potential dioxin-like compounds, have been studied. Cell cultures would be the most practical approach to this, as it is easier to expose cultures than fish, especially in the case of species that are difficult to maintain in the laboratory.

Fish cell cultures have also been used as a bioasssay tool to screen for the presence or absence of CYP1A-inducing activity and to measure the potency of the mixture extracted from environmental samples (132, 151, 152). For these purposes, fish cells seem appropriate, because the goal is to study the possible exposure and the impact of dioxin-like compounds on fish. In addition, some fish cell lines might be more sensitive than many mammalian cell lines. For example, although the magnitude of EROD induction by TCDD was always higher in H4IIE cells than in RTL-W1 cells, the EC50 was always lower (140, 145, 150). This means that a significant induction of EROD activity was seen at lower TCDD concentrations, making RTL-W1 cells a slightly more sensitive but perhaps less robust bioassay tool.

Several different types of environmental samples have been evaluated in bioassays with fish cell cultures. These include extracts from celluloses (153), fish livers (151), semi-permeable membrane devices (SPMD; 154), lake waters (155), and various sediments (155–157). In recent years, various methods have been developed to calculate the REPs from the EROD-induction data obtained after applying environmental extracts to fish cell cultures (156, 158). The data can be used to derive a bioassay TEQ (151), without analysing the sample chemically.

The Use of Fish Cells in Toxicity Testing of Complex Environmental Samples

The ecotoxicological evaluation of environmental chemical mixtures is an area of expanding possibilities for the use of fish cell systems. Industrial effluents and wastewaters contain mixtures of toxicants, such as pesticides, heavy metals, pharmaceuticals, industrial products and a variety of other substances. Basal cytotoxicity tests with fish cells, as well as tests using more specific endpoints such as genotoxicity or CYP1A induction, are most valuable in the toxicity assessment of environmental samples. The cell tests have an advantage over fish tests in that they provide for specific, rapid and cost-effective screening. In addition, they require only small sample volumes, which is of particular advantage when it comes to samples obtained, for example, via SPMD or by bioassay-directed fractionation.

The regulatory procedures used to evaluate the toxic hazard of complex mixtures are based on the chemical-analytical identification of substances and/or the biological testing of whole sample toxicity. The first approach is used in the European legislation on waste, whereby the wastes are classified mainly according to physicochemical properties and the chemicals present (159). Because the chemical-analytical identification of chemical substances is time-consuming and costly, toxicity identification and evaluation (TIE) and bioassay-directed fractionation (BDF) procedures, which combine the chemical-analytical with the biological approach (160), were developed in the USA. The bioassays employed in TIE typically include *in vivo* fish and cladoceran tests (161).

Fish cells represent a promising *in vitro* alternative to the use of fish in TIE and BDF procedures (36, 79, 156, 162, 163). *In vitro* systems represent a logistical advance over *in vivo* tests and even cladoceran testing, because the volume of each fraction required is small and the culture plates can be prepared in advance, allowing the evaluation of hundreds of fractions in a rapid and economic way. A further advantage of cell systems over fish tests is the array of endpoints that can be easily and rapidly measured with the *in vitro* test, thus leading to a much more specific toxicity characterisation of the sample than a lethality test. The endpoints used for environmental samples include basal cytotoxicity (77, 164), CYP1A induction (156, 157, 165–168), metallothionein induction (169), genotoxic responses (T. Braunbeck and co-workers, unpublished data; S. Nehls & H. Segner, unpublished data; 62, 170–172), and cytopathological alterations (79, 173).

The use of cell tests as an alternative to chemical analysis and compound identification was proposed by Castaño and co-workers, who demonstrated the suitability of this approach for a variety of industrial effluents (162). Short-term toxicity tests on *Daphnia magna*, and cytotoxicity tests with RTG-2 cells, were used as toxicity monitoring systems for water samples and fractions (174). Bioassay-directed sample fractionation permitted the detection of a toxic chemical by HPLC. The appearance of excitatory toxicity as a sublethal endpoint (for example, the increase of an energetic cellular parameter, such as ATP content) compared to non-exposed cells in sediments, effluents, and biological samples, was the basis for the detection of ecotoxi-

cological effects related to a fish canning factory (175).

