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Eawag News

Zebrafish as ecotoxicity sensors

Editorial

Rik Eggen, a molecular biologist, was head of the Environmental Toxicology Department prior to his appointment as Deputy Director of Eawag.

Zebrafish – the aquatic mouse

When the zebrafish or zebra danio *(Danio rerio)* was first described in 1822, no one could have foreseen its subsequent rise to prominence. Today, this blue-and-silver-striped fish is not only popular among aquarists – the species has also been "discovered" by scientists and is commonly found in research laboratories. The fish, only 5–6 cm in length, originally aroused the interest of medical scientists and developmental biologists who were looking for a vertebrate model. Researchers were attracted by the numerous advantages of zebrafish – they are hardy and easy to keep, reproduce prolifically, develop from fertilized egg to larva in only 24 hours and are ideally suited for genetic analysis.

At Eawag, the idea of using the zebrafish as a model organism in environmental toxicology arose about eight years ago. At that time, researchers were alarmed by reports of endocrine-disrupting chemicals and suspected that substances of this kind could be responsible for the increased prevalence of fish with malformed reproductive organs. It was already possible to detect endocrine disruptors in water samples with the aid of *in vitro* tests; these methods – still used today – involve single-celled organisms (bacteria, yeasts), cell cultures or cell components. What was lacking, however, was a model organism that could be used to investigate the causal links between endocrine disruptors and gonad abnormalities. As zebrafish appeared to be highly suitable for this purpose, we started to build up our fish lab at the beginning of 2001. In collaboration with the developmental biologist Stephan Neuhauss of Zurich University and the fish physiologist Helmut Segner of Bern University – both of whom had already been working with zebrafish for some time – we studied the precise mechanisms of action of endocrine-disrupting chemicals as part of the National Research Programme NRP 50 "Endocrine disruptors: relevance to humans, animals and ecosystems" and in an EU-funded project. Some of our key findings are presented in this issue of *Eawag News*.

Our environmental toxicological research in zebrafish is also focusing on the development of new toxicity tests. One of our goals is to reduce the use of adult animals for toxicity testing. At the same time, these tests should elucidate the mode of action of the substances concerned. It therefore makes sense to define molecular endpoints rather than relying – as has been the case to date – on observations of relatively unspecific criteria, such as morphological changes or mortality rates. The molecular *Danio rerio* teratogenicity test (MolDarT), developed at Eawag and already evaluated for applicability in practice, marks a major step in this direction. With the identification of new molecular biomarkers, the MolDarT test system can be extended to include additional modules at any time.

Several years ago, only a few environmental toxicological research groups were working with zebrafish; now, however, the number is increasing rapidly. In addition, zebrafish are replacing rodents in many life-science laboratories, so it is not surprising that these fish have been dubbed "aquatic mice". Eawag will continue to use zebrafish, e.g. at the new centre for applied ecotoxicology which is to be operated jointly with the Federal Institute of Technology Lausanne (EPFL). For one thing is certain: the potential of these model organisms for research in environmental toxicology has yet to be fully exploited.

Cover photo: Zebrafish *(Danio rerio)*. © Manu Heim, Eawag.

Content

Lead Article

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eawag aquatic research

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Miscellaneous

Martina Bauchrowitz, biologist and editor of Eawag News.

Zebrafish as models

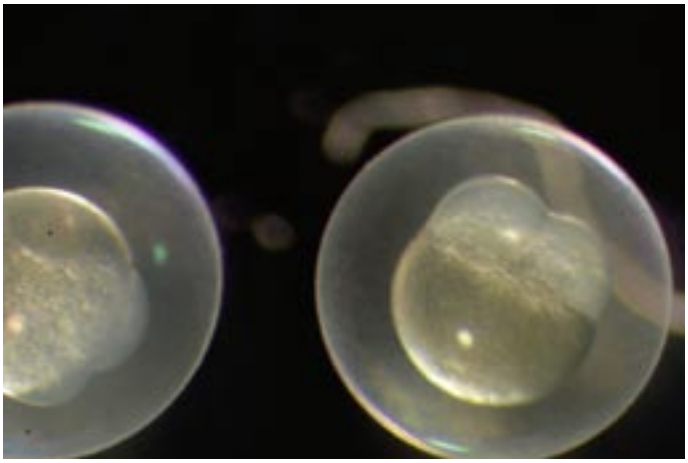
Around 8 years ago, research on zebrafish began in the Environmental Toxicology Department. Since then, an active team led by molecular biologist Rik Eggen has been working with this new model organism. Now the researchers have an opportunity to give an account of their work.

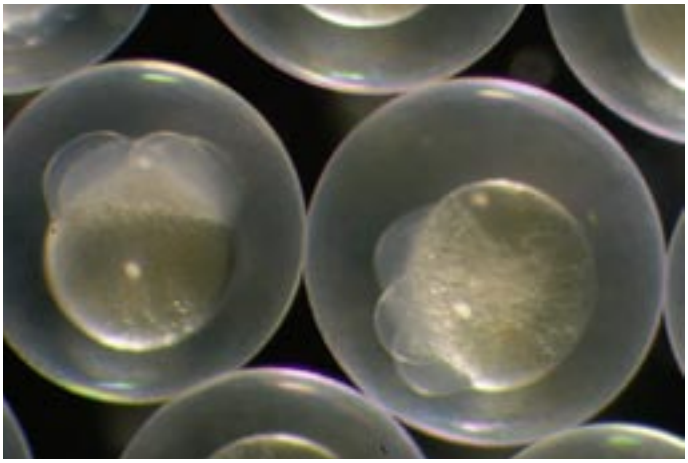
"The idea had been knocking around in my head for a long time," says Rik Eggen, former head of the Environmental Toxicology Department and now Deputy Director, as he enthusiastically explains how the zebrafish came to be established as an ecotoxicological model system at Eawag. In the late 1990s, rather than continuing to work with single-celled organisms (e.g. bacteria, yeast and algae) or cell cultures, he was looking for a more advanced, whole-animal model. In the literature, he had read about zebrafish, which at that time were making waves particularly in medicine and developmental biology. As Eggen points out, these fish, which are only 5–6 cm long, offer a variety of advantages. They have a short generation interval and are sexually mature after 3–4 months; the females lay large numbers of eggs, which are fertilized externally by the males; and the transparent embryos develop entirely outside the mother's body (see photographs), making it easy to detect any morphological abnormalities.

Warm water preferred. Finally, in 2001, the first batch of 20 zebrafish was acquired. Today, Eawag's aquariums house some 600 zebrafish, and fertilized eggs are used on a daily basis for ecotoxicological experiments. Lab technician Karin Rüfenacht has been involved from the outset (see the article on p. 8). The welfare of the fish is very important to her: although it doesn't often happen that one of the fish in her care has to be killed, if it does, she is still saddened after all these years. Fortunately, zebrafish are a fairly undemanding species and easy to keep: their requirements, as regards water, feed and tank size, are modest. However, they do have a preference for warm water – after all, they are indigenous to tributaries of the Ganges in India, Bangladesh and Pakistan. Taxonomically, the zebrafish *(Danio rerio)* is a member of the carp family (Cyprinidae) – one of the largest families of freshwater fish, with more than 1400 species.

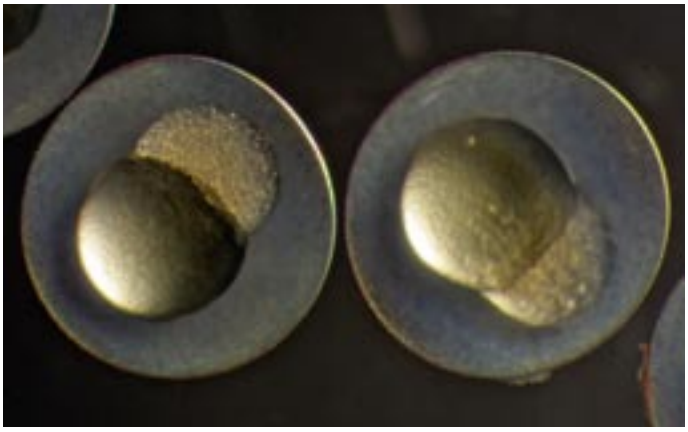
A new toxicity test. The first doctoral student at Eawag to work with zebrafish was Jane Muncke. Together with her then-supervisor Nina Schweigert, she had the task of developing a molecular zebrafish assay to screen for environmental toxicants in water (see the article on p. 10). Muncke explains: "People simply assumed that the effects of toxic substances, as observed in toxicity tests involving single-celled organisms or cell cultures, are also manifested in vertebrates in just the same way. But it's completely unclear whether that's true – and actually it's not to be expected." Indeed, two factors that significantly influence the toxicity of chemicals need to be considered: firstly, the uptake of substances from the environment is much more complex in vertebrates than in single-celled organisms and, secondly, vertebrates are in a position to transform chemicals by metabolism. "That's why we were determined to develop a vertebrate toxicity test.

The fertilized egg cells divide: the 2-, 4-cell and …





Photos: Steve Baskauf 2002, http://bioimages.vanderbilt.edu/



The early blastula stage (about 2.3 hours post fertilization): a mass of cells forms at one end of the yolk sac.

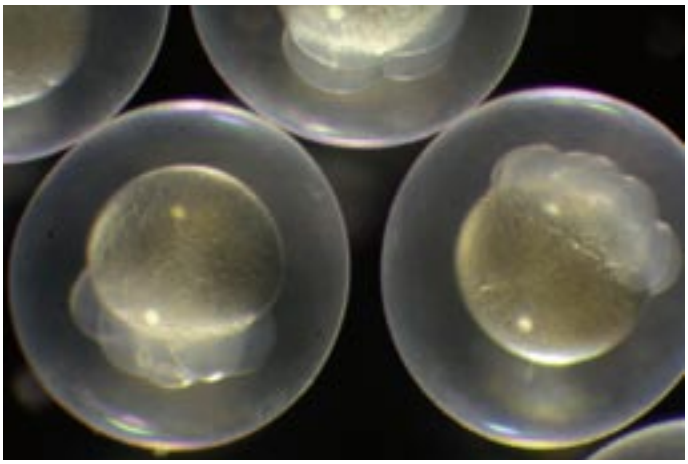
Other major plus points are the fact that the assay doesn't require the use of adult fish and also provides an insight into the mechanisms of action of chemicals at the molecular level."

What causes abnormalities in Lake Thun's whitefish? The first opportunity to test the newly developed zebrafish toxicity bioassay in practice arose quite recently in connection with the Lake Thun project. "The lake harbours a mystery," says Anja Liedtke. "Nobody knows why, over the last few years, so many whitefish with malformed reproductive organs have been found." The biologist, who joined Eawag after completing her thesis at the Helmholtz Centre for Environmental Research (UFZ) in Leipzig, is studying whether these deformities are caused by pollutants and in particular by endocrine disruptors (see the article on p. 13). For this purpose, as well as other toxicity tests, she has used the zebrafish assay.

Effects of endocrine disruptors on fish. The problem of gonadal abnormalities in fish is not, however, confined to Lake Thun. In the 1990s, an increasing number of reports concerning the feminization of male fish appeared around the world. Scientists suspected a direct link between the occurrence of endocrine disruptors (see Box) – which were being detected ever more frequently in surface waters – and sex reversal in fish. Rik Eggen comments: "The fact that, in the end, this causal relationship can only be demonstrated if you actually work with fish was another important reason why Eawag chose zebrafish as a model organism." At that time, it was considered to be particularly likely that estrogens – the female sex hormones – were a target for endocrine disruptors. Eggen's group, therefore, used zebrafish to explore various aspects of this subject as part of the National Research Programme (NRP 50) on "Endocrine Disruptors – Relevance to Humans, Animals and Ecosystems" – a programme funded by the Swiss National Science Foundation and involving a total of 26 project teams [1].

Sexual differentiation in fish. In order to identify the causes of changes in the reproductive organs, it is first necessary to gain a better understanding of the process of normal sexual differen-

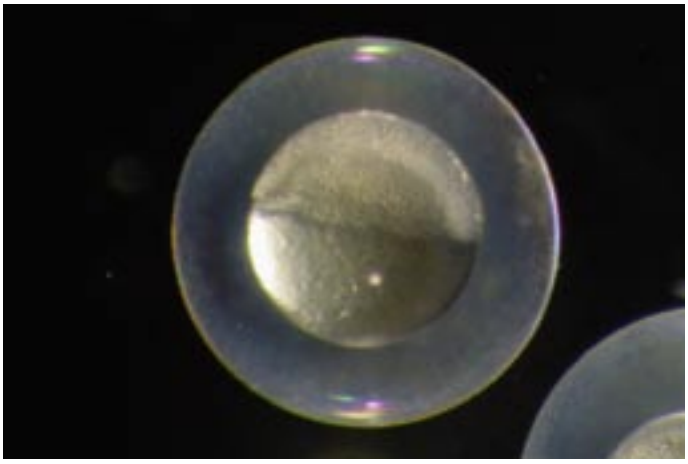
… 8-cell stages.



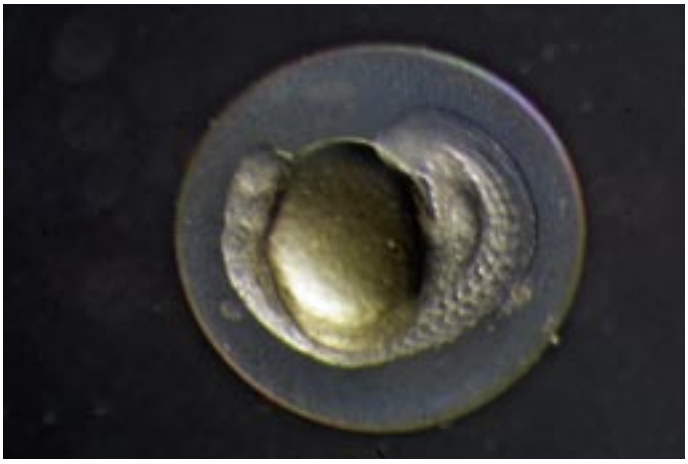
What are endocrine disruptors?

