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FACULTY OF FISHERIES AND PROTECTION OF WATERS

The role of toxicity tests on early life stages of fish in assessing the toxicity of substances and preparations

Úloha testů toxicity na raných vývojových stadiích ryb při posuzování toxicity látek a přípravků

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I thereby declare that I wrote the Ph.D. thesis myself using results of my own work or collaborative work of me and colleagues and with help of other publication resources which are properly cited.

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Chapter 1

General Introduction

1.1. Protection of aquatic environment and toxicity tests on fish

To maintain a good quality of water is the priority and contemporarily the limiting factor for maintaining life on the Earth. In order to fulfil its function as a part of the environment, water must be permanently protected from contamination with toxic or otherwise dangerous substances. If we admit it or not, aquatic environment becomes the final depository of substances and preparations used by man during his life or, which arise as intermediate products or waste during their production. It is therefore necessary to know the rate of risks which the individual components represent for the aquatic environment. When assessing a risk which a given substance or preparation represents for the aquatic environment, it is necessary to work with results of biological assays on aquatic organisms.

Toxicity tests are necessary in water pollution evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota. For example, the interaction of chemical factors and the toxic effects of complex matrices cannot be determined. Different kinds of aquatic organisms are not equally susceptible to the same toxic substances nor are organisms equally susceptible throughout the life cycle. Even previous exposure to toxicants can alter susceptibility (Tarzwell, 1958, 1971).

Toxicity tests are useful for a variety of purposes that include determining: a) suitability of environmental conditions for aquatic life, b) favourable and unfavourable environmental factors, such O_2, pH, temperature, salinity, or turbidity, c) effect of environmental factors on waste toxicity, d) toxicity of wastes to a test species, e) relative sensitivity of aquatic organisms to an effluent or toxicant, f) amount of waste treatment needed to meet water pollution control requirements, g) effectiveness of waste treatment methods, h) permissible effluent discharge rates, i) compliance with water quality standards, effluent requirements, and discharge permits (Franson et al., 1989).

From the aquatic organisms of choice, fish are the most frequently used organisms in toxicity tests, as they represent the highest positioned organisms in the aquatic environment and they are contemporarily the last link in the food chain. Fish toxicology has its particularities which differ from mammalian toxicology. Fish are very closely associated with the environment where they live, and the majority of toxic substances are consequently absorbed into the organism via skin and gills. Only lesser amount of such substances is taken up via the digestive tract. Therefore, the Playa's CCC rule (concentration, complexation, competition) is applied in fish toxicology to an important extent. The amount of a substance in water (its concentration) influences expressively its effect. Bioavailability of substances for fish strongly depends on the form of their occurrence (simple ions or complexes, dissociated or undissociated forms of occurrence) and on their solubility. Generally, complex forms of various elements are less toxic. This concerns either metals (toxic are above all simple ions), or non–metals (biologically active are above all simple ions such as CN^- and F^-, and not cyanocomplexes or fluorocomplexes). For this reason, when assessing the toxicity and effects of substances and preparations on fish, it is necessary to know not only the concentration, but also the form of occurrence which is closely related to water quality (pH, ANC4.5, COD, BOD5 and others). Another factor applied in toxicology is the exogenic or endogenic competition. Exogenic competition can be exemplified on the relationship of nitrites and chlorides and their adsorption via chloride cells (eosinophilic cells) in gills. Protection of fish against the uptake of nitrites by increasing the concentration of chlorides in water is a result of such competition. To exemplify endogenous competition, e.g. the competition between zinc and cadmium in filling the SH groups of amino acids can be reported.

A very important difference between fish and mammalian toxicology is given by their differing relationships to the ambient temperature. Metabolism intensity in fish as in

poikilothermic organisms depends on water temperature and on the duration and intensity of sunlight. These factors (together with other ones, such as water saturation with oxygen) contemporarily affect the intensity of detoxicating mechanisms (biotransformation and excretion of toxins or their metabolites from the organism).

From the above it is evident, that fish are very valuable and up to now clearly not substitutable testing material which can reflect the effects of the tested substances on their organisms, helping to record the risks emerging from the contamination of the aquatic environment.

Toxicity tests are the basic method to assess the effect of the evaluated substances on fish.