Fish cell tests have been employed in bioassay-directed fractionation studies on environmental samples to identify CYP1A-inducing toxicants (156, 176). Villeneuve and co-workers sampled the Lincoln creek, an urban stream in Milwaukee, by using a biomimetic SPMD exposed for 30 days (176). Organic extracts from the triolein phase were used for analytical PAH determination and parallel EROD measurements. Brack and co-workers, in a series of papers (156, 177), undertook the identification of toxicants present in sediment probes in a river formerly impacted by industrial effluents. By using a whole biotest battery for bioassay-directed fractionation, it was shown that this river sediment carries various toxicants of high potency. Part of the contamination pattern that proved phytotoxic and cytotoxic could be attributed to fractions containing aromatic hydrocarbons. With an EROD assay with rainbow trout liver cell cultures (RTL-W1), elegant fractionation procedures permitted the separation of various halogenated and non-halogenated fractions, both of which showed CYP1A-inducing activities. One problem when applying the cellular EROD assay to environmental samples is complex interference by the many toxicants in the samples; for example, the sample may contain EROD-inhibiting compounds, or toxic effects may be initiated in the higher range of the EROD induction curve. To overcome this problem, Brack *et al.* (156) suggested toxicity equivalents on the basis of fixed effect levels. Changes in EROD activity in RTL-W1 cells were used in the bioassay-directed fractionation of a contaminated sediment extract in a German industrial region. Using fixed-effect-level TEQs, high dioxin-like activity was found in lipophilic sediment fractions containing the prototypic arylhydrocarbon receptor agonists, polychlorinated biphenyls and polycyclic aromatic hydrocarbons.

The Use of Fish Cells in Mechanistic and Biomarker Studies

For making sound hazard assessments and predictions of toxic effects, a sufficient understanding of toxic mechanisms is indispensable. To study the impact of chemical substances on biological processes at the cellular level, isolated cells are recognised as valuable models (178). This will become even more important in the future as the new concepts of genomics and proteomics become incorporated into the process. Proteomics, a leading-edge technology that provides a comprehensive, quantitative and qualitative picture of protein expression and its changes, has the potential to have a significant impact on our ability to identify toxic hazards. This could, in turn, form the basis of more-predictive risk assessments, while also improving our current understanding of the mechanisms of toxic action. There is currently very little information regarding protein expression profiles in fish at any level. Full-scale proteomic analysis is too new to be used routinely in fish and aquaculture research, and reports on fish proteomes have only just begun to appear in the scientific literature (179). Fish cells make toxicological assessments relatively simple, as automated and high-throughput technologies can be much more easily adapted to *in vitro* work than to cumbersome research with whole fish. Proteomics could bolster aquatic toxicology when fish cells are used, circumventing the reported drawbacks to proteomics technology, such as a difficulty in obtaining homogeneous tissue samples (180, 181).

For mechanistic studies, in particular, primary cells from a variety of fish tissues have been used. In the following sections, some examples are given of situations in which isolated fish cells were found to be very useful experimental models, with the potential to reduce, replace and refine *in vivo* experiments with fish.

Xenobiotic metabolism

Xenobiotic biotransformation strongly influences the fate and toxicity of a foreign compound in the organism. Due to the complexity of the biotransformation process and its regulation, *in vitro* systems are the preferred models for obtaining initial information on the metabolism of xenobiotics (182). Since biotransformation shows marked species differences, the use of piscine *in vitro* systems is essential in fish toxicology.

The metabolic enzyme repertoire of the available fish cell lines is poorly characterised. The RTG-2, BF-2, FHM, PLHC-1, RTL-W1 and BB cell lines have retained at least rudimentary CYP1A expression, and are therefore able to convert compounds such as BaP to metabolites which are more soluble in water (183, 144, 184). For biotransformation enzymes other than CYP1A, almost no information has been published to date.

