The MolDarT – a new toxicity test

Many toxicity tests involve the use of adult fish and take lethality as the sole criterion for assessing the toxic effects of a substance, leaving the underlying toxic mechanisms unclear. We have therefore developed a bioassay that provides information on the molecular effects of toxicants – and does not require the use of adult fish.

More than 100,000 commercially used chemical compounds are currently registered in the European Union [1]. Under the new EU REACH legislation (Registration, Evaluation and Authorisation of Chemicals [2]), which came into effect in June 2007, existing chemicals and all new substances, starting materials and intermediates exceeding an annual production volume of 1 tonne have to be tested for toxic effects. These requirements cover an estimated 30,000 substances. For toxicity studies, the Organisation for Economic Co-operation and Development (OECD) recommends the use of appropriate model organisms and a series of tests for the assessment of acute and chronic toxic effects. These involve the exposure of adult fish to contaminants in water for periods of 96 hours (acute) or at least 14 days (chronic toxicity). The toxicological criterion applied is lethality – a non-specific, integrative endpoint, which depends on the concentration and toxicity of the compounds tested. In vivo tests of this kind are
time-consuming and raise animal welfare concerns. In addition, although they reveal the lethal (acute toxic) dose, they do not provide any further information on the mechanism of action of the substances concerned. Our aim, therefore, was to develop a test that could not only be performed rapidly and without using adult animals, but would also allow the effects of chemicals to be studied at the molecular level.

**Seeking to combine the advantages of in vivo and in vitro tests.** For several years now, efforts have been made to identify the molecular targets of toxic chemicals and thereby gain a better understanding of their mechanisms of action [3]. Such tests – normally carried out in vitro on single-celled organisms (bacteria, yeast), cell cultures or cell components, rather than in vivo, using multicellular organisms – are designed to detect specific molecular effects. For example, the yeast estrogen screen (YES [4]) assay uses genetically modified yeast cells to test chemicals for estrogenic effects, which are indicated by a change in colour.

The key advantages of in vitro test systems include generally short exposure periods, the use of small quantities of living material and the avoidance of animal experiments. The disadvantage, however, is the lack of biological relevance. It is simply assumed that the effects observed in single-celled organisms likewise occur in vertebrates, even though the complex uptake and metabolic transformation of substances from the environment are lacking in in vitro systems – both processes that significantly influence the toxic effects of chemicals on higher organisms. An ideal system, therefore, would combine the positive aspects of traditional in vivo tests with those of molecular in vitro assays.

**Zebrafish used as a model organism.** In ecotoxicology, the DarT (Danio rerio teratogenicity) assay [5] has been used for a number of years to assess the acute toxicity of chemicals. Unlike other fish tests, it involves eggs and embryos rather than adult fish. Zebrafish (Danio rerio) eggs develop very rapidly: about 48 hours post fertilization, the embryos are fully developed and ready to hatch. A wide variety of tissues are present in rudimentary form, and differentiation of certain organs is already well advanced. The DarT test is used to study disorders of development, e.g. detachment of the tail from the yolk, and embryo mortality occurring after exposure to chemicals. The procedure for the DarT test is specified in a DIN standard [6].

In addition, the zebrafish has been widely studied in the life sciences in recent decades. It was one of the first vertebrate species to have its genome fully sequenced, and many molecular biological methods are available which were specifically developed for the zebrafish. There are thus many good reasons for choosing this species as a model organism. The novel test system developed at Eawag is essentially based on the DarT assay, but also screens for molecular effects that are detectable in the subacute toxicity range. For this reason, the new system is called the molecular Danio rerio teratogenicity test (MolDarT [7, 8]).

**How does the MolDarT work?** The endpoints used in the MolDarT are genes whose expression is either up- or down-regulated by certain chemicals. The expression of these genes is assessed at the mRNA level, the intermediate stage between gene and protein. Comparison with an unexposed control allows us to determine whether exposure to chemicals had an influence on mRNA abundance and hence on expression of the biomarker gene.