Toxicity tests on fish are used mainly for the following:
Ecotoxicity assessment of substances and preparations
Determination of ecotoxicity of wastes
Assessment of medicaments designated for fish, for their registration
Assessment of tolerance to medicaments before their use
Assessment for toxicity of water when investigating the accidental fish kills
Study of the effects of the tested substances on the aquatic environment (experimental purposes)

Different test procedures to evaluate the hazard potential of anthropogenic xenobiotic on fish have been proposed and are applied in practice. In the simplest tests acute toxicity is determined, and mostly concentrations leading to mortality within a short time only are of interest. However, from the ecotoxicological point of view, mortality is of minor significance as most xenobiotic usually occurs in subacute concentrations in the environment. To reveal actions of low substance concentrations, finer test procedures have to be applied; effects on ecotoxically important factors such as growth and reproduction are of interest. Long-term tests, i.e. life–cycle tests extending over one or two generations, are indicated to scrutinize a substance thoroughly. Such tests have been conducted occasionally (Bresch, 1991). But the life cycle toxicity test is considered by most aquatic toxicologists to bet the ultimate test in establishing long–term „safe“environmental concentrations of toxic chemicals for both vertebrate and invertebrate aquatic populations. Act of 1976, which required the Environmental Protection Agency (EPA) and industry to evaluate the environmental impact of new chemicals before commercial production, and the manufacture or marketing of an estimated 1000 new chemicals each year created the need for a more rapid, less costly, and less risky vertebrate test than the fish life cycle test for determining safe environmental concentrations of toxic chemicals. During life cycle tests with several species of fish and a variety of toxicants, certain developmental stages have consistently been more sensitive than others. The possibility of focusing research efforts on these more sensitive stages promises success in searching for quicker and less costly ways of predicting chronic toxicity of chemicals to fish. Several investigators proposed that chronic toxicity to fish might be predicted by use of shorter tests with early developmental stages. In studies with selected toxicants, these early stages were shown to be among the most sensitive in the life cycle (Pickering and Thatcher, 1970; Pickering and Gast, 1972; McKim et al., 1978). It was emphasized that, with a relative short exposure (several months) of the embryo-larval and early juvenile stage of fish to a toxicant, an estimate of the maximum acceptable toxicant concentrations (MATC) could be obtained without a complete life cycle test. Reviews of life cycle toxicity test data on freshwater fish, from more than 60 chronic tests with more than 40 organic and inorganic chemicals, show that tests with early life stages of four species of fish can be used to estimate the MATC within a factor 2 in most cases (McKim, 1977).

1.2. The most used toxicity tests on fish

1.2.1. Juvenile toxicity tests

This concerns test during which the fish that have not yet reached their sexual maturity are exposed to a series of concentrations of the tested substance. According to the duration of exposure, these tests can be of **acute toxicity** (acute toxicity test usually lasts for 24 to 96 hours) or of **subchronic toxicity** (fish juvenile growth test lasting for 28 days).

Acute toxicity test

This method is replicate of the OECD 203 Guideline for Testing of Chemicals "Fish, Acute Toxicity Test". The principle of this method is the exposure of fish to tested substance preferably for a period 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours. Test procedure uses static, semi-static or flow-through methods. There are recommended fish species as follows for the tests: zebrafish (*Danio rerio*), fathead minnow (*Pimephales promelas*), common carp (*Cyprinus carpio*), ricefish (*Oryzias latipes*), guppy (*Poecilia reticulata*), bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*). Fish aren´t fed until 24 ours before the test is started and during the test. Test duration is preferably 96 hours. The basic result of this test is the concentration which kills 50% of the fish during 96 hours exposure (96hLC50).