Primary cultures of fish hepatocytes appear to have a higher biotransformation capacity than fish cell lines (144, 185). Fish hepatocytes in primary culture maintain CYP1A at rather stable levels during *in vitro* incubation (41). It has been shown that the metabolic profile of, for example, BaP is comparable in fish *in vivo* and in fish hepatocytes *in vitro*, at least qualitatively (186, 187), and that most of the reactions observed *in vivo* in fish, such as glucuronidation, sulphation, acetylation and oxidation, also took place *in vitro* (188). However, important differences between *in vivo* and *in vitro* metabolism have also been observed. For instance, when isolated trout hepatocytes were incubated with ani-

line, neither hydroxylated acetanilide nor its conjugate were formed, although these metabolites were present in the bile and urine of the fish exposed *in vivo* (188). The opposite situation is exemplified by a study on 2,4-dichloroaniline, in which hepatocytes *in vitro* not only produced the glucuronide conjugate, as did the fish *in vivo*, but also additionally generated the toxicologically relevant hydroxylamine metabolite (189). Clearly, comparative *in vitro/in vivo* studies would be necessary to permit more-accurate judgements on the potential of piscine *in vitro* systems for metabolic studies.

Immunotoxicology

Evaluating effects on the competence of the fish immune system is important in ecotoxicology (190, 191), as this could be a key to achieving the ecotoxicological goal of understanding the actions of toxicants on fish populations. A very general hypothesis is that fish populations could be reduced by toxicants through toxicant-mediated impairment of the immune system and a consequent increase in the susceptibility of fish to disease. The discipline of studying the impact of xenobiotics on specific parameters of the fish immune system is fish immunotoxicology, and the knowledge gained can be used to assess the risk of environmental contaminants to the health of fish populations in aquaculture, as well as in ecotoxicology.

Although fish immunotoxicology is performed at all levels of organisation (191), studies on immune cell cultures have proved to be a valuable approach, either as a complement to studies at other levels of organisation or in their own right. Primary cultures have been used most frequently, and these have usually been prepared from fish that have been exposed to a potential immunotoxicant.

After this *in vivo* (or *ex vivo*) exposure, the primary cultures are used to assess the competence of specific immune cells. Leucocyte, or sometimes more specifically, lymphocyte or macrophage, primary cultures are prepared from either peripheral blood, the head kidney (pronephros) or spleen. They can then be analysed in several ways. Their proliferative capacity can be assessed by challenging them with a T-cell mitogen (concanavalin A) or a B-cell mitogen (lipopolysaccharide). As an example of such an approach, Carlson *et al.* (192) found that both T-cell and B-cell proliferative responses were reduced in spleen leucocytes from medaka that had been injected with BaP. Another use of the primary cultures is to assess their activity as non-specific cytotoxic cells (NCC). NCC activity was reduced in channel catfish that had been injected with high concentrations of PCB 126 (193). Finally, primary cultures can be used to assess macrophage function. For example, macrophages from mummichogs near a pulp mill had less phagocytic activity than macrophages from fish downstream (194).

A different way of using primary cultures is to prepare them from unexposed fish and to add potential immunotoxicants to the cultures. The strength of this completely *in vitro* approach is its value for studying mechanisms of immunotoxicity. As an example of such studies, Duchiron *et al.* (195) found that adding lindane to rainbow trout peripheral blood leucocyte cultures stimulated the secretion of macrophage activating factor by acting on intracellular cAMP levels. Such mechanistic studies would be greatly aided by the availability of more continuous cell lines that expressed specific immune cell functions. Recently, some progress has been made in this direction. A rainbow trout spleen cell line, RTS11, has been developed, which grows well and expresses many macrophage-specific activities (196, 197). Most remarkably, catfish cell lines have been obtained that maintain the properties of B lymphocytes, T lymphocytes, natural killer-like cells, cytotoxic T lymphocytes and macrophages (198, 199). These cell lines should be valuable tools in the future for use in fish immunotoxicology, and ultimately, in ecotoxicology.