Endocrine disruptors are substances that interfere with the endocrine system – and in particular the sex hormones – of humans and animals. To date, only a small proportion of the roughly 100,000 chemicals currently on the market have been tested for possible endocrine activity. However, a number of known endocrine disruptors are frequently detected in surface waters. These include natural and synthetic estrogens (e.g. the active ingredient of oral contraceptives), several musk compounds from artificial fragrances, some UV filters used in sunscreens, certain antioxidants in cosmetics and preservatives in foods, a wide variety of industrial chemicals, and dioxins (see separate Box).

Summing up the current state of knowledge and recommending future measures at the end of the National Research Programme (NRP 50) on "Endocrine Disruptors – Relevance to Humans, Animals and Ecosystems", the Consensus Platform "Endocrine Disruptors in Waste Water and in the Aquatic Environment" [2] concludes that estrogenic steroid hormones are to be viewed as the main cause of any estrogen effects in aquatic organisms. Accordingly, the working group, comprised of representatives from industry, regulatory authorities, professional associations and research, calls for the introduction of a quality objective for estrogen activity in the aquatic environment – particularly for sensitive receiving waters in which treated wastewater is insufficiently diluted.



The early gastrula stage (about 5.3 hours post fertilization): formation of the three germ layers (ectoderm, endoderm and mesoderm) from which the organs develop.



24-hour-old embryo: the notocord is already detectable.

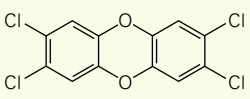
tiation. It was generally assumed in the literature that zebrafish develop female sex organs if estrogen levels in the brain are high at a certain developmental stage, with male gonads developing in the presence of low estrogen concentrations. In this process, a specific enzyme involved in estrogen biosynthesis was believed to play a key role. This hypothesis was tested by doctoral student Evi Kallivretaki. Her findings show that the process is in fact considerably more complex, probably requiring the interaction of sex hormones with various other factors in a fine-tuned combination (see the article on p. 16).

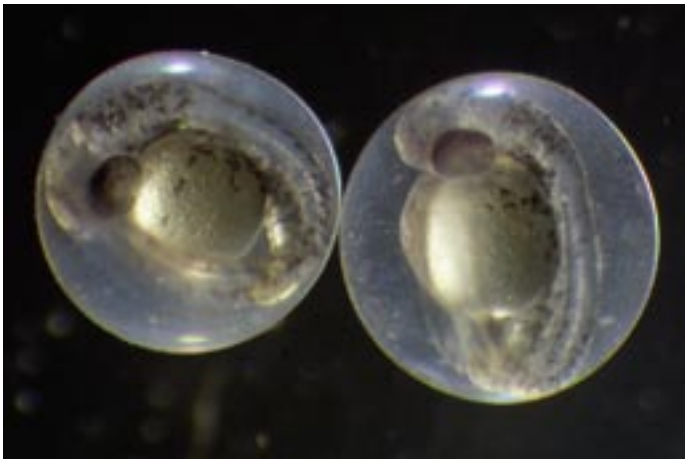
Without estrogen receptors, no lateral line. Estrogens can generally only serve their functions in individual organs if they are recognized by estrogen receptors in the cells, triggering the expression of specific genes. But what happens if the formation of estrogen receptors is suppressed during the sensitive phase of embryogenesis when the reproductive organs develop? Doctoral student Mirjam Fröhlicher recalls: "We were pretty surprised to see that, without estrogen receptors, zebrafish only swim in circles and are obviously no longer able to navigate properly." Fröhlicher, who is currently writing up the results of her study, suspected that the abnormal swimming behaviour was due to changes in the lateral line – the sensory organ that enables the fish to orient themselves. This turned out to be true: the circling zebrafish were shown to lack neuromasts, which are the sensory cells in the lateral line system (see the article on p. 18). In addition, the important role played by estrogens in the development of the lateral line was confirmed by a further experiment: zebrafish in which one of the two key genes involved in estrogen biosynthesis had been suppressed developed significantly fewer neuromasts than normal fish (see the article on p. 16). Overall, these findings strikingly demonstrate the fact that estrogens not only play a role in the reproductive system but also, as previously shown by other studies, influence the development of sensory organs.

Actions of dioxins. Another way of finding out more about the relevance of endocrine disruptors was to expose zebrafish directly to substances of this kind. Among the chemicals chosen for this study were dioxins (see Box), for which contradictory findings as to estrogenic effects had been reported in the literature. Ksenia Groh, a doctoral student at Eawag who had previously studied at Moscow University, emphasizes: "Dioxins are extremely hazardous substances, which can interfere with the endocrine system even at very low concentrations. I was interested in what precisely happens at the molecular level." Groh investigated how dioxins act on an important regulatory element in the endocrine system, namely the key gene in estrogen biosynthesis, which is predominantly active in the brain of the zebrafish and is normally controlled by estrogens. In her study, Groh was able to shed light on the discrepancies in the literature data. She discovered that

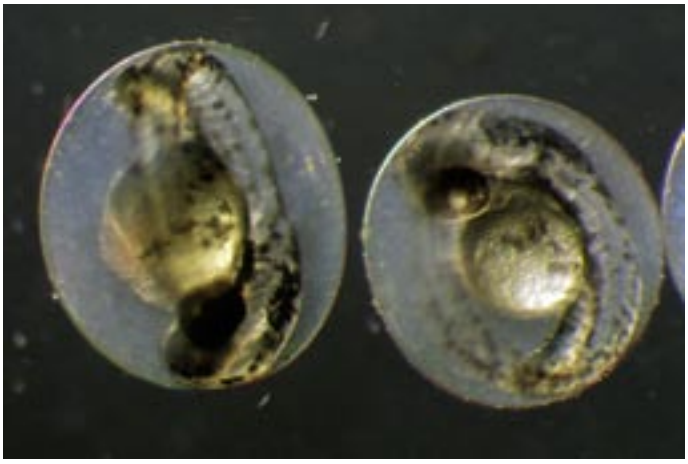
Dioxins

Chemically, dioxins fall into two classes – compounds with a molecular structure of the dibenzo-p-dioxin type and others of the dibenzofuran type. By far the most toxic of these compounds is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which became notorious as the "Seveso dioxin". In 1976, large quantities of this substance were released when a reactor exploded at a chemical plant in Seveso (northern Italy). Dioxins are formed as unwanted by-products during incineration processes and the synthesis of certain chemicals; however, they are also produced by natural events such as volcanic eruptions and forest fires. As these substances are extremely persistent, they accumulate in the environment.





2-day-old embryos: the rudimentary eyes are clearly visible.



3-day-old embryos shortly before hatching.

exposure to dioxins modifies the expression of the key gene, leading to disruption of the endocrine system (see the article on p. 20).

Genome analysis. Another advantage of the zebrafish as a model organism is the fact that it was one of the first vertebrate species to have its genome fully sequenced. As Jules Kemadjou explains, this knowledge is a prerequisite for the genome analysis. Kemadjou gained his first experience of the technique of genome analysis in zebrafish while working on his PhD at Karlsruhe University. This method involves the use of gene chips, on which thousands of genes are arrayed. At Eawag, Kemadjou is now studying the effects of oil on zebrafish embryos. With the aid of gene chips, he aims to elucidate the processes occurring at the molecular level – i. e. to identify the genes that are activated or repressed when zebrafish are exposed to crude oil (see the article on p. 24).

Proteome analysis. "It's a similar concept, but the two methods are fundamentally different." Marc Suter, a chemist specializing in mass spectrometry, sketches out the differences: while genomics is concerned with the entire complement of genes, proteomics focuses on the proteins. The method investigates how protein expression is induced or inhibited in a given situation. Despite the complexities involved, proteome analysis has made considerable progress in recent years thanks to advances in analytical techniques – for example, proteins are now generally identified by mass spectrometry. Suter recently spent a year on sabbatical at the Scripps Research Institute in La Jolla, California, working at the laboratory of John Yates, one of the leading experts in the field of proteomics. Suter's aim was to gain further insights into this method with a view to introducing it for ecotoxicological research on zebrafish at Eawag. The success of this project – including further enhancements of proteome analysis – is confirmed by the initial results that have since been achieved in Eawag's laboratories (see the article on p. 27).

Pieces falling into place. Rik Eggen sums up: "In recent years, we have not only learnt a lot of new things and answered fundamental questions but also made some surprising discoveries. And, as always in science, that opens up another range of fascinating questions." Zebrafish have certainly proved to be extremely valuable as model organisms and will continue to be used in Eawag's ecotoxicological research. The know-how that has been built up – in relation to the molecular zebrafish bioassay or techniques of genome and proteome analysis, for example – will doubtless be exploited not only at Eawag but also at the new centre for applied ecotoxicology that is to be jointly run by Eawag and the Federal Institute of Technology (EPF) Lausanne (see the article on p. 35). ○ ○ ○

3-day-old fish shortly after hatching.

[1] http://www.nfp50.ch
[2] Consensus Platform (2008): Endocrine Disruptors in Waste Water and in the Aquatic Environment, 15 pp., www.nrp50.ch/uploads/media/finaldocumentwater_english.pdf

Well looked after

Karin Rüfenacht and Kerstin Dannenhauer, technicians in the Environmental Toxicology Department, are responsible for rearing zebrafish.

About 600 zebrafish *(Danio rerio)* are kept in aquariums at Eawag. These little creatures provide the fertilized fish eggs that are required on a daily basis for ecotoxicology experiments.

8.30 a.m. The lights come on in the fish lab. This is the signal for the zebrafish to start breeding. The females lay their eggs in a dish placed at the bottom of the aquarium, and the males do their best to fertilize as many as they can. At this time, the fish need complete quiet, since a disturbance of any kind would make them stop spawning and fertilizing immediately. A male can fertilize the eggs from two females, with one female laying between 100 and 200 eggs.

10.00 a.m. Now our work can begin. First of all, we remove the dishes containing the eggs, collect them in a sieve and wash off any food particles and fish faeces. The fertilized eggs are then separated from the unfertilized ones and delivered to the researchers who are planning to carry out experiments that day. Fertilized eggs are transparent, while unfertilized ones are white.

10.15 a.m. Once every 2–3 months, we use some of the fertilized eggs to raise a new generation of zebrafish. One day after fertilization, the eggs are bleached with a diluted sodium hypochlorite solution, so as to kill off pathogens sticking to the surface. We then allow the eggs to develop in quarantine tanks (15–20 eggs per tank). After just one day, the zebrafish hatch, but they remain in the quarantine tank for another two weeks. During this period, they are raised on a special diet of baby fish food. The juveniles are already sexually mature after 3–6 months.

10.30 a.m. Every morning, the fish are fed newly hatched brine shrimp *(Artemia)*. This means that each morning we start incubating a new batch of *Artemia* eggs, so that the larvae can be used for feeding the next morning. Older *Artemia* – brine shrimp die after only 40 hours and then decay – and unhatched *Artemia* eggs would be indigestible or could at worst kill the zebrafish.

10.45 a.m. During and after feeding, we inspect the zebrafish very carefully: Are there any signs of disease? Are all the fish intact,

The zebrafish lay their eggs in a dish filled with marbles.

Photos: Ruedi Keller, Zurich

Live brine shrimp larvae, known as *Artemia,* are the zebrafish's favourite food.

To ensure that fresh fish feed is always available, two new batches of *Artemia* eggs are incubated each day.

or have some of them been injured while fighting amongst themselves? Or are there even dead fish floating in the tank? As soon as any diseased or dying fish are discovered, we remove them from the tank and put them down using a high-dose anaesthetic. The worst disease to which zebrafish are susceptible is so-called fish tuberculosis. Affected fish can be recognized by their swollen belly, protruding scales and bulging eyes. As fish tuberculosis is extremely contagious, we may have to sacrifice all the fish in a tank. This is something we don't find it easy to do – after all, we have raised the zebrafish from the cradle, as it were.

11.00 a.m. As we have a duty to report to the Veterinary Office of Canton Zurich, we keep precise records of how many fish die under natural or unnatural conditions, and how many are used for our experiments.

3.30 p.m. We don't go back to the fish lab until the mid-afternoon. This is when the zebrafish get their cocktail of vitamins to fortify them, as well as their newly hatched brine shrimp. Once again, we start incubating a flask of *Artemia* eggs so that we'll have a fresh supply of larvae the following afternoon. At the weekend, incidentally, the fish are usually fed less regularly.

4.00 p.m. Every day, we refill the tanks with fresh water and once a week we also clean them by hand. This involves removing food debris and fish faeces and changing about 20 % of the water. Finally, the dishes used by the fish for laying their eggs every morning are put into the aquariums again. This completes our daily routine in the fish lab, and after a final check we say goodbye to our fish.

10.30 p.m. Late in the evening, the lights go off in the fish lab and the zebrafish settle down for the night. ○○○

Jane Muncke, environmental scientist, completed her doctoral thesis on this topic in the Environmental Toxicology Department at the end of 2006.

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

Zebrafish prefer a warm environment. The aquarium room is kept at a constant temperature of 29 °C.