The 96hLC50 value for fish belongs to data that enable to label specific risk of individual chemical substances and chemical preparations for aquatic organisms as follows:

R 50: Very toxic to aquatic organisms LC (EC, IC)50 $\leq$ 1 mg.l^{-1}

R 51: Toxic to aquatic organisms 1 mg.l^{-1} < LC (EC, IC)50 $\leq$ 10 mg.l^{-1}

R 52: Harmful to aquatic organisms 10 mg.l^{-1} < LC (EC, IC)50 $\leq$ 100 mg.l^{-1}

Fish juvenile growth test – (subchronic toxicity test)

This test is designed to assess of prolonged exposure to chemicals on the growth of juvenile fish. The method is a replicate of the OECD 215 Guideline for Testing of Chemicals "Fish, Juvenile Growth Test". Principle of this test is the exposure of juvenile fish in their exponential growth phase to a range of sublethal concentrations of the test substance dissolved in water preferably under flow-through, or, if not possible, under appropriate semi-static (static-renewal) conditions. The test duration is 28 days. Fish are fed daily. The food ration is based on initial fish weights and may be recalculated after 14 days. At the end of the test, differences of weight gain of fish of particular groups are calculated and compared with control. Effects on growth rates are analysed using a regression model in order to estimate the concentration that would cause an x % variation in growth rate, is EC (e.g. EC10, EC20 or EC30). Alternatively, the data may be compared with control values in order to determine the lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC). There is usually used juvenile rainbow trout (*Oncorhynchus mykiss*) for this test. It is possible to use other well documented species, for example zebrafish (*Danio rerio*) and rice fish (medaka, *Oryzias latipes*).

1.2.2. Toxicity tests on embryo and larval stage of fish

Tests are concerned, during which the fish are exposed to the substance tested since the developmental stage of a fertilized egg till:

a) consumption of yolk sac (so-called embryonic test),

b) reaching the juvenile stage of development (so-called embryolarval test).

Short-term toxicity test on embryo and sac-fry stages

This method is replicate of the OECD 212 Guideline for Testing of Chemicals "Fish, Short-term Toxicity Test on Embryo and Sac-fry Stages".

Principle of this method is the exposure of life stages from the newly fertilized egg stage to the end of the sac-fry stage to a range of concentrations of the test substance dissolved in water. Within the protocol a choice is possible between a semi static and flow-through procedure. The test begins by placing fertilised eggs in the test chambers and is terminated just before the yolk sac in any of the test chambers has been completely absorbed or before mortalities by starvation start in controls.

During the test, observations on hatching and survival of organisms are made at least twice a day and noticed. Dead embryos and larvae are removed and the abnormal appearance (abnormality of body and/or pigmentation, and the stage of yolk-sac absorption etc.) and also abnormalities of larvae behaviour (e.g. hyperventilation, uncoordinated swimming etc.) are noticed.

At the end of the test, measurement of individual lengths and individual weights is recommended.

Lethal and sub-lethal effect are assessed and compared with control values to determine the lowest observed effect concentration LOEC) and hence the no observed effect concentration (NOEC). Alternatively, they may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LCx/ECx, where x is a defined % effect)

Fish species recommended for this test are as follows: rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio),* common carp *(Cyprinus carpio),* ricefish *(Oryzias latipes),* fathead minnow (*Pimephales promelas*), goldfish (*Carassius auratus*) and bluegill (*Lepomis macrochirus*). The aim of test is to assess effects of the tested substance on early life stages of fish.

The following remarks to the methodology are stated upon our own experience:

- In conclusion of the test, it is recommended to carry out microscopic examination of the tested organisms (embryos), in order to register better the appropriate occurrence of abnormalities.
- In contrary with the methodical approach to initiate the exposure of eggs in 30 min to 8 hours after fertilization, we recommend to initiate the test 24 hours after fertilization, when it is already possible to distinguish unfertilized eggs and to eliminate them from the test.
- Prolongation of the test after resorption of the yolk sac without feeding the larvae did not prove useful, as the changes observed in both the control and the experimental larvae caused by starvation were serious enough, so that it was not possible to identify any impacts of the tested substance.

Early-life stage toxicity test

This method is replicate of the OECD 210 Guideline for Testing of Chemicals, „Fish, Early-life Stage Toxicity Test".

Principle of this method is the exposure of early-life stages to a range of concentrations of the test substance dissolved in water preferably under flow-through conditions, or where appropriate, semi-static conditions. The test is begun by placing from the newly fertilized eggs in the test chambers and is continued at least until all the control fish are free-feeding. During the test, observations on hatching and survival are made and noticed at least twice a day. Dead embryos and larvae are removed and the abnormal appearance (abnormality of

body and/or pigmentation, and the stage of yolk-sac absorption etc.) and also abnormalities (e.g. hyperventilation, uncoordinated swimming etc.) are noticed.