Toxicant uptake and effects at the gill barrier

Perhaps the most fundamental difference between fish and mammals is that fish possess gills. The delicate epithelial structures are designed to facilitate exchange functions by comprising as much as 60% of the total body surface area, by having a very short water–blood diffusion distance (2–5μm), by receiving the entire cardiac output, and by having a counter current flow arrangement between water and blood. The gills are multifunctional, performing the bulk of gas exchange, ion transport, acid–base regulation, osmoregulation, nitrogenous waste excretion, and the excretion of metabolites from xenobiotic transformation. Multiple cell types facilitate these functions, including so-called "chloride cells" or "mitochondria-rich cells" (MRCs) for ion transport, "respiratory" or "pavement cells" (PVCs) for both gas exchange and ion transport, mucous cells for secretion of protective mucus, and neuroepithelial cells for sensory or respiratory control functions. The dominant cell types are the PVCs, comprising typically about 85% of the total. Clearly, the gills are the first point of contact with waterborne toxicants, the major route of uptake for virtually all of them, and the key target organ for lethal damage for many of them. Indeed, the cause of systemic death in an acute toxicity test (LC50 test) is very often critical damage to gill structure and/or to one or more of the above-listed functions. This concentration of essential physiological functions into one organ — an organ that at the same time is in intimate physiological contact with the

surrounding water — explains the high sensitivity of fish relative to mammals to waterborne toxicants (see review by Wood [200]).

In consequence, when developing *in vitro* methodology for testing the toxicity of chemicals to fish, the gills and gill function are logical targets to choose. The development of methods for the primary culture of gill epithelial cells on permeable filter supports (see Figure 1) represents a potentially important advance for *in vitro* ecotoxicological assessment. Two methods are now available for the freshwater rainbow trout (full methodological details are given in Kelly *et al.* [201]), one which yields a pure PVC epithelium (54), and one which yields an epithelium consisting of about 85% PVCs and 15% MRCs, as found *in vivo* (202). The significance of these preparations is manifold. Firstly, the apical surface can be exposed to freshwater (for up to 48 hours), and the basolateral medium to a blood-like culture medium; therefore, unmodified environmental water samples can be tested, and the chemical speciation of the toxicants is preserved (i.e. no complications arise from the addition of serum or culture medium). Secondly, the preparations offer the possibility of assessing not just cellular viability, but also epithelial viability as an assay endpoint. Thus electrophysiological properties (for example, transepithelial resistance, transepithelial potential), ion transport properties (for example, radio isotopic and "cold" ion fluxes), and barrier properties (for example, tritiated water permeability, polyethylene glycol-4000 permeability, the latter a measure of paracellular permeability) can all be measured (203, 204). Thirdly, the preparations can be used to screen for toxicant bioavailability, by measuring either the amount of toxicant which binds to the epithelium (for those where the gill itself is the target), or the amount of toxicant which transits from the apical side to the basolateral compartment per unit time (for those where internal systemic actions are the target). Fourthly, the availability of preparations with PVCs only, or with PVCs plus MRCs, offers the potential to separate toxicant actions on the two cell types. Lastly, a comparable PVC epithelial preparation (from the sea bass), which tolerates apical seawater exposure, has also been developed (205), offering the potential to comparatively assess toxicants in seawater and freshwater.