Andri Bryner, Eawag

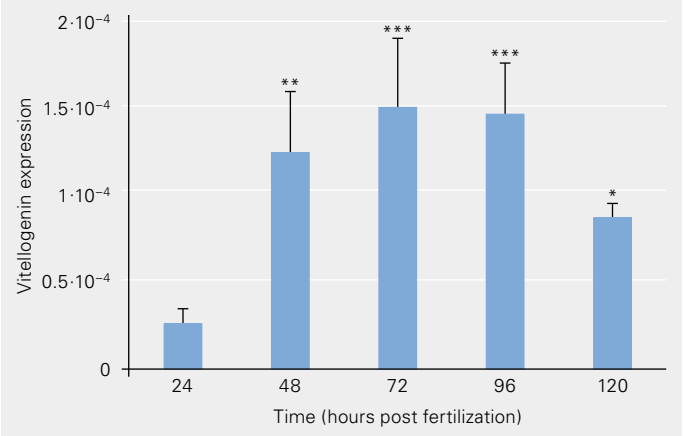
time-consuming and raise animal welfare concerns. In addition, although they reveal the lethal (acute toxic) dose, they do not provide any further information on the mechanism of action of the substances concerned. Our aim, therefore, was to develop a test that could not only be performed rapidly and 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.

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 *** = $p<0.001$.



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

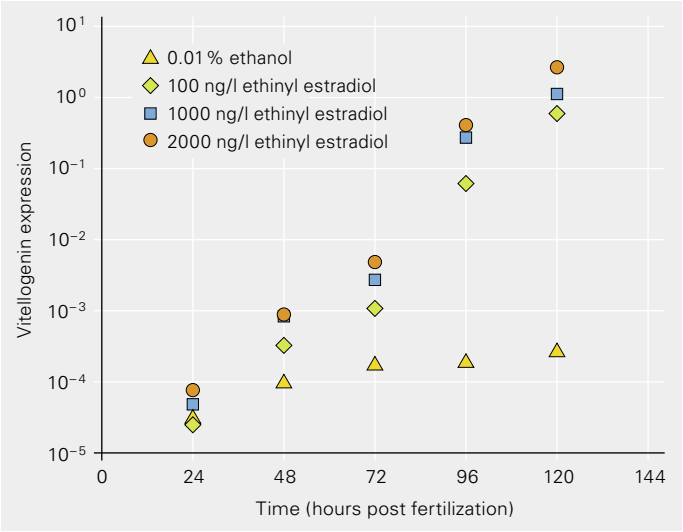


Fig. 2: Expression of the biomarker gene vitellogenin 1 as a function of time in zebrafish embryos exposed to various concentrations of ethinyl estradiol (EE2). The standard deviation is not greater than 30 %.

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. ○ ○ ○

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Anja Liedtke, biologist, scientific associate in the Environmental Toxicology Department.

Mysterious deformities in Lake Thun's whitefish

Since 2000, gonadal alterations have been observed in large numbers of whitefish from Lake Thun. Despite intense research efforts, the causes of these abnormalities have yet to be explained. Possible explanations include genetic factors, pathogens and environmental pollutants. To test the pollutant hypothesis, Eawag is using for the first time a zebrafish bioassay developed in its own laboratories – as described in this progress report.

Lake Thun harbours a mystery: a large proportion of its whitefish have developed deformed gonads (testes, ovaries; see Box and Fig. 1). Since the first appearance of the abnormal fish in 2000, this unique phenomenon has been closely studied, but the causes are as yet unknown. According to one theory, the malformations are caused by a pollutant or mixture of pollutants. Chemical analyses were therefore carried out in an effort to identify the compounds responsible in the lake. However, it was soon concluded that chemical studies are not sufficient, as they only permit the detection of substances that are already known. Our aim is now to test environmental samples from Lake Thun on model organisms, and only to proceed with further chemical characterization in cases where such samples produce a biological effect. For this purpose, we are using for the first time the MolDarT zebrafish bioassay, which was developed at Eawag (see also the article by Jane Muncke on p. 10).

Fig. 1: Normal and altered gonads in whitefish from Lake Thun:
A) Normal ovaries.
B) Asymmetrical ovaries.
C) Hermaphroditism: ovarian and testicular tissue on the same gonad strand.
D) Normal testes.
E) Testes divided into different compartments.
F) Testes fused with the peritoneal wall. Size of the gonads: about 1/3 of the body length, here around 7–10 cm.

Photos: D. Bernet, University of Bern

Broad-based sampling. To obtain a comprehensive picture of Lake Thun, we collected samples over 2 years (2005 and 2006) from all the compartments with which whitefish come into contact in the course of their development:

- sediment, where fish larvae develop;
- plankton, the main food source for whitefish; and
- water, the ambient medium that provides a permanent habitat.

In addition, we studied whitefish muscle, since absorbed pollutants could accumulate in this tissue over time.

All the samples require specific processing before they can be characterized in a biological test system. In general, the active substances are extracted from the samples using a mixture of organic solvents, producing a liquid extract.

Wide variety of deformities in whitefish from Lake Thun

About 40 % of male and 26 % of female whitefish in Lake Thun exhibit deformities of the testes or ovaries [1]. For example, sex organs have been found to be severely underdeveloped, or occurring singly rather than in pairs, and in some cases also fused with the peritoneal wall. Many testes and some ovaries are constricted or divided into different compartments. There have also been cases of hermaphroditism, where an individual fish has testicular and ovarian tissue, or even both types of tissue on the same gonad strand.

Pollutant inputs into Lake Thun. In the media, two historical sources of pollutants are frequently cited as possible causes of the gonadal abnormalities: between 1920 and 1963, large quantities of munitions were dumped by the Swiss army (e. g. at Beatenbucht), and during work at the Lötschberg base tunnel, treated wastewater from the NEAT construction site has been released into the River Kander, which flows into Lake Thun.

Although it is unlikely that these localized inputs have affected whitefish throughout the lake, we specifically also collected sediment samples from Beatenbucht and intended to assess water quality in the Kander below the release point. Special sampling devices were to be left in the Kander for several months, allowing any pollutants contained in the water to become concentrated. Unfortunately, both in 2005 and in 2006, these instruments were swept away by the current, so that no results are available from this site.

Initial indications from yeast assay. As no other organs apart from the gonads are affected in whitefish, we supposed that the deformities could be due to endocrine disruptors. This group comprises both natural sexual hormones (estradiol and testosterone) and synthetic substances such as ethinyl estradiol, an oral contraceptive. However, it also includes substances that have endocrine effects even though they are not classified as sex hormones, e. g. bisphenol A and phthalate. The samples from Lake Thun were analysed for endocrine disruptors using the YES/YAS (Yeast Estrogen Screen/Yeast Androgen Screen) system – a bioassay based on genetically modified yeast cells.

The water and muscle tissue samples yielded no specific evidence of endocrine disruptors. However, estrogenicity was detected for the plankton and sediment samples collected in 2006. Striking results were also obtained with the extracts from the sediment samples collected in 2005: although no estrogenic effects were detectable, the majority of the yeast cells died in each case, suggesting generally high levels of toxicity. In our subsequent investigations, we therefore focused on plankton and sediment.

Analysis of plankton samples with MolDarT. Does the estrogenic effect observed with the plankton samples also occur in a complex organism closely related to the whitefish? To answer this question, we used the molecular zebrafish bioassay known as MolDarT. It is quite conceivable that the substance responsible cannot even be absorbed by the fish or is metabolized before it can exert its effects.

With the MolDarT system, we expose zebrafish eggs and larvae to plankton extracts for five days and then assess whether effects are detectable on the molecular level. In this particular case, we studied two biomarker genes – the vitellogenin gene, which is activated in the presence of estrogenic substances, and a gene from the cytochrome P450 family *(cyp19),* whose expression is increased by dioxin-like substances. For this experiment, the plankton collected in 2005 was homogenized, with plankton from Lake Brienz serving as a control. Lake Brienz was originally chosen because it lies not far above Lake Thun, the two waterbodies are joined by the River Aare and are thus exposed to similar environmental conditions.

Following exposure to the plankton samples, both the estrogen and the dioxin biomarker genes are expressed in zebrafish larvae. Surprisingly, we found that both genes are also activated by the plankton extract from our control waterbody, Lake Brienz (Fig. 2). From this finding we conclude that, while plankton samples from both lakes most probably contain estrogenic and dioxin-like substances, the plankton is not the cause of the deformities observed in whitefish from Lake Thun.

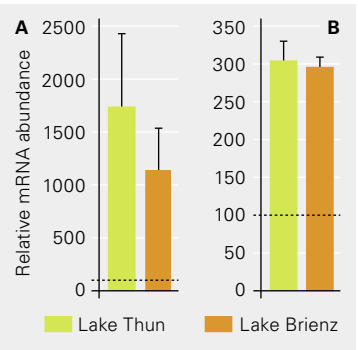

Fig. 2: Activity of the estrogen biomarker vitellogenin gene (A) and the dioxin biomarker cytochrome P450 1A1 gene (B) after exposure of zebrafish embryos to plankton samples from Lake Thun and Lake Brienz. Gene expression is given as relative mRNA abundance, normalized to the negative controls (solvent only), which were set at 100 % (horizontal line).

How toxic are the sediments? To investigate the toxicity of sediments, contact assays were performed in which 15 zebrafish eggs (1 hour post fertilization) were exposed to sediment samples in an air-saturated medium [2]. This method is designed to simulate the natural environment in which fish development occurs. Pure quartz sand and sediments from Lake Brienz were used as negative controls.

After 48 hours – the natural hatching time – the zebrafish were assigned to one of three categories: "hatched", "unhatched" or "dead" (Fig. 3). The results for the negative control with pure quartz sand indicate that the conditions in the sediment contact assay were not optimal overall, with 35 % of the fish embryos failing to survive the 48-hour exposure period. Similarly high mortality rates are also observed with the sediment samples from Lake Thun and the controls from Lake Brienz. Apparently the embryos die as a result of insufficient oxygen in the medium.

Nonetheless, an important conclusion can be drawn from this experiment: the development of the embryos is markedly delayed. While not a single hatched larva was found on the lake sediments, 20 % of the embryos in the negative control with pure quartz sand had already hatched at this point. The sediments from Lake Thun and Lake Brienz presumably contain undesirable substances that slow down zebrafish development.

Altered gene expression in zebrafish larvae after exposure to lake sediments. The zebrafish embryos that had not yet hatched at 48 hours were left on the sediment for another 4 days. Following this exposure, the expression of selected genes in the surviving fish larvae was assessed with the aid of the MolDarT system (Fig. 4) [3]. Once again, vitellogenin and cytochrome P450 1A1

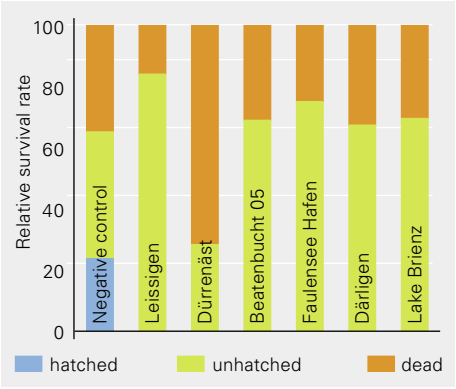

Fig. 3: Survival/hatching rate for zebrafish embryos after 48-hour exposure to sediment samples collected at five different spawning sites in Lake Thun in 2005.
100 % = total number of live embryos after 24-hour exposure.

were used respectively as estrogen and dioxin biomarker genes. In addition, three other genes were studied:

- the heme oxygenase 1 gene, which is induced by stress conditions of any kind;
- the recombination activation gene 1 *(rag1),* which is involved in immune system development; and
- the metallothionein 2 gene, which plays a role in metal regulation.

Unfortunately, in each case, only 1 or 2 fish larvae survived the 6-day incubation on the sediment samples from 2005, which had already displayed a high level of toxicity in the yeast assay. It was therefore not possible to carry out molecular tests. By contrast, fewer fish died on the sediment samples from 2006, and sufficient material was available for gene expression to be analysed. In 2006, our sampling focused on Beatenbucht, with samples being collected from five different sites in this bay.

Our findings (Fig. 4) show that the vitellogenin gene was not activated – i.e. the estrogenic effects exhibited by the sediment samples in the yeast assay could not be confirmed in the zebrafish. Transcription of the heme oxygenase gene was also unchanged. At the same time, expression of the cytochrome gene was stimulated and transcription of the recombination activation and metallothionein gene was repressed. This suggests that the sediment contains a dioxin-like substance and compounds that disrupt metal metabolism and the development of the immune system.

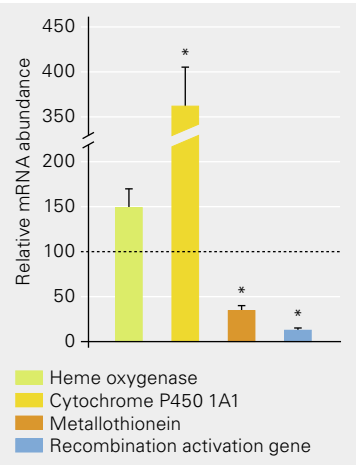

Fig. 4: Activity of four biomarker genes (see text for details) after exposure of zebrafish embryos to sediment samples collected from Beatenbucht on Lake Thun in 2006. Gene expression is given as relative mRNA abundance, normalized to the negative controls (solvent only), which were set at 100% (horizontal line). Asterisks = significant differences.

Next steps. In view of the effects detected in the bioassays, we are currently pursuing the chemical characterization of the potential pollutants. For this purpose, the complex sample extracts are separated into different fractions, e.g. by the polarity and molecular size of their constituents, and again subjected to bioassays. Fractions with positive test results are to be further investigated, ideally until a pure substance or a narrowed-down mixture of substances produces the same effects in the bioassay as the original extract.

Even though this would not demonstrate unequivocally that the gonadal deformities are indeed attributable to the pollutants identified – after all, the bioassays involve yeast cells and zebrafish rather than whitefish – the evidence would be fairly compelling.