In practice, the MolDarT is relatively simple to carry out: groups of approx. 50 freshly fertilized zebrafish eggs are kept in Petri dishes containing solutions of chemicals in water. As the MolDarT involves exposure to concentrations in the subacute range, the exposed eggs/larvae do not differ from the controls in morphology or behaviour. After an exposure period of 120 hours (5 days), total mRNA is isolated from the embryos, and real-time PCR is used to assess relative mRNA abundance for the target genes. To correct for losses occurring during isolation and processing of the mRNA, expression levels are internally normalized. For this purpose, expression of the biomarker genes is calculated relative to a gene that is expressed regardless of cell type, cell stage and external influences, and is thus not affected by pollutants (a so-called housekeeping gene).

**First biomarker gene in MolDarT: vitellogenin.** We wished to establish whether vitellogenin 1, a gene that has already been extensively studied, could serve as a biomarker for estrogenic substances in the MolDarT system. The vitellogenin gene codes for an egg yolk protein, and its activity is regulated by endogenous estrogens. It is normally only expressed in adult females. However, it can also be induced in males if they are exposed to estrogenic substances in the environment [9].

Before the initial exposure tests, we first investigated whether, after fertilization, the vitellogenin gene is also expressed in zebrafish eggs and embryos prior to sexual differentiation.

At 4 hours post fertilization, vitellogenin mRNA cannot yet be detected, but after 24 hours the activity of the vitellogenin gene

![Fig. 1: Natural expression of the biomarker gene vitellogenin 1 in the course of zebrafish embryo development. The values marked with asterisks differ significantly from the first measurement after 24 hours; * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.](image)
is increased, and it remains at a relatively high level during the period between 48 and 120 hours post fertilization (Fig. 1).

**Vitellogenin gene activated by estrogens and other endocrine disruptors.** How do vitellogenin expression patterns compare with these findings when zebrafish eggs are exposed to estrogens and other endocrine disrupting substances? For our series of experiments, we used estrogenic substances that are frequently detected in the aquatic environment.

We found that the synthetic hormone ethinyl estradiol, which is used in oral contraceptives, does indeed stimulate gene activity, with expression of the vitellogenin gene depending on the concentration of the substance in the exposure solution and on the duration of exposure (Fig. 2). Comparable results were obtained with the natural estrogen estradiol. In addition, the vitellogenin gene was induced by bisphenol A, a compound used in large quantities in the manufacture of polycarbonate plastics. Bisphenol A is one of the most widely produced chemicals worldwide.

The estrogenic effects of ethinyl estradiol, estradiol and bisphenol A had previously been demonstrated in the YES assay. For other known endocrine disruptors, such as the fungicide cyproconazole, induction of the vitellogenin gene could not be detected in the MolDarT. Although cyproconazole showed positive results in the YES assay, it is presumably metabolized in zebrafish eggs, yielding non-estrogenic metabolites. This shows that, as an in vivo test, the MolDarT is closer to real-life conditions than an in vitro test such as the YES assay.

**MolDarT: a modular assay, adaptable to specific requirements.** In principle, it is possible to investigate as many biomarker genes as desired in an exposure test, with the MolDarT being adapted to new findings and individual research questions. At Eawag, four modules have so far been developed (see also the article by Liedtke on p. 13):
- estrogenicity (vitellogenin 1 gene),
- immunotoxicity (recombination activation gene),
- metal toxicity (metallothionein 2 gene), and
- toxicity of polycyclic aromatic hydrocarbons and dioxins (gene coding for cytochrome P450 aromatase).

With the MolDarT, screening for specific toxic effects can be carried out rapidly, at low cost, using small amounts of cellular material and above all without the need for adult animals. Even though ecological relevance remains to be demonstrated for the biomarker genes studied to date, the MolDarT is a potentially suitable tool for evaluating the ecotoxicological risks of the many chemicals that await assessment following the introduction of the new EU REACH legislation. In addition, the MolDarT can also be used in the ecotoxicological assessment of environmental samples of unknown composition.

![Fig. 2: Expression of the biomarker gene vitellogenin 1 as a function of time in zebrafish embryos exposed to various concentrations of ethinyl estradiol (EE2). The standard deviation is not greater than 30%.](image-url)