The development of these preparations has been quite recent, and was driven by the need to understand basic gill function. To date, they have been largely used for this purpose (for example, 203, 204, 206–210), and very little toxicological work has been conducted. To our knowledge, only three studies have been published in which these cultured epithelia have been used for ecotoxicological assessments (85, 211, 212). The conclusions from the studies (212) indicate their great potential. Cultured trout gill cells have an inducible CYP1A activity (213), and the same is true of cultured trout gill PVC epithelia (212). The non-induced activity (measured as EROD activity) in the epithelia was non-detectable or very low. When EROD activity was measured in intact epithelia, a clear sidedness of the resorufin distribution was observed, with higher concentrations in the basolateral compartment than in the apical compartment (212). This probably indicates that carriers in the cell membranes export the metabolites from CYP1A metabolism from the cells and that there is a difference in carrier profile between the various epithelial membranes.

The use of fish cells to assess effects of prolonged exposure — a case study

Nowadays, acute exposure is not usually as common as chronic low-dose exposure in the environment. The typical exposure situation is often characterised by the long-term exposure of populations to low levels of toxic chemical mixtures. The assignment of effects to a specific chemical cause may not be possible, and acute effects may be absent. Long-term effects may be suspected and may, in fact, exist, but are very difficult to prove. Many of the anxieties voiced by environmentalists involve these non-specific low-dose effects.

A promising tool for investigating the effects on fish of prolonged exposure to low levels of toxins is the approach proposed by Mothersill *et al.* (46), which uses fish fins or skin explants *in vitro*. The system involves the harvesting of skin or fin from live or just-killed fish. The skin or fin is used to set up multiple explant cultures. These are plated singly into tissue culture flasks and can be grown for 10–15 days, or more than a month, if required. The culture remains viable without medium changes for approximately four weeks, and further careful treatment enables it to be maintained for a further three weeks or even longer. The system has great potential for investigating long-term or delayed effects, because the explants can be treated and the effects studied in cells growing from the tissue several generations after the original exposure. The explants or the cell outgrowths can be used for histological, immunocytochemical, molecular biological or electron microscopical (EM) investigations. Quantitative image analysis has been successfully applied to the explants and to the cellular outgrowths (46). The technique has been successfully used to determine the *in vitro* effects of several compounds, including heavy metals and endocrine disruptors, and of ionising and UV radiation. *In vivo* exposure experiments performed in parallel with *in vitro* exposures at similar doses, have confirmed that the technique provides data which are in the same dose range. The potential for the system as an *in vitro* test is that cell communi-

cation, differentiation, polarity and tissue architecture can be maintained during exposure of the cells. This means that spatial information can be readily obtained and quantified. The resulting explant outgrowth can then be analysed, along with the explant fragments themselves, if desired.

The endpoints for effects can include: growth inhibition/stimulation and proliferating cell nuclear antigen (PCNA) positivity in the outgrowths; numbers of mucous cells, dead cells, apoptotic cells, necrotic cells; heat shock protein expression; metallothionein expression; EM changes (for example, endoplasmic reticulum stress, mitochondrial changes, lysosomal activity or microtubule status); and delayed death and bystander signal production can be assayed by using medium transfer protocols to a clonogenic fish cell line.

This system, similar to the gill system above, has the potential for exposure of the cells directly to the test water in a way that necessitates the retention of the barrier function of the trout skin. The most remarkable aspect of this system is that the explants last for a long time in culture; this offers the possibility of studying chronic, low-dose, sublethal and delayed effects. It also permits long-term mechanisms to be investigated.

A drawback of the technique is that it is only semi-quantitative. While quantitative information can be obtained in terms of, for example, frequency of apoptotic cells or level of PCNA expression, it is not possible to standardise the number of cells in the explant or the number of cells in the outgrowth at the time of treatment. This leads to a considerable variation in results between explants from the same set-up, making standard errors large. This can be overcome by using large numbers of replicates, but this is costly and time-consuming. The increasing use of quantitative image analysis improves the results in terms of determining numbers of responding cells, but this is difficult to relate to the number of exposed cells or to the dose per exposed cell.