This project is being carried out jointly by the Centre for Fish and Wildlife Health (FIWI) at Bern University and Eawag. It is part of the National Research Programme NRP50 on "Endocrine Disruptors: Relevance to Humans, Animals and Ecosystems", sponsored by the Swiss National Science Foundation. The project is also supported by the Faulensee fish farm, the Bern Fishery Inspectorate, and the Water and Soil Protection Laboratory of Canton Bern.

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Rik Eggen, a molecular biologist, was head of the Environmental Toxicology Department prior to his appointment as Deputy Director of Eawag. Co-authors: Evangelia Kallivretaki and Helmut Segner (University of Bern).

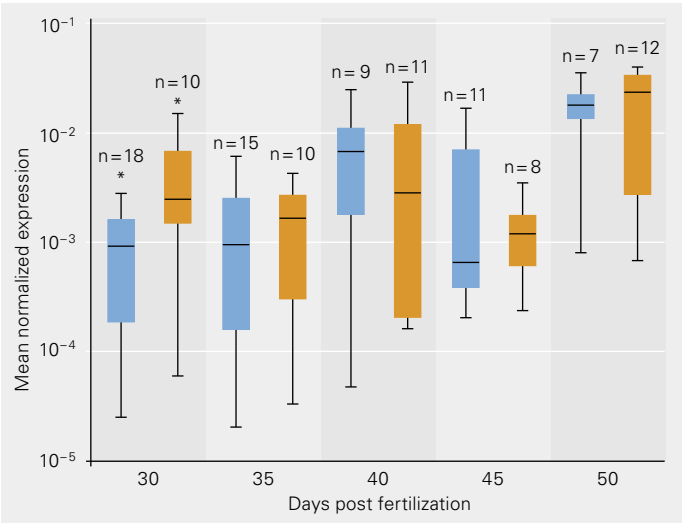
Role of estrogens in organogenesis

In estrogen biosynthesis, the key enzyme is cytochrome P450 aromatase. It catalyses the crucial step, i.e. the conversion of androgens to estrogens, thereby determining estrogen concentrations. We used zebrafish to investigate whether aromatase controls the development of the sex organs and the lateral line.

Estrogens are steroid hormones found in all aquatic and terrestrial vertebrates [1]. While several types of estrogens are naturally present in organisms, 17β-estradiol is the form responsible for nearly all biological activities. Estrogens are involved, in particular, in controlling sexual differentiation, maturation and reproduction. However, these hormones also serve numerous functions in the development and differentiation of other organs, such as the nervous system. To ensure that these diverse tasks can be successfully performed, estrogen levels need to be carefully balanced and regulated in the various stages of life.

Estrogens are synthesized from cholesterol via a series of intermediate products. While several enzymes are involved in estrogen biosynthesis, the level of estrogen concentrations is determined by the final step of the process, in which androgens are converted to estrogens [2]. This key reaction is catalysed by the enzyme cytochrome P450 aromatase. In zebrafish, this protein is encoded by two genes, with *cyp19a1* being expressed primarily in the ovaries and *cyp19a2* mainly in the brain. Both genes are potential targets for micropollutants that occur widely in the environment, possibly interfering with estrogen-regulated processes and thus acting as indirect endocrine disruptors. Using zebrafish as a model organism, we studied the role of aromatase and hence of estrogens in the development of the sex organs and of the lateral line, a sensory organ enabling fish to detect changes in pressure [3]. The insights gained enhance our understanding of the mechanisms of action of endocrine disruptors.

Fig. 1: Expression of the *cyp19a2* gene in the brains of juvenile male (blue) and female (orange) zebrafish. The upper and lower limits of the boxes represent the highest and lowest mRNA amounts measured, while the line within each box indicates the mean (± standard deviation). n = number of individuals classified by sex. * = significant difference in mRNA levels between males and females at day 30 post fertilization ($p < 0.012$).



Does *cyp19a2* determine sexual differentiation of the gonads?

Zebrafish are undifferentiated gonochorists: they are born as females with initially non-functional ovaries, which subsequently develop either into mature male or female gonads. At the start of our studies, it was generally assumed that sexual differentiation of the gonads depends on the levels and patterns of aromatase expression in the brain. What would this mean in practice? The more frequently the *cyp19a2* gene is transcribed, the more aromatase is present in brain cells and the higher the estrogen concentrations – a situation leading to the development of female gonads. By contrast, male gonads would form when estrogen levels are low. We tested this hypothesis by analysing zebrafish throughout the process of gonadal differentiation, i.e. between day 30 and day 50 post fertilization. The fish heads were used to quantify aromatase expression (based on gene transcripts/mRNA abundance) and to characterize the distribution of aromatase protein in the brain. At the same time, the fish bodies were used to determine the status of gonadal development. Animals that were undergoing transition to testes or already had clearly developed testes were classified as males, while those with non-functional ovaries were counted as females. Until about day 50 post fertilization, female gonads retain the capacity to develop into testes. It is only possible to determine the sex of the fish with certainty when oocyte maturation begins.

***cyp19a2* not differentially expressed in males and females.** Our analysis revealed that aromatase transcripts were present in zebrafish heads throughout the test period, with mRNA abundance varying considerably within the sex groups and showing a slight increase over time irrespective of sex group. However, aromatase transcript levels were not correlated with sex (Fig. 1). The *cyp19a2* gene is thus continuously expressed – in a non-sex-dependent manner – during sexual differentiation. A similar pattern was observed for protein quantity (data not shown). In addition, the distribution of aromatase protein in the developing fish brain was identical for males and females. The enzyme is, as already known for adult fish, mainly produced in parts of the forebrain (telencephalon and diencephalon) and in the pituitary.

Our findings suggest that sexual differentiation in zebrafish is not, as previously assumed, solely regulated by *cyp19a2* expression and estrogen concentrations. This process appears to be much more complex and probably depends on the interaction of various genes, and possibly other factors, working in a fine-tuned combination. Bearing this in mind, we are currently seeking to unravel the sexual differentiation process in a project funded by the Swiss National Science Foundation (SNF).

Does *cyp19a1* affect development of the lateral line? Estrogens play an important role not only in sexual differentiation, but also in the development of the sensory organs, as has been shown, for example, in fish. Consistent with these results is our observation that the *cyp19a1* gene is expressed in the lateral line. This pressure-sensitive organ, usually visible as faint lines around the eyes and along both flanks, allows the fish to localize movements in its vicinity (see also the article by M. Fröhlicher on page 18). The receptors in the lateral line organ, known as neuromasts, are each composed of a group of sensory hair cells. To determine whether *cyp19a1* is in fact involved in the development of neuromasts and the lateral line system, we experimentally inhibited the expression of this gene by means of the "knockdown technique" (see Box on page 18). This involves the injection of morpholino oligonucleotides into developing zebrafish eggs, where they hybridize with the endogenous *cyp19a1* transcripts. As a result, protein synthesis is blocked and aromatase activity in the neuromasts is suppressed.

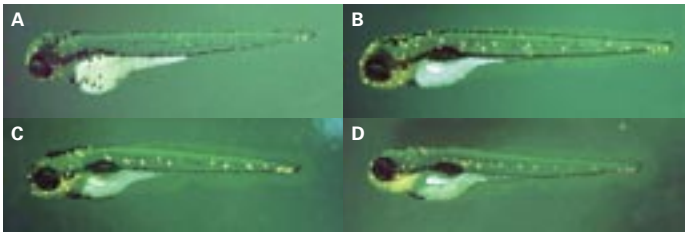
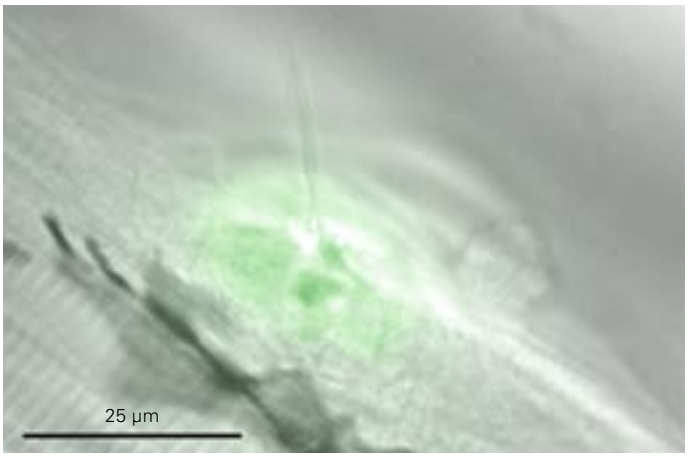


Fig. 2: Effects of morpholino knockdown on neuromast numbers in the lateral line system. A: embryo injected with *cyp19a1* morpholino; B: untreated control embryo; C: embryo injected with control morpholino; D: embryo injected with *cyp19a2* morpholino.

Neuromast numbers reduced by repression of *cyp19a1*. Our experiment showed that far fewer neuromasts were formed in the knockdown zebrafish (Fig. 2A) than in untreated fish (Fig. 2B), or in fish injected with a control morpholino (Fig. 2C) or a morpholino directed against *cyp19a2* (Fig. 2D). We therefore conclude that the aromatase gene *cyp19a1* is indeed involved in the development of the lateral line system – a surprising new finding. This means that endocrine disruptors not only act on the reproductive system but also affect quite different developmental processes in organisms.

Sensory hair cells of a neuromast. Overlay of a light micrograph and a fluorescence micrograph in which the hair cells were stained with a fluorescent dye.



Mirjam Fröhlicher, Eawag

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Mirjam Fröhlicher, biologist and doctoral student in the Environmental Toxicology Department.

Estrogen receptor knockdown in zebrafish

Estrogens, the female sex hormones, can only exert their effects when they are recognized by estrogen receptors. But what happens if the production of these receptors is suppressed? We studied this question with the aid of knockdown zebrafish. A particularly striking feature of their behaviour was the fact that they persistently swam in circles, lacking the lateral line organ that zebrafish normally use to orient themselves.

Substances that affect the hormonal system – so-called endocrine disruptors – are found in surface waters worldwide. Even at low concentrations, these compounds can have detrimental effects on aquatic organisms. Endocrine disruptors may not only take the place of natural hormones in stimulating the production of certain proteins; they may also disturb the activity of endogenous hormones. One crucial factor is when the target organisms come into contact with endocrine-disrupting compounds. For example, if exposure occurs during embryonic development, when the reproductive organs and the brain are in the process of differentiation, it can lead to permanent defects and functional disorders, such as the feminization of entire fish populations [1].

The majority of endocrine disruptors – specifically those that are similar to estrogens, the female sex hormones – are recognized in the organism by estrogen receptors (cf. Fig. 1 on p. 20). In the same way as endogenous estrogens, the estrogen-like substances bind to and activate the receptor. Thus altered, the activated complex migrates to the cell nucleus, where it binds to specific DNA sequences, the estrogen responsive elements. These are located in the control regions of certain genes whose expression is promoted by binding of the receptor complex [2].

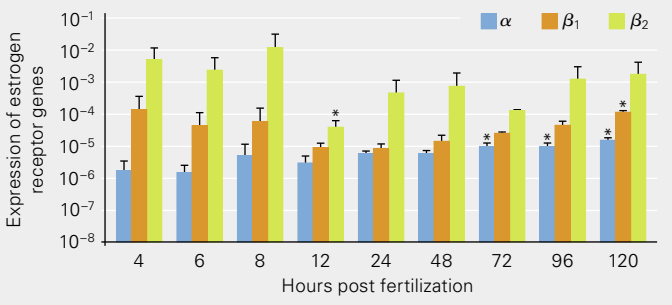
However, what happens if the formation of estrogen receptors is artificially suppressed, particularly during embryogenesis? To examine this question, we decided to work with zebrafish embryos. Our study is part of a wide-ranging project concerned with the estrogen system at the molecular level – fundamental knowledge that is required to improve our ability to assess the effects of endocrine disruptors (cf. the articles by Rik Eggen on p. 16 and Ksenia Groh on p. 20).

The knockdown technique

Using the morpholino technology, it is possible to block the synthesis of specific proteins for a certain period. The knockdown method involves the injection of a short synthetic oligonucleotide – the so-called morpholino [3]. This is injected into the yolk sac of the zebrafish egg during the four-cell stage and then diffuses into the cells. The morpholino sequence is chosen so as to be complementary to the region around the start codon of the target gene. As a result, the morpholino hybridizes with the mRNA transcripts of the gene, thereby preventing protein synthesis. After about five days, the organism is able to degrade the foreign oligonucleotide, and protein synthesis is resumed. The *knockdown* is not to be confused with the *knockout* procedure, in which the mutation is integrated into the genome and transmitted to offspring.

To demonstrate the specificity of morphological effects occurring after injection of the morpholino, a control morpholino is also used. This is a standard oligonucleotide which does not find a complementary sequence in the organism to bind to.

Fig. 1: Expression of the α, β_1 and β_2 estrogen receptor genes during embryonic development in zebrafish. Significant differences are indicated by asterisks ($p < 0.05$).



β_2 Estrogen receptor gene strongly expressed in zebrafish embryonic development. Three distinct estrogen receptors are found in zebrafish – the α, β_1 and β_2 types. In a preliminary experiment, we aimed to establish which of the three estrogen receptor genes is most strongly expressed in embryonic development. Our analysis showed that the β_2 gene is most frequently transcribed in the first five days post fertilization, so mRNA abundance was found to be greatest for this gene (Fig. 1). We therefore decided to switch off the β_2 receptor gene and had a specific β_2 morpholino synthesized for this purpose (see Box: «The knockdown technique»).

β_2 mRNA was found, incidentally, to be more abundant in the first few hours post fertilization than after 12 hours (Fig. 1). However, this is explained by the presence of maternal mRNA transcripts in the fish eggs. Endogenous transcription only begins in the fish embryos after 12 hours, and concentrations of native β_2 mRNA then rise steadily.