The use of fish cells to examine the mechanistic basis for tolerance of fish to sublethal copper exposure — a case study

The identification of applications for fish cells which are difficult or impossible to achieve with *in vivo* models could reduce whole-animal use in aquatic toxicology. The use of cell cultures enables experimental conditions to be more readily standardised (214), and also avoids complex hormonal and metabolic considerations (215). Two recent investigations (211, 216) have provided evidence that cell cultures have a distinct advantage in more-mechanistic toxicological studies. These studies centre on the effects of sublethal copper on protein synthesis rates in rainbow trout skin, liver and gill *in vivo*, and their corresponding cell types *in vitro* (i.e. rainbow trout skin cell primary cultures, hepatocytes and gill epithelial cells, respectively), and also intracellular Na^+ concentration in gill epithelial cells. Both studies suggest that the reduction of cellular energy demands is instrumental in the tolerance of sublethal copper and also clearly demonstrate that this conclusion could not be derived from investigations *in vivo*.

The protein synthesis rates of rainbow trout skin, liver and gills *in vivo* are closely correlated with the corresponding cell type *in vitro* (211, 216). Also, intracellular Na^+ concentrations in whole gills are faithfully reproduced in the gill epithelial cells (211). However, it is not possible to determine oxygen consumption rates in different tissues from a single measurement made *in vivo*. Thus, combining either tissue-specific respiratory demand with protein synthesis or gill intracellular Na^+ regulation cannot be performed with precision in whole animals. Herein lies the first major advantage of using cellular models in mechanistic investigations; simultaneous measurements *in vitro* avoid the use of different animals or groups of animals (217). Furthermore, the ability to combine homologous measurements of protein synthesis, intracellular Na^+ concentration and oxygen consumption with the use of specific inhibitors of protein synthesis or Na^+/K^+-ATPase activity (cycloheximide and ouabain, respectively), permits the derivation of the energy costs of each process. Indeed, it is when using specific inhibitors that the second major advantage of cell cultures becomes apparent. Although cycloheximide and ouabain have been used *in vivo* (218, 219), without careful evaluation of the action of these metabolic inhibitors, one must consider whether any observed effect is due to general toxicity, rather than to the inhibition of a specific process. An advantage of using isolated cells is that the validation of the inhibitor effect can be achieved much more easily *in vitro* than *in vivo* (220, 221).

Although, in most cells types, protein synthesis consumes a large amount of cellular energy (reviewed in 220), in gill epithelial cells, the majority of the cellular respiration is accounted for by maintaining intracellular Na^+ concentration (211). Again, data generated *in vitro* suggest an energy-related basis for sublethal copper tolerance, with respect to maintaining intracellular Na^+ concentration. However, there is an important difference between the energy budgets for protein synthesis and for maintaining intracellular Na^+ concentration. The exponential nature of the protein synthesis rate/synthesis cost model illustrates that the complete protein synthesis energy budget is composed of fixed and variable components (220, 222). Higher protein synthesis rates are associated with lower synthesis costs,

since the fixed component makes an ever-decreasing contribution to total energy expenditure. There is no evidence to suggest that this is the case for intracellular Na^+ regulation. Instead, reducing the energy demand for maintaining intracellular Na^+ in copper-exposed gill epithelial cells may be achieved by the de-coupling of membrane function and metabolic activity (223); this is most likely to be achieved by membrane channel arrest (211, 224).

Previously, it has been proposed that toxicity arises from pollutant interaction with basic cellular functions (65) and, in the case of sublethal copper, this does appear to be corroborated by the findings reviewed here. In conclusion, it is suggested that cellular models afford a degree of physiological resolution (particularly in the case of gill epithelial cells; 43), which it is not possible to achieve with whole animals. Thus, when applied in a mechanistic sense, there appear to be significant possibilities for the use of *in vitro* models to increase our understanding of adaptive responses by aquatic organisms.

Fish Cells Versus Mammalian Cells

The question may arise as to whether fish cell systems are needed for ecotoxicological purposes or whether mammalian cells would have the same diagnostic power. The main advantage of mammalian cells is that a larger number of established cell lines are available, and that many of them are better characterised than are the currently available fish cell lines.