Estrogen receptor knockdown: low hatching rate and circular swimming pattern. What concentrations of morpholino oligonucleotides are most effective for knocking down β_2 estrogen receptor expression? We showed that concentrations between 25 and 50 µM produced clearly detectable morphological alterations without at the same time placing an undue burden on the zebrafish embryos. Toxic effects were, however, seen with morpholino concentrations of 100 µM and above.

Zebrafish embryos injected with 25 or 50 µM β_2 morpholino exhibited unusual behaviour, swimming incessantly in a circular pattern. In addition, only 30 % of the embryos injected with 50 µM β_2 morpholino had hatched at 72 hours post fertilization (Fig. 2).

Sensory organs for orientation lacking in knockdown zebrafish. We suspected that the abnormal swimming behaviour was due to alterations in the lateral line. This system is responsible for the sense of «distance touch», enabling zebrafish to detect the slightest changes in current and vibrations and thereby orient themselves. The sensory organs of the lateral line are the neuromasts. At the centre of these structures is a collection of hair cells,

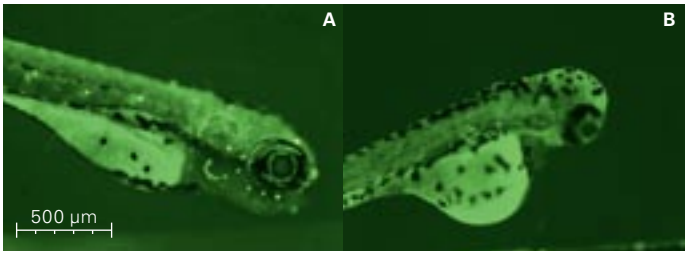
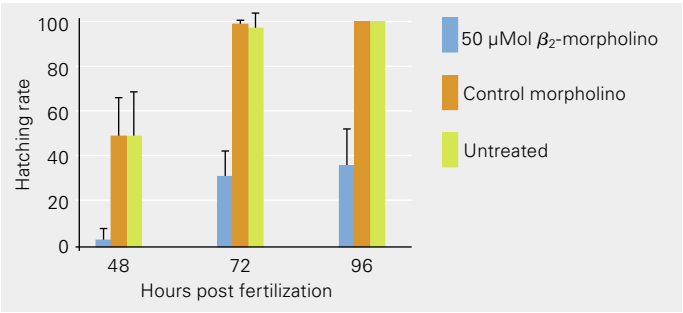


Fig. 3: Neuromasts in the lateral line of zebrafish injected with the control morpholino (A) as seen under the microscope. These organs are not found in the knockdown zebrafish injected with 25 µM morpholino (B).

connected at the base to nerve cells that pick up incoming signals. The hair cells are surrounded by support cells. Normally, zebrafish have neuromasts on the head, around the eyes and along a line on either side of the body, which can be readily visualized using a fluorescent dye (Fig. 3A). But this is not the case in knockdown zebrafish: in these constantly circling fish, neuromasts are either completely lacking or non-functional and therefore cannot be stained (Fig. 3B).

Importance of estrogen receptors during organ development. Our findings clearly indicate that the β_2 estrogen receptor plays an important role in embryonic development, and that estrogens control not only sexual differentiation and reproductive function but also general morphogenesis. As well as the hatching rate, the estrogen/estrogen receptor interaction influences the formation or functioning of neuromasts, which are essential for normal swimming behaviour. Our next research goal is now to identify specific genes that are up- or downregulated in knockdown zebrafish.

Fig. 2: Hatching rate for knockdown zebrafish (injected with 50 µM β_2-morpholino) 48, 72 and 96 hours post fertilization compared with untreated fish and embryos injected with a control morpholino not capable of binding.



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Ksenia Groh finished her doctoral thesis on this topic at the end of 2006 and works now as post-doctoral fellow in the Environmental Toxicology Department.

Estrogen-related actions of dioxins

Dioxins are widespread pollutants. Like estrogens, these chemicals are suspected to interfere with the hormonal system of wildlife. The reproduction of fish, for example, is severely affected. To improve assessment of the risks posed by these contaminants to the aquatic environment, we analyzed whether the expression of a typically estrogen-controlled target gene might be modified by dioxins.

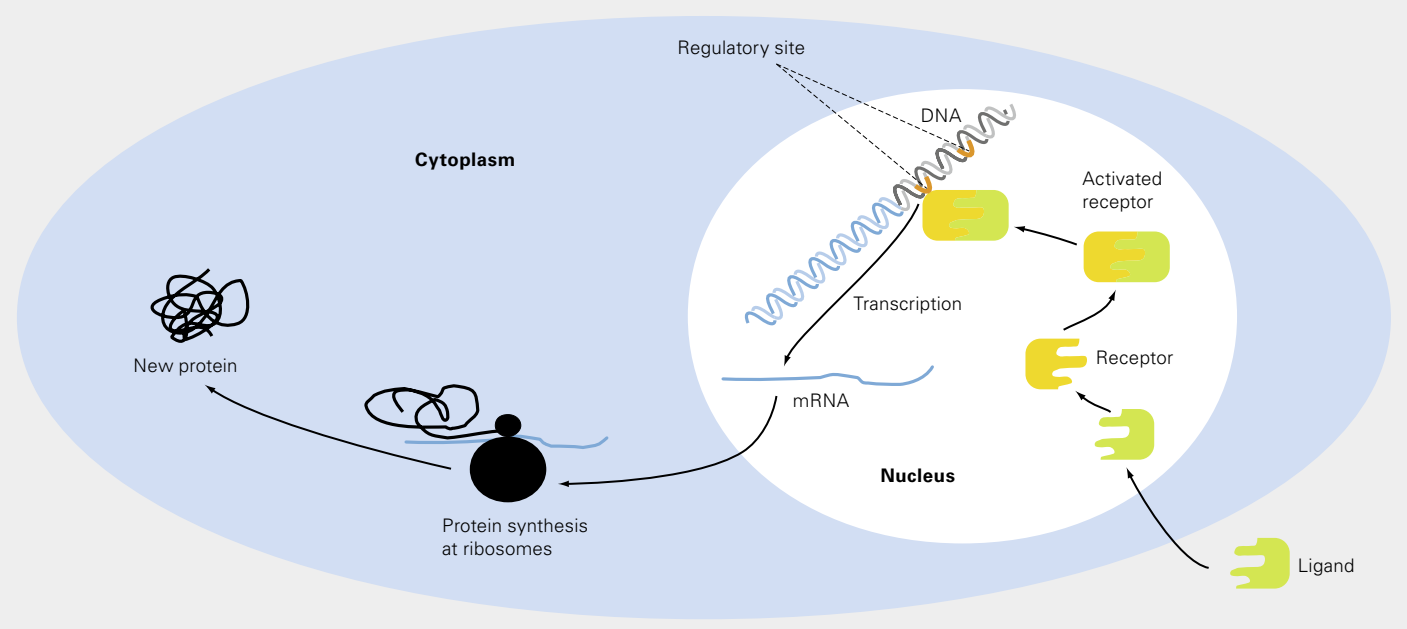
In recent years, diverse developmental and reproductive abnormalities have been reported in fish, i. e., deformed gonads, feminised males, or females adopting male characteristics. These malfunctions can be linked, at least partially, to exposure to so-called endocrine disrupting chemicals – natural or synthetic compounds widely present in the environment that can mimic or disrupt hormone action. So far, about 1000 substances have been identified or are suspected to act as endocrine disruptors [1]. Among these are estrogen- and dioxin-like chemicals.

The classical modes of action of estrogen- and dioxin-like substances are quite similar (Fig. 1): After binding of the ligands to the estrogen or dioxin receptor, the activated complex will initiate transcription of specific target genes by attachment to so-called estrogen- or dioxin-responsive elements in their promoters.

One potential target gene may be *cyp19* encoding the enzyme cytochrome P450 aromatase which catalyzes the final step in the biosynthesis of estrogens. Interference with aromatase gene expression can alter the rate of estrogen production, unbalance the local and systemic levels of estrogens and thus lead to disruption of biological processes controlled by estrogen. In order to predict the consequences of potential effects of estrogen- and dioxin-like chemicals on this system, we aimed at understanding the molecular mechanisms that govern the transcriptional control of this key gene. Therefore, we analyzed the expression of the aromatase gene of the model teleost species zebrafish *(Danio rerio)*.

Zebrafish carry two aromatase genes. Zebrafish, like many other teleosts, have two aromatase genes: *cyp19a* predominantly

Fig. 1: Mode of action of estrogen- and dioxin-like chemicals (simplified schema).



expressed in gonads and *cyp19b* mainly found in the brain. These genes play a critical role in fish development and reproduction. In particular, aromatase's brain form *(*cyp19b*)* is presumed to be involved in the regulation of neuroendocrine functions of estrogens such as development of the brain or shaping of male and female behaviour. In comparison with other genes, three short DNA sequences have been predicted in its promoter which might bind the activated estrogen or dioxin receptor complexes and therefore be involved in regulating the expression (Fig. 1). One of these sites has recently been shown to act as a functional estrogen responsive element [2] whereas the functionality of the other two sites, potential dioxin responsive elements, has not yet been unambiguously confirmed [3, 4].

Consequently, our specific research questions were:

► Are the two dioxin responsive element in the brain aromatase gene functional and the gene hence regulated via the classic pathway shown in figure 1?

► Do dioxins affect the action of estrogens via crosstalk between the receptors and thus modify aromatase expression?

Combining *in vivo* and *in vitro* tests. To answer these issues, we combined *in vivo* exposure of zebrafish larvae and *in vitro* assays using promoter reporter gene constructs. For the *in vivo* exposure studies, zebrafish larvae were reared from 17 to 20 days post fertilization in a medium containing an estrogen or a dioxin or a mixture of both substances. At the end of the exposure period the larvae were sacrificed and processed either for mRNA or protein quantification.

Since the brain aromatase gene is mainly expressed in glial cells, we used a human glial cell line [2] as *in vitro* test system. Glia are a specific type of brain cells that provide support and nutrition to the neurons. For our studies, the cells were transfected with a combination of different DNA constructs:

► Reporter constructs: We made use of luciferase as reporter gene, i.e. this gene has been cloned either under the control of the wild-type or a mutated (lacking both dioxin responsive elements) brain aromatase promoter. If gene expression is activated, the transfected cells become luminescent.

► Expression constructs: To test whether the promoter is activated via binding of estrogens or dioxins to their respective receptors, all components have to be present in the cells. Therefore, the reporter constructs were co-transfected with expression constructs containing the coding sequences of the zebrafish receptor proteins which are constitutively expressed.

Transfected glial cells were lysed after 48 hours of estrogen or dioxin exposure.

The dioxin responsive elements in the brain aromatase gene are not functional. Our first experiment consisted of exposing developing zebrafish larvae to the prototypic dioxin receptor ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD is known as the most potent activator of the dioxin receptor pathway. However, there was no effect neither on the amount of aromatase mRNA (Fig. 2A) nor on the presence of aromatase protein in brains (Fig 2B).

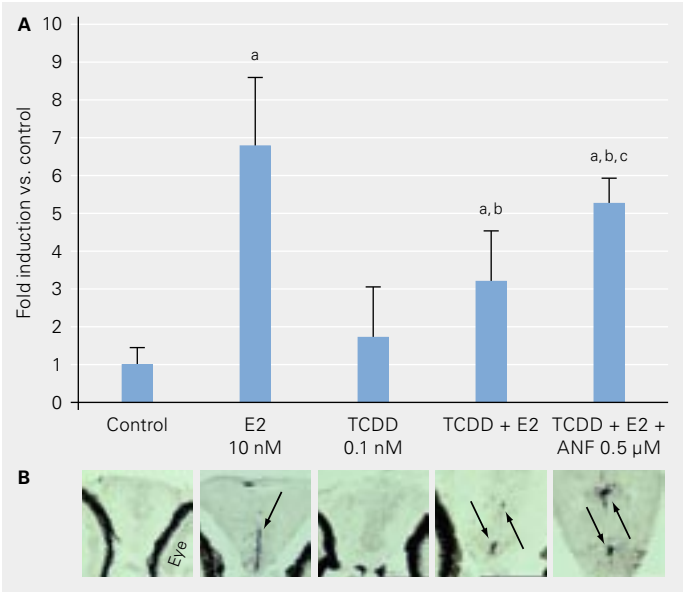


Fig. 2: Effects of exposure to estrogen and dioxin receptor ligands on brain aromatase in zebrafish larvae.
A: Amount of mRNA. The results are expressed as mean of three independent experiments with duplicate measurements ± standard deviation relative to solvent control. a = results significantly different from solvent control; b = results significantly different from E2 treatment; c = results significantly different from co-treatment with E2 and TCDD; $p < 0.05$ in all cases.
B: Presence of aromatase protein in the brain detected with aromatase antibodies. Immunoreactive glial cells are marked.

Using the *in vitro* test system, we analysed how the wild-type aromatase promoter and its mutated form without dioxin responsive elements react to TCDD exposure. The glial cells had been co-transfected with expression constructs containing the sequence of the dioxin receptor complex. Both promoters left uneffected, although the control promoter with a known dioxin-responsive element was activated under the same conditions (Fig. 3).

Additionally, we showed that short DNA fragments containing dioxin responsive elements similar to the one predicted in the brain aromatase promoter were unable to bind to the activated dioxin receptor complex (data not shown). Taken together, these observations let us conclude that both dioxin responsive elements predicted in the zebrafish brain aromatase promoter are not functional and that dioxins do not directly regulate this gene via the classic pathway involving ligand-receptor signalling.

Crosstalking receptors: The anti-estrogenic action of TCDD. Next, we aimed at examining whether a possible crosstalk between the estrogen and dioxin receptor complexes may effect the expression of the brain aromatase gene. We observed that dioxins can lead to both estrogenic and anti-estrogenic effects on the expression of zebrafish, depending on the presence or absence of estrogens.