The idea that chemicals exerting their acute cytotoxicity through interference with basal cellular functions should result in more-or-less similar effect levels in all cell types is reflected in the basal cytotoxicity concept of Ekwall (65, 66). This gave the underlying rationale to the MEIC project. In fact, a comparative cytotoxicity analysis of the results from the international MEIC study demonstrated a high level of correlation of the cytotoxicities of the 50 MEIC compounds in cells from humans, rodents and fish (57, 58). Fish cells predicted the human cytotoxicity of the MEIC chemicals with an r^2 of 0.87–0.91, in parity with the prediction by mammalian cells (r^2 = 0.87–0.91).

The conclusion from the MEIC study is that, when it comes to acute basal cytotoxicity, any cell system would do, be it piscine or mammalian. Accepting this conclusion means that fish cells should have a wider application in general cytotoxicity testing than is currently appreciated. Several practical considerations favour the use of fish cells. They can be stored at 4°C for long periods without deterioration in quality or changes of incubation media. They can be transported between laboratories on ice, and can be seeded in plates or in vials. Incubation can be carried out at room temperature and atmosphere, i.e. under conditions present in every laboratory. Specialised culture facilities are not needed, although access to a clean air bench is an advantage. Non-sterile environmental samples can be tested without sterility problems and previous treatments that may change the characteristics of the sample, because of the low incubation temperature compared to that used for mammalian cells. In addition, fish cells can be exposed to aquatic environmental samples at various osmolarities (from 200mosmol to 600mosmol [225]), something that can be done in mammals only with renal cells.

However, when it comes to species-specific or taxa-specific toxicity processes, only fish cells can provide a suitable *in vitro* model. For instance, species differences in xenobiotic metabolism are an important argument for the use of species-specific *in vitro* cell systems. Also, the MEIC study clearly showed that a species-specific cell system better predicts *in vivo* toxicity for the species of concern: human cells *in vitro* predicted acute systemic toxicity to humans *in vivo* better than rodent cells and fish cells (55, 56). The better performance of human cells was explained by their response to compounds such as digoxin, which undergoes species-specific metabolism.

Several fish-specific toxicity mechanisms can only be reflected *in vitro* by using fish cells. Interactions at the branchial epithelium are a central toxicological mechanism in fish, for which no corresponding mammalian cell system is available. Gill epithelia cultured *in vitro* can be used to target this kind of toxicity mechanism. Additionally, since the epithelia tolerate direct exposure to environmental water samples, it is possible to investigate *in vitro* the impact of water quality on bioavailability and toxicity. The way that temperature affects toxicity, as an experimental variable, is a clear example of the advantages of using fish cells, as is their choice for use in genotoxicity studies, because of their greater sensitivity. Another example is oestrogen-induced vitellogenin synthesis in fish hepatocytes, which is a "clean" and straightforward response to oestrogenic activity, with few, if any, interferences. The determination of "fish-specific" TEF values is another example of fish cells being superior to mammalian cells.

Conclusions and Recommendations

Conclusions

1. Currently, fish toxicity testing is mainly based on *in vivo* tests. The *in vivo* tests are of ethical concern, and they suffer from technical limitations and low cost–benefit efficiency. Their ecotoxicological relevance is doubtful, since they are not able to detect a number of relevant toxic effects.

2. To date, the use of fish cells in ecotoxicology has mainly been focused on the measurement of: a) cytotoxicity, both basal and selective (cell-specific); b) genotoxicity; and c) effects on cell-specific functions and parameters, including studies on biotransformation and the induction of biomarkers, or mechanisms of toxicity. *In vitro* systems based on fish cells have been found to be valuable:

   i) For the toxicity ranking and classification of chemicals, including quantitative structure–activity relationship studies: These types of studies are largely based on the measurement of basal cytotoxicity, where fish cells perform as well as mammalian cell systems. The more-practical handling of fish cells even favours the replacement of mammalian cells for some purposes.