The natural estrogen 17β-estradiol (E2) strongly upregulated the expression of zebrafish brain aromatase mRNA and protein in zebrafish larvae (Fig. 2A + B). E2 also activated the zebrafish brain aromatase gene promoter in the *in vitro* cell system when cells were co-transfected with vectors constitutively expressing both the estrogen and dioxin receptor proteins (Fig. 4A). These results are in agreement with previous studies [2]. Co-exposure of E2 with TCDD decreased the E2-induced expression of zebrafish brain aromatase. This anti-estrogenic effect was observed both *in vivo* (Fig. 2) and *in vitro* (Fig. 4A). Experiments with the mutant of the zebrafish brain aromatase promoter (deficient in the dioxin responsive elements) and with the control promoter containing only an estrogen responsive element showed the same expression pattern. Therefore, downregulation appears to occur independently of dioxin responsive elements predicted in the promoter of zebrafish brain aromatase.

The anti-estrogenic effect of TCDD could either be partially *(in vivo*, Fig. 2 A + B) or fully (*in vitro*, Fig. 4A) rescued by the addition of a dioxin receptor antagonist, α-naphthoflavone (ANF). An antagonist is a substance that binds to the same receptor but does not activate it. We concluded, therefore, that the dioxin receptor is involved in this downregulation mechanism of TCDD.

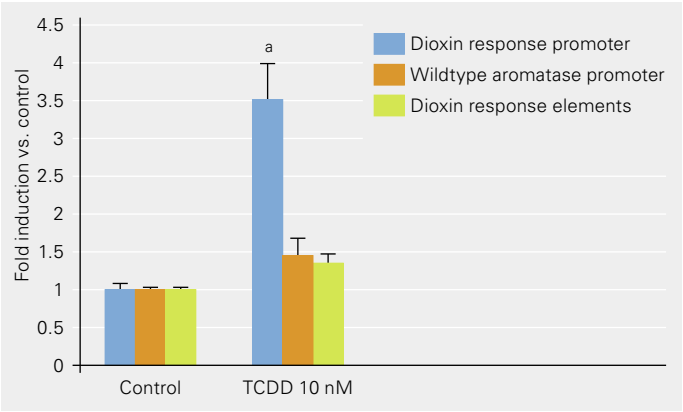
E2 produced by aromatase in the brain is an important neurotrophic and neuroprotective factor. It can be hypothesized that exposure to TCDD can disrupt the normal E2-induced expression of aromatase and hence decrease the amount of estrogens synthesised in the brain.

Crosstalking receptors: The estrogenic action of TCDD. Only a slight upregulation of zebrafish brain aromatase promoter activity was observed in the presence of zebrafish estrogen receptor and dioxin receptor proteins in glial cells treated with TCDD (Fig. 4B). This effect could be blocked by co-treatment either with the estrogen receptor antagonist ICI 182,780 (ICI) or with the dioxin receptor antagonist ANF, suggesting the involvement of both receptors in this process (Fig. 4B).

The fact that only the estrogen responsive element on the promoter is involved in this type of estrogen receptor and dioxin receptor interaction was confirmed by the experiments with the mutant of the zebrafish brain aromatase promoter lacking both predicted dioxin responsive elements, and with the control estrogen-responsive promoter, containing just an estrogen responsive element. The two latter constructs exhibited the same pattern of response to treatment with the estrogen and dioxin receptor ligands as the construct containing the wild-type promoter of zebrafish brain aromatase (Fig. 4B).

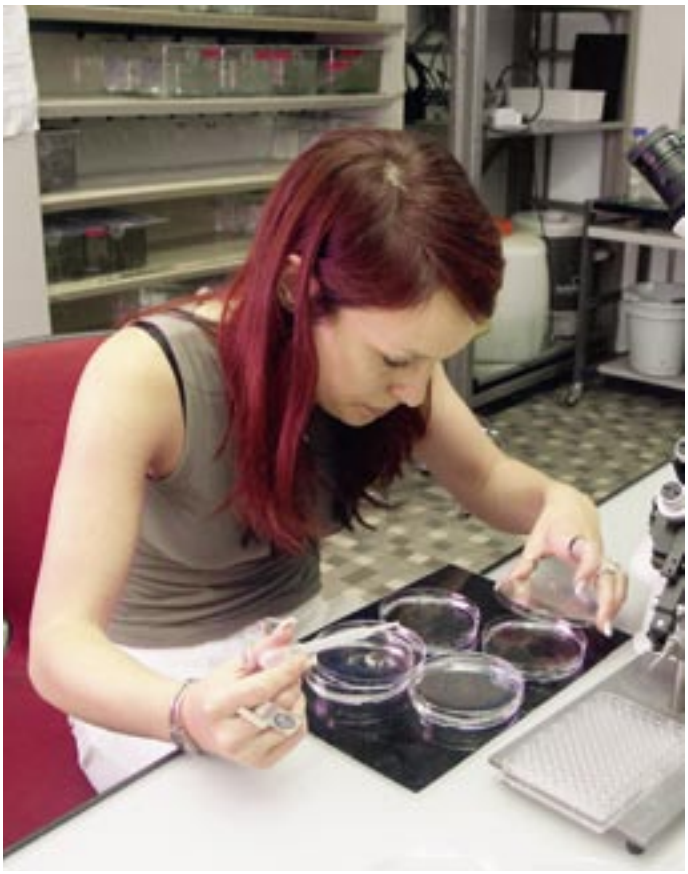
It was recently shown that activated human dioxin receptor can directly associate with the unliganded estrogen receptor, leading to gene activation via the estrogen responsive element [5]. Our findings indicate that this mechanism of estrogen/dioxin receptor interaction might be conserved in fish. It is important to note that the weak estrogenic effect of TCDD could only be detected *in vitro* in the absence of an estrogen receptor ligand. Thus,

Fig. 3: Response of brain aromatase promoter either containing or lacking its potential dioxin responsive element to treatment with TCDD. A vector with a functional dioxin-responsive promoter from rainbow trout was used as positive control. The glial cells were co-transfected with expression vectors coding for the zebrafish dioxin receptor. Results are expressed as mean of three independent experiments with triplicate measurements ± standard deviation relative to the non-dioxin treated control. a = result significantly different from solvent control ($p < 0.01$).



The fertilized eggs are separated from the unfertilized eggs.

Andri Bryner, Eawag



the inability to observe upregulation of the expression of zebrafish brain aromatase by TCDD *in vivo* in zebrafish larvae (Fig. 2A) can be explained by the presence of endogenous estrogens, which preclude potential estrogenic actions of dioxins. However, the estrogenic pathway of TCDD might still function *in vivo* at specific life stages or in specific cell types when estrogens are absent or present at a very low level.

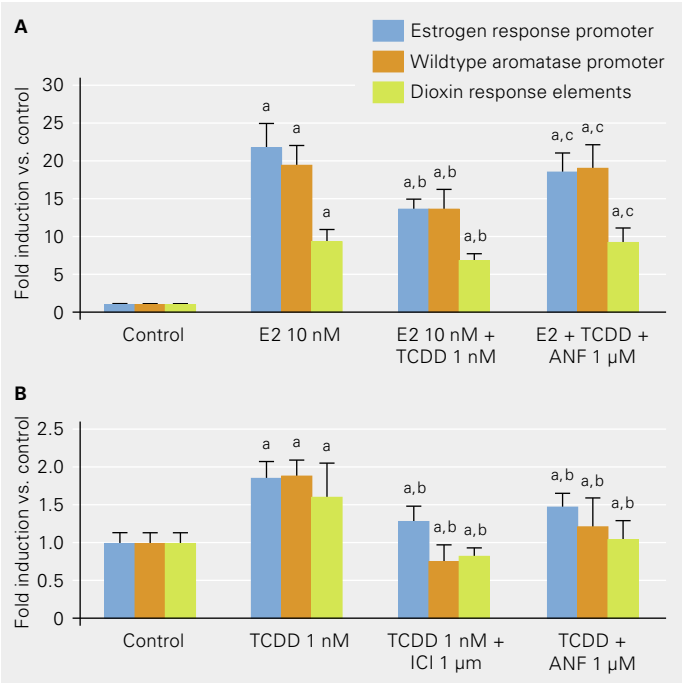
In conclusion: External estrogens and dioxins perturb the delicate endogenous estrogen balance. Our analysis of the regulation of the zebrafish brain aromatase gene led to the assumption that estrogen- and dioxin-like endocrine disrupting chemicals can interfere with the expression of this gene in different ways [6]:

► Estrogenic endocrine disrupting chemicals which possess the ability to bind to and activate estrogen receptor have the potential to disrupt the function of zebrafish brain aromatase by causing the unprogrammed upregulation of its expression.

► Both dioxin responsive elements predicted in the promoter of the aromatase gene are not functional, probably due to the low degree of conservation of their consensus sequence. This example shows how important it is to directly analyze the functionality of transcriptional regulatory sites predicted by sequence comparison analysis. Thus, the dioxin-like endocrine disrupting chemicals are not likely to affect the expression of zebrafish brain aromatase via the classic pathway involving both the dioxin receptor complex and a dioxin-responsive element.

► However, dioxins are able to affect expression of genes containing a functional estrogen-responsive element in their promoters. These actions of dioxins may be either estrogenic (leading to stimulation of the estrogen-receptor and activation of gene expression via estrogen responsive element) or anti-estrogenic (leading to attenuation of normal E2-induced upregulation of transcription), depending on the absence or presence of estrogen receptor ligands. Thus, at different stages of development, or in different target organs, which might differ in estrogen content, the actions of dioxins may be different. Similarly, the actions of dioxins in the environment can also be quite different depending on the presence or absence of estrogenic substances in the same exposure solution. ○○○

Fig. 4: Response of brain aromatase promoter either containing or lacking its potential dioxin responsive element to treatment with estrogen and dioxin-like substances. A vector with a functional estrogen-responsive promoter was used as positive control. The glial cells were co-transfected with expression vectors coding for the zebrafish estrogen and dioxin receptors. a = results significantly different from solvent control in (A) and (B); b = results significantly different from E2 (A) and TCDD treatment (B); c = results significantly different from co-treatment with E2 and TCDD; $p<0.01$ in all cases.



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Jules Kemadjou,
biologist and scientist
in the Environmental
Toxicology Department.

Effects of crude oil on zebrafish embryos

Oil disasters occur every few years. In most cases, the acute effects on wildlife are dramatic. But even decades later, oil – or its water-soluble components – remain in the aquatic environment and seem to be persistently toxic. Our goal was therefore to assess the effects of oil on fish under subacute conditions. Using zebrafish embryos as a model system, we were able to identify hundreds of genes that showed altered expression on exposure to oil.

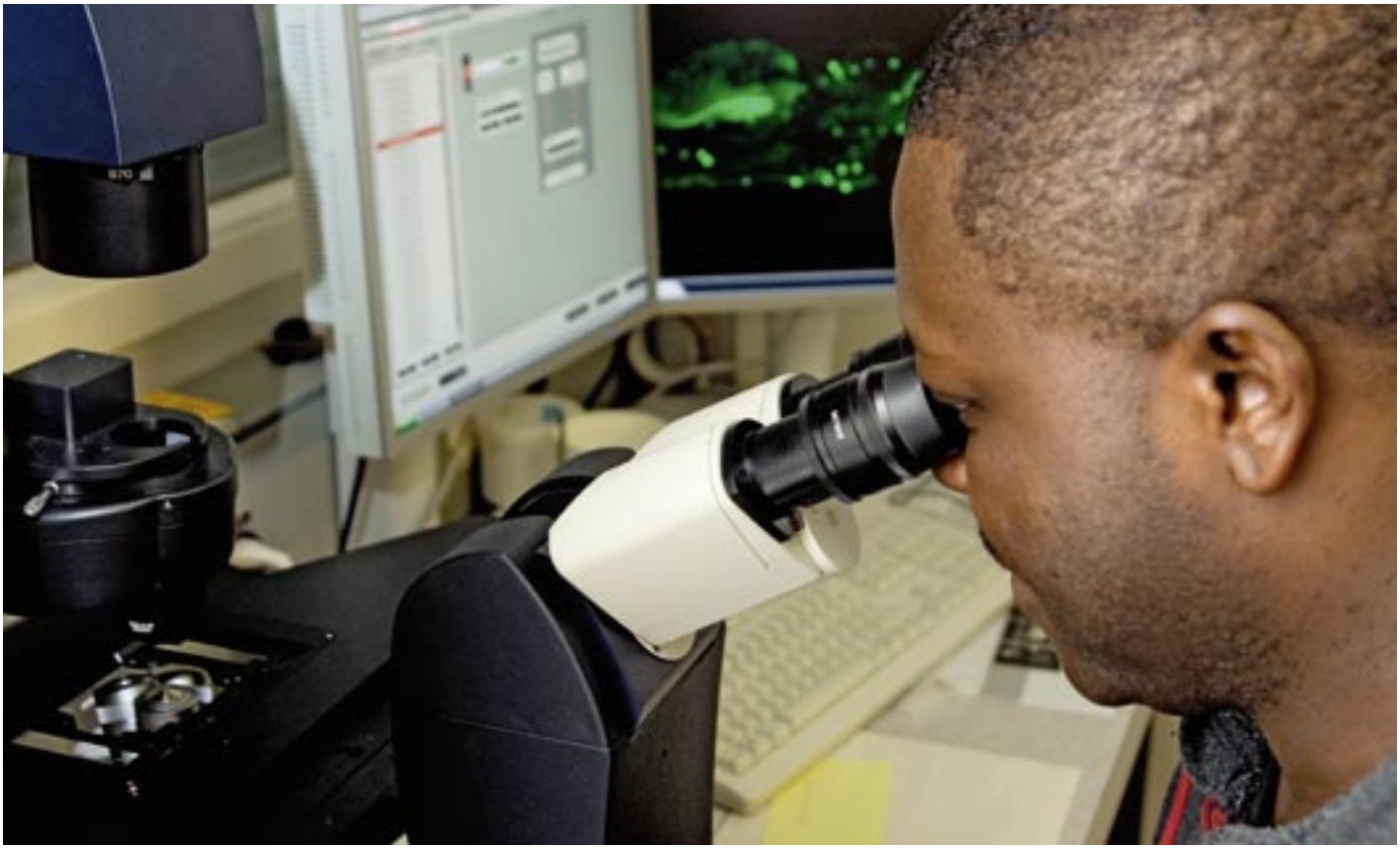
It is not long since the last oil disaster made the headlines. At the end of 2007, about 220 000 litres of oil spilled from a ship that struck a bridge in San Francisco Bay. This spill was rated as medium-sized in the long history of accidental releases. While it was nowhere near as large as the 1989 Exxon Valdez disaster in Alaska – where 41 million litres leaked into the ocean – every acute spill poses a threat to wildlife. In addition, most of the oil found in seawater can be traced back, not to acute releases, but to natural seeps or diffuse releases from human activities [1], which represent a chronic hazard to the aquatic environment. In an effort to elucidate the effects of oil on fish, we used zebrafish as a model organism. Specifically, we were interested in analysing the – as yet largely unknown – effects of oil on fish development. Since most assays using zebrafish rely on changes in fish morphology that display little discrimination between different toxicants, we decided to perform a toxicogenomic study. This approach makes it possible to identify specific genes that are either up- or down-regulated on exposure to toxic substances [2].

Morphological changes caused by oil during zebrafish embryogenesis. We chose to work with Norwegian crude oil because Norway is an important European oil producer. Moreover, since under real-life environmental conditions some of the constituents will be soluble in water, we prepared a water accommodated fraction of the oil [3].

As a first step, we established the concentrations of experimental toxicants which, while avoiding cell death or embryo mortality, ensure that at least 40 % of the exposed embryos will

Fig. 1: Morphological changes after exposition with crude oil or the water accomodated fraction.
A: untreated control, B: dorsal curvature, C: kinked tail, D: oedema and kinked tail,
E: elongated heart, F: untreated control, normal heart.

Ruedi Keller, Eawag



The zebrafish are examined for abnormalities under the microscope.

show morphologically visible effects. Hence, crude oil was tested in dilutions ranging from 0 to 1000 parts per million (ppm) and the water accommodated fraction in concentrations of 0–100 %.

The optimum concentrations were 30 % for the water accommodated fraction and 100 or 1000 ppm for the oil, although the morphological changes were much less marked with 100 ppm. Among the major visible signs observed with these exposure regimes were an elongated heart, oedema, dorsal curvature of the body axis and a bent tail, while control embryos developed normally (Fig. 1).

Gene expression considerably altered in developing zebrafish exposed to oil. After these preliminary tests, we started the toxicogenomic experiments. Zebrafish at different developmental

Number of genes affected by crude oil or the water accomodated fraction.

		Age of embryos during exposure		
		4–28 hours post fertilization	24–48 hours post fertilization	96–120 hours post fertilization
100 ppm crude oil	total number of genes affected	634	639	719
	upregulated	326	353	387
	downregulated	308	286	332
1000 ppm crude oil	total number of genes affected	564	752	983
	upregulated	315	533	428
	downregulated	249	219	555
30 % water accommodated fraction	total number of genes affected	46	40	230
	upregulated	9	9	86
	downregulated	37	31	144
Number of genes affected by both the crude oil and the water accomodated fraction		7	16	3

stages were treated with crude oil or the water accommodated fraction. We tested

- 4-hour-old embryos, at the beginning of gastrulation (i. e. formation of different cell types);
- 24-hour-old embryos, at the stage when the overall body plan is laid down;
- 96-hour-old fish, after hatching is completed.

The duration of exposure was 24 hours in each case, i. e. from 4 to 28 hours, from 24 to 48 hours and from 96 to 120 hours. Using so-called microarrays (see Box), we detected several hundred genes which, on exposure to 100 or 1000 ppm crude oil, showed significantly altered expression levels compared with untreated zebrafish embryos (Table). Most strikingly, the number of genes exhibiting a significant response to 100 ppm was found to be as high as with exposure to 1000 ppm. This assay can thus detect responses to toxicant concentrations that do not cause acute morphological effects.

In contrast to crude oil, the water accommodated fraction affected far fewer genes overall (Table). It seems that compounds present in the crude oil but not in the water accommodated fraction act synergistically, leading to an increase in the toxic effects. While only around 40 genes are affected in unhatched zebrafish embryos, 230 genes showed altered expression levels at a later stage, shortly after hatching (Table). This might be due to the fact that organ development has not yet proceeded far enough in the unhatched embryos.

Effects on genes specifically expressed during embryogenesis or toxicant defence. In order to find suitable biomarkers, we focused on the small number of genes that were affected by the crude oil and by the water accommodated fraction: this applies to 7 genes during early embryogenesis, 16 genes in the 24- to 48-hour-old embryos and 3 genes in the hatched embryos. The low similarity between the toxicogenomic profiles observed is a further indication of the high stage specificity of toxicogenomic effects.

Among the altered genes, we identified those that are either specific to embryogenesis or involved in toxicant defence. One up-regulated gene, for example, codes for a protein that plays an important role in blood cell formation. Higher transcript levels were also detected for *Cyp1a1*, a gene encoding a member of the cytochrome P450 superfamily of enzymes, which is primarily involved in toxicant defence.

Zebrafish embryos: an effective model system for the assessment of pollutants. Our preliminary work demonstrates that zebrafish embryos can serve as a specific and highly sensitive whole-animal model for monitoring the toxicogenomic impact of chemicals [4]. Although vertebrate cell lines and other *in vitro* test methods are of considerable value in assessing the toxicological effects of drugs and pollutants, they cannot entirely replace whole-animal test systems. Zebrafish embryos represent a low-cost and ethically acceptable vertebrate model that will not only be useful in the toxicological evaluation of the tens of thousands of compounds to be tested under the EU REACH programme but can also help to estimate the developmental toxicity of novel compounds at an early stage of drug development. Furthermore, zebrafish embryos are especially suitable for evaluating the effects of pollutants during cell differentiation and morphogenesis which are impossible to detect in cell cultures or other *in vitro* systems.

To obtain a complete read-out of crude oil toxicity, we next plan to examine proteomic profiles during the early life stage of zebrafish development. Analysis of an organism's proteome permits the detection of subtle changes in individual protein levels in response to environmental stressors. This provides insights into underlying mechanisms of toxicity, potentially leading to the discovery of new, general biomarkers of exposure that could be used as molecular assessment tools. ○ ○ ○

Toxicogenomics using microarrays

Microarrays or gene chips are collections of microscopic DNA spots, commonly representing single genes, arrayed on a solid matrix. Gene chips make it possible to monitor the expression levels of thousands of genes simultaneously. The gene chips used in our study comprise the zebrafish genome.

After exposure to pollutants, zebrafish embryos are sacrificed to extract total messenger RNA (mRNA), i.e. transcripts of all the genes that were active during exposure. In parallel, mRNA is isolated from untreated embryos. Subsequently, the two mRNA fractions are translated into complementary DNA (cDNA) by the enzyme reverse transcriptase, and the cDNA is labelled with a red (treated embryos) or green (untreated embryos) fluorescent dye. These fluorescent probes are mixed and hybridized to a single microarray, which is finally scanned in a microarray scanner. Fluorescence is visualized after excitation with a laser beam of a defined wavelength. The relative intensities of each fluorophore can be used in ratio-based analysis to identify up-regulated and down-regulated genes.

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Marc Suter, chemist and group leader in the Environmental Toxicology Department.
Co-authors: Ksenia Groh, Victor Nesatyy

Exposure to pollutants revealed by protein patterns

Proteome analysis is not only a promising method in the medical field. For almost 10 years, it has also been applied in environmental toxicology. This approach reveals which proteins are induced or suppressed when organisms are exposed to environmental pollutants. At Eawag, the method has now been established for zebrafish.

As fish are generally unable to avoid the effects of adverse chemical or physical changes in their habitat, they are considered sentinel species for the condition of surface waters. If fish are exposed to chemical stressors, the appropriate defences are activated in the body: specific genes are expressed and the associated proteins are synthesized. Thus, for example, metallothioneins (metal-binding proteins) are produced when water is contaminated with heavy metals, or the synthesis of proteins from the cytochrome P450 family is stimulated in the presence of organic pollutants.

The reaction of fish to altered environmental conditions can be studied with the aid of proteomics. This approach involves analysing the proteome – the total set of proteins expressed by a genome in a cell, tissue or organism – so as to identify proteins that are induced or suppressed. This attractive but complex method (see Box) has been significantly improved in recent years, but it still poses a number of challenges [1]. Our aim was to further refine the method and establish it for purposes of ecotoxicological research at Eawag. We focused on the zebrafish since its genome has been fully sequenced and therefore all the proteins are theoretically identifiable.

Identifying more proteins. A serious methodological difficulty for proteome analysis is the fact that usually only a small proportion of all the proteins whose expression is altered as a result of contact with pollutants can be identified. This is because the proteins produced in response to chemical stress are often only present in small quantities and are masked by housekeeping proteins occurring in higher concentrations, e.g. structural proteins.

In our experiments with zebrafish, it was shown that the majority of the total of 900 proteins identified belonged to the family of vitellogenins (egg yolk proteins), preventing the detection of other important proteins lost in the noise. We therefore investigated whether it is possible to increase the number of proteins that can be identified by removing the yolk sacs from the zebrafish larvae prior to protein extraction. After all, vitellogenins occur mainly in this organ formed by the mother. This measure proved to be highly successful, as the list of proteins subsequently identified included virtually no vitellogenins, and the total number of proteins detected rose from 900 to 1200.

We managed to obtain an even larger number of identifiable proteins – 2700 in total – through biological fractionation of the extract. In this method, a commercially available extraction kit is

Principle of proteome analysis

Proteome analysis characterizes the entire set of proteins present in a type of cell, tissue or organism under specified conditions and at a given time. In our laboratories, we are interested in protein expression patterns in zebrafish that are either untreated or exposed to pollutants. To analyse these patterns, proteins are extracted from the fish after the incubation period, separated and sequenced. The method traditionally used for this purpose is two-dimensional gel electrophoresis: individual spots are excised from the gel, and the proteins they contain are sequenced. However, with this technique, only 50–100 proteins can be identified. Today, therefore, we use an approach known as multidimensional protein identification technology (mudPIT) [2], which involves liquid chromatography coupled with mass spectrometry. Here, the proteins are first digested with trypsin to produce smaller fragments. The resultant peptides are separated by liquid chromatography on two connected columns with different properties and successively eluted from the columns with increasing salt concentrations (NH4Ac) (Fig. 1). Finally, the amino acid sequences of the peptides are determined in a mass spectrometer. The relevant proteins can then be identified by comparing these sequences against the zebrafish sequence database. Between 8 and 10 mudPIT experiments are required to identify 95% of the detectable proteins (Fig. 2A).

used to separate membrane, nuclear and cytoplasmic proteins before they are further analysed (Fig. 2B).

Improving reproducibility and reducing the time required. Other problematic aspects of proteome analysis have been poor reproducibility and the substantial investment of time required to identify the greatest possible number of proteins induced or suppressed by pollutants. About ten mudPIT experiments (see Box) are needed to identify 95 % of the detectable proteins [3]. Since an experiment of this type takes 24 hours, at least 10 days would be required for the analysis of one single sample. However, the amount of time required was not the only unsatisfactory aspect: in addition, the ten replicates often yielded widely varying results (Fig. 2A). We therefore tried to find a way of improving the method in this respect.

Classical mudPIT analyses only take into account doubly and triply charged peptides, which are abundant after tryptic digestion of the protein extract. To date, singly charged molecules have been excluded from sequencing, as they very often include matrix ions with no protein sequence information. However, it has now been shown that, with our method, it is indeed worthwhile taking singly charged ions into account. Considering the example of a peptide from the heavy chain of myosin (motor protein of muscle fibres), Fig. 3 illustrates that the singly charged molecule is only slightly weaker than the doubly charged, whereas the triply charged ion is not visible without zooming in on 374.3 m/z (m/z = mass-to-charge ratio of a molecule). However, if the sequence of a peptide is to be determined as unequivocally as possible, the signal must be clearly distinguishable from the noise. Accordingly, the number of proteins identified when only doubly and triply charged ions were taken into account was substantially lower than when singly charged peptides were also included (Fig. 2C).

Overall, the inclusion of singly charged peptides in our analytical method proved advantageous. Not only was reproducibility markedly improved, but 3–4 replicates are now sufficient to allow 95 % of detectable proteins to be identified (Fig. 2D). The time required to analyse a sample is thus reduced to 3–4 days.