   ii) For toxicity measurements on environmental samples: For this type of sample, fish cells offer a number of technical advantages over mammalian cells, as well as over *in vivo* fish tests, particularly for bioassay-directed fractionation studies, which are of increasing importance in environmental toxicology. The rapid, differentiated effects assessments provided by fish cells, as well as their need for only small sample volumes, represent distinct advantages over *in vivo* assays.

   iii) For toxicity characterisation of chemicals and environmental samples: Fish cells *in vitro* provide a bioanalytical tool for the assessment of differentiated effects, and the possibility of screening in a rapid and cost-effective way, for a wide range of endpoints (for example, cytotoxicity, genotoxicity, dioxin-like activity, endocrine disruptor activity). Fish cells can be used to develop relevant ecotoxicological endpoints (for example, the prediction of genotoxic effects in terms of genetic diversity).The introduction of genomics and proteomics will further enhance the potential value of fish *in vitro* systems.

   iv) For studies on toxic modes of action: Fish cells *in vitro* can address aspects of toxic mechanisms that are difficult to study *in vivo*; for example, chemical-induced receptor activation, attacks on the permeability properties of the gills, or temperature effects. A further advantage of the *in vitro* system is that it is based on a relatively uniform cell type. Compared to mammalian cells, fish cells are the models of choice for investigations on fish-specific toxicokinetic and toxicodynamic processes.

3. The reduction and complementation of *in vivo* ecotoxicity tests by *in vitro* assays could greatly improve the quality of ecotoxicological hazard assessments. The *in vitro* systems offer the possibility of the more-differentiated assessment of effects, involving the use of more endpoints, and could provide the necessary mechanistic understanding for effect prediction and the systematic classification of chemicals.

4. However, a disadvantage of fish cell systems, which they share with other cell systems, is their lower absolute sensitivity compared to the fish used in *in vivo* tests. The absolute sensitivity of tests is of relevance, for example, in the determination of environmentally safe levels of chemicals. This, however, is usually not done on the basis of a single test, but the results from several tests (32).

5. The data presented in this report clearly show that fish cell assays offer vast opportunities for improved (better, faster, cheaper) hazard identification in environmental risk assessment. The challenge now is to translate this knowledge into concrete actions on the standardisation and validation of fish cell tests, as a prerequisite for their incorporation into ecotoxicity testing strategies.

Recommendations

In order to promote the application of fish cell systems and to reduce the number of fish used in ecotoxicity testing, the following recommendations should be considered.

1. A better characterisation of the available fish cell systems is needed. For most of the fish cell systems used to date, only incomplete data are available on their culture requirements, sensitivities, responsiveness to specific chemical groups, physiological repertoires, etc. This hampers their directed and selective use.

2. The most promising fish cell systems should be further developed and standardised. Until now, no generally accepted standards on the use of fish cells for ecotoxicity testing are available. Optimised and standardised protocols, as well as prediction models, would be essential for prevalidation/validation studies.

3. *In vitro* data on ecotoxicologically relevant compounds should be generated with such standardised protocols, since many of the compounds tested with fish cells are not really relevant in ecotoxicology.

4. Efforts should be undertaken to obtain more standardised *in vivo* fish data for use in *in vivo/in vitro* comparisons. This could be achieved by using the *in vivo* data submitted to the European Chemicals Bureau, provided that their confidential use was respected.

5. ECVAM should establish a Task Force, which should select the most promising fish cell systems and develop a strategy for their optimisation and future application.

6. With regard to the EC White Paper, *Strategy for a Future Chemicals Policy*, it should be evaluated whether the combination of fish cell tests with other, rather sensitive tests, such as the acute *Daphnia* immobilisation test or the algal growth test, may be an appropriate approach to reducing the use of fish in chemicals testing, while still maintaining sensitive hazard classification. Considering the large number of chemicals for which little or no information about their ecotoxic potential is available, and which must be tested in the near future, such a strategy could not only save a large number of fish, but would also reduce the costs involved.

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