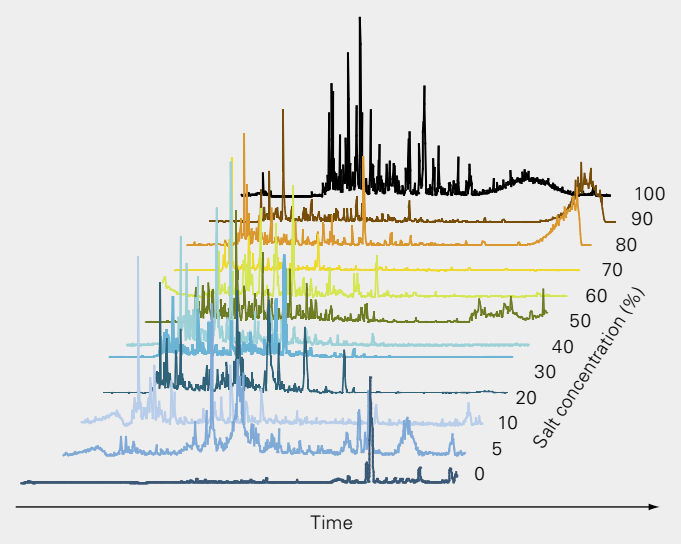
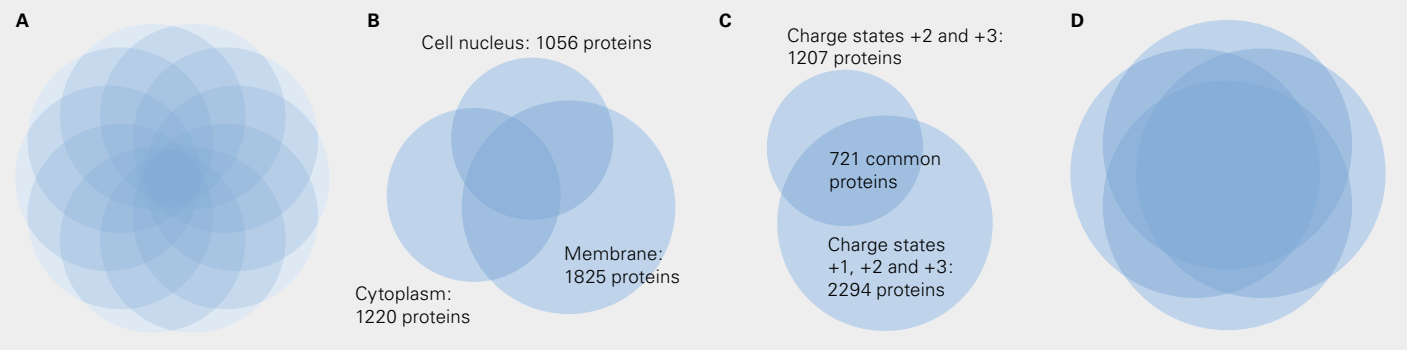


Fig. 1: Peptides are separated by liquid chromatography on two columns connected in series and then eluted with increasing salt concentrations.

Initial experience with the refined method. The establishment of environmental proteomics is challenging, but has now been largely completed at Eawag. This approach is extremely attractive for our ecotoxicological research, as it not only provides insights into underlying mechanisms of toxic action but also makes it possible to identify proteins that could serve as biomarkers of exposure to specific pollutants or groups of pollutants. Among the biomarkers already employed today are the two above-mentioned detoxification enzymes and the egg yolk protein vitellogenin, which is induced in the event of exposure to endocrine disruptors [4].

Initial experiments have shown that zebrafish exposed to 1 µM cadmium show significantly altered protein expression patterns compared with untreated fish. For example, myosin heavy chain concentrations were elevated compared with controls. In

Fig. 2: The number of proteins that can be identified by proteome analysis. Each circle represents a single mudPIT experiment. The more the circles overlap, the greater the reproducibility. A: On average, 8–10 mudPIT experiments are required to analyse the proteome. B: Biological fractionation allows more proteins to be identified. C: Inclusion of singly charged peptides increases the number of proteins identified. D: With our technique, only 3–4 mudPIT experiments are now needed for proteome analysis.



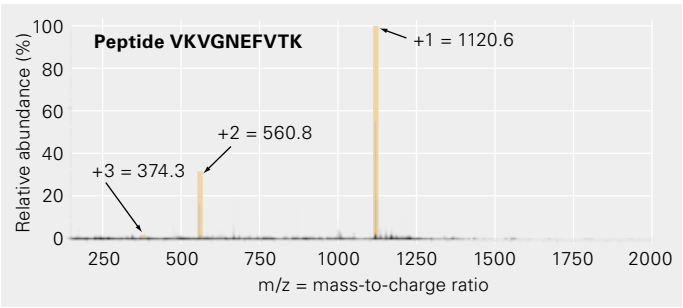
Martina Bauchrowitz, Eawag

Eawag scientist Victor Nesatyy at the mass spectrometer.

addition, we were able to observe the same effect at the genetic level: expression of the myosin gene was induced in zebrafish larvae that had been incubated with crude oil containing heavy metals. In both cases, the fish also exhibited curved tails – a classic response to toxins.

Similarly, proteome analysis was used to confirm that exposure to the female sex hormone estradiol leads to an increase in vitellogenin. We are currently also using this method in GENEZIS, a project funded by the Swiss National Science Foundation, which is investigating mechanisms of sexual determination and differentiation in zebrafish. Now that the method has been established at Eawag, it could even – without any major difficulties – be applied to other organisms, such as bacteria or green algae. ○ ○ ○

Fig. 3: Mass spectrum of a peptide from the heavy chain of myosin (motor protein of muscle fibres). See the text for details.



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Eawag spin-off: sound management of surface runoff

Two years ago, Michele Steiner overcame his cold feet and set up his own company: wst21 – water, strategy and technology in the 21st century.

For Michele Steiner, an ETH-educated environmental engineer, the decision to leave the "sheltered" environment of Eawag research was anything but easy. However, at the end of 2005 – encouraged by his supervisor at Eawag, Professor Markus Boller – Steiner took the plunge and founded his own company, wst21. Although he had a lot to learn in a wide variety of new fields – from marketing and corporate design, through choosing a legal form for the company, securing an entry in the Commercial Register and taking out insurance, to drumming up business and drawing up contracts – Steiner relished the challenges and felt well equipped to cope with them, thanks to the experience he had gained as a student and research scientist.

wst21 – water, strategy and technology in the 21st century. The key aim of the wst21 business is to combine the development of technical solutions and strategies for urban water management. As well as roof and facade water, the main focus is on road runoff. Currently, for example, wst21 is carrying out monitoring at a treatment facility for road runoff in Attinghausen. Here, water draining from a section of the St Gotthard motorway about 2 km in length is collected. The company is studying whether the performance and dimensions of the installation are optimal, so that cost savings can be made when facilities of the same type are installed in the future. In addition, wst21 is monitoring the operation and disposal arrangements, offering answers to questions such as: When do the layers of sand and adsorber material need to be regenerated or disposed of? How often do the pipes need to be cleaned and sludge pumped off? As well as working under contract to public authorities at the communal, cantonal and federal level, wst21 provides services for engineers, planners and private clients.

Knowing your own abilities and learning from others are fundamental to successful collaboration.

Michele Steiner

Successfully going it alone. Before the spin-off was set up, Eawag offered the budding entrepreneur technical and administrative support. In addition, the start-up benefited from low-rent office facilities and infrastructure on the Eawag site for the first two years. These advantages were highly appreciated, and Michele Steiner intends to maintain contacts with the institute and its researchers in future. With a team now comprising four members, wst21 has been based at the TECHNOPARK Zurich since December 2007.

www.wst21.ch

Martina Bauchrowitz

A road runoff treatment facility on the St Gotthard motorway: using the monitoring data obtained here, wst21 is developing a scientific basis for specifying the dimensions of future installations.

A wealth of experience

Michele Steiner joined Eawag in 1997. His first project, a literature study, concerned the evaluation of potential adsorbents for the removal of heavy metals, such as copper and zinc, from roof and facade runoff. The topic of his subsequent thesis was experimental testing of the efficiency of these materials and their practical applicability. A filter system consisting of granulated ferric hydroxide mixed with lime sand proved to be particularly suitable and has since been successfully used in a number of applications, e.g. for the new copper-clad building at the Federal Office of Metrology (METAS) site in Wabern (Canton Bern). In a postdoctoral project, Michele Steiner also investigated the treatment of contaminated runoff from motorways and other busy roads. The aims of this study were to develop technical processes offering a high level of performance within a relatively small area, and also to elaborate monitoring programmes that would allow the performance of the innovative systems to be evaluated.

New centre for applied ecotoxicology

The risks posed by chemicals should be more closely studied in Switzerland – this was the conclusion reached by the Federal Council and Parliament. In cooperation with the Federal Institute of Technology Lausanne (EPFL), Eawag is now establishing a centre for applied ecotoxicology.

In its report on independent toxicology research in Switzerland, issued in May 2007, the Federal Council concluded that the existing resources and structures within the university sector are not adequate to permit the development of a scientific basis for the assessment of chemical-related risks to health, safety and the environment. This problem has become more acute since the closure in June 2001 of the Institute for Toxicology in Schwerzenbach, which had been jointly operated by the Federal Institute of Technology (ETH) Zurich and the University of Zurich. Having received the approval of the Swiss Parliament in October 2007, Eawag can now, in partnership with the EPFL, establish a centre for applied ecotoxicology.

Close to research, teaching and practice. With public funding of CHF 2 million per year, the centre for applied ecotoxicology will serve three essential functions:

- Clearing house: the centre is to monitor national and international developments in applied ecotoxicology and regularly discuss current and emerging issues and potential solutions with representatives of practice and academia.
- Research and development: efforts will focus on the development of, for example, cost- and time-saving test methods for detecting ecotoxic effects, methods of chemical analysis, and user-friendly systems for modelling the risks of chemical compounds.
- Information platform: the centre is to publish important findings in scientific journals and also to make information available online, in a newsletter, in media contributions and in the form of continuous education events for professionals and students. In addition, as a public service contact point, it will answer technical queries.

Based in Dübendorf and Lausanne. The development of the ecotoxicology centre was planned and overseen by a national task force led by Professor Rik Eggen, Deputy Director of Eawag. The new institution is based at Eawag in Dübendorf (approx. 6 staff) and at the EPF Lausanne (approx. 3 staff). While research at Dübendorf will focus on questions of aquatic ecotoxicology, the Lausanne site will be mainly concerned with terrestrial ecotoxicology. The ecotoxicology centre will also carry out contract research but will not compete with the private sector. Instead, it will offer services in cases where neutral expertise or specific capabilities are not otherwise available. ○ ○ ○

Martina Bauchrowitz

Three questions for the head of the new ecotoxicology centre

The head of the new centre for applied ecotoxicology is biologist **Almut Gerhardt,** who took up this post on 1 June 2008. As well as pursuing an academic career in aquatic ecotoxicology, Almut Gerhardt has also set up and managed her own company, LimCo International.

What makes this new position attractive for you?
The opportunity to develop something new has a pioneering dimension, which appeals to me. I'm also attracted by the task of conducting applied ecotoxicological research and at the same time developing concrete products, like sensitive tests for the assessment of chemicals and for use in ecotoxicological water monitoring. Last but not least, we can pass on practice-oriented knowledge to students and users, thus defining future directions.

What makes the new centre's activities different from those of universities or the private sector?
By being embedded in the ETH Domain, the centre is right at the heart of research. It can pick up findings directly and develop practical products on these sound foundations. I'm thinking in particular of toxicity tests, risk assessment software or educational materials. In contrast, universities' strengths lie in fundamental research, and product development at private companies is exclusively market-oriented. The new independent centre simply has greater freedom in that respect.

What research projects do you intend to start as soon as possible?
Existing projects concerned with effect-oriented assessment of toxic substances are to be continued and expanded. At the same time, we want to launch new multidisciplinary projects. One specific example is the development of a so-called multimetric sensor platform as an early-warning system for routine monitoring of waterbodies. This should make it possible to measure biological, chemical and physical parameters simultaneously and combine them with ecological parameters.

In Brief

12 September 2008: Eawag Info Day

From source to tap – good-quality drinking water for today and tomorrow

The quantity and quality of drinking water depend on the sources it is drawn from. This year's Info Day will be concerned with various aspects of this relationship, considering questions such as: What is the importance of surface waters for groundwater protection? Does climate change affect water resources? And how can the risk of geogenic contamination be assessed at the global level? In addition, the key findings of the cross-cutting Eawag project Wave21 (Drinking water in the 21st century) will be summarized at this event. Presentations will also cover new methods for evaluating drinking water safety and removing organic trace contaminants, as well as modern approaches to drinking water treatment. Conference language: German. infotag@eawag.ch

Expedition on the Yangtze

For the first time, an international scientific team was recently granted permission by the Chinese government to study water quality on the Yangtze. Among the foreign researchers participating were Eawag's Beat Müller and Michael Berg. Hundreds of water and sediment samples were collected for analysis, and the results are remarkable: although the pollutant loads are in some cases very heavy, the concentrations in China's "main artery" are generally comparable to those found in other major rivers worldwide. Another goal of the expedition was to detect any surviving white-flag dolphins – unfortunately, however, this species (also known as the Yangtze River baiji) is probably already extinct. www.eawag.ch/jangtse_EN

New postgraduate course

«Integrated Water Resource Management»

One of the UN Millennium Development Goals is to halve, by 2015, the proportion of people without access to safe drinking water. To attain this goal, there is a global need for experts taking an integrated approach to water-related problems and poverty reduction. For this reason, a postgraduate course in Integrated Water Resource Management has been offered since 2007 by the Bern University of Applied Sciences, Architecture, Wood and Civil Engineering, in partnership with various other universities, federal institutions, NGOs and Eawag.
Further information: www.ahb.bfh.ch/ahb/en/Weiterbildung/ndk

Events

Eawag Info Day
12 September 2008, Eawag Dübendorf
Vom Gewässer ins Glas – gutes Trinkwasser für heute und morgen

Peak courses at Eawag Dübendorf
7 October 2008
Der Einsatz von umweltpsychologischen Massnahmen für Verhaltensänderungen im Umweltbereich

29/30 October 2008
Wo ist Heizen und Kühlen mit Abwasser möglich und sinnvoll?

11/12 November 2008
Ökotoxikologie-Kurs coetox Basis-Modul

5/6 February 2009
Evolutionsökologie im Gewässerschutz

Details: www.eawag.ch/veranstaltungen