After absorption of yolk-sac, when they become able to feed actively, larvae are fed by given freshly hatched brine shrimp (*Artemia salina*), by size-graded zooplankton or by quality starter feed. It is optimal for growth and development of larvae when they have access to live food. When feeding the live zooplankton, there is a danger of infection transfer and from this point of view, feeding the freshly hatched artemia appears to be optimal. During this phase of the test the uptake of feed by the larvae and their behaviour is to be examined and dead larvae are to be removed. Samples of embryos/larvae are taken every 3–5 days during the whole test to check their length and weight growth and ontogenic development, as well as to register the occurrence of malformed specimens.

The experiment is terminated in a moment when the control or any of the experimental fish reach juvenile period.

Test is terminated, when fish reach the juvenile period. During, and at the conclusion of the test, samples of embryos and larvae are collected to monitor ontogeny development, occurrence of malformations, rate of length and weight. All the monitored parameters including mortality are compared with control and assessed lethal and sub-lethal effect, NOEC and LOEC concentrations. Similarly to the embryonic test, obtained results may be analysed using a regression model in order to estimate the concentration that would cause a given percentage effect (i.e. LC/ECx, where x is a defined % effect).

For this test these fish species are recommended: rainbow trout (*Oncorhynchus mykiss*), zebrafish (*Danio rerio), c*ommon carp *(Cyprinus carpio),* ricefish *(Oryzias latipes),* fathead minnow (*Pimephales promelas*), goldfish (*Carassius auratus*) and bluegill (*Lepomis macrochirus*).

From the point of view of the duration and carrying out the test on early developmental stage of fish it is very important to define as precisely as possible the developmental periods which the experimental organisms undergo during the test. It is evident from the review of Peňáz (2001); terminology in this branch of ichthyology is not unified. In contrary, this review showed that of numerous and even controversial contributions to development of new or existing theories of fish ontogeny, the main discrepancies appear either as the result of different approaches in philosophical generalisations or of the different accent given to particular developmental events, which have been chosen as boundaries for ontogeny periods. Peňáz in his review gave example on using selected literature sources; it was showed that extremely different definitions of the embryonic and larval periods have been in use.

This review clearly shows how differently various authors understand the period of embryonic development of the fish and it is evident, that the embryonic period is understood by various groups of scientists as follows:

A) period from activation to hatching,
B) period from activation to onset of exogenous feeding,
C) period from activation to completed yolk depletion.

Similar differences are in understanding the larval period and the review of Peňáz shows that the larval period can be understood by various scientists as follows:

A) period from hatching to onset of oral feeding,
B) period from hatching to terminated yolk sac absorption,
C) period from hatching to elimination of temporary structures and appearance of definite adult organs,
D) period from hatching to attained of sexual maturity,

E) from the onset of oral feeding to elimination of temporary structures and appearance of definitive adult organs,
F) from the termination of yolk sac absorption to elimination of temporary structures and appearance of definite adult organs.

It is evident from the above that the specification of limits between the particular developmental periods differs so much that in some cases, some periods are eliminated. In a study reporting the results of a test on fish during their early development, it is therefore necessary to specify unequivocally the limits of periods concerned by the test. Otherwise, wrong conclusions could be drawn and the results could be wrongly interpreted.

Therefore I consider necessary to specify how the embryonic and larval periods of development are understood in our tests. When evaluating the ontogeny of experimental organisms, we followed the paper of Penaz et al., (1983), who described nine embryonic (E1 – E9), six larval (L1 – L6) and two juvenile stages (J1 – J2). Peňáz (2001) and other authors report that the larval period commences with transition to exogenous feeding, or more accurately expressed, with the acquisition of the ability to ingest orally and digest intestinally. The most conspicuous and best recognisable morpho-physiological features important to detect this stage are: perforated mouth with movable mandible, perforated anal orifice, intestinal lumen and peristalsis, gall-bladder, bile secretion, and also characteristic food seeking behaviour.

This, predominantly morphological, approach to separate both periods is critised because it is not enough holistic, not taking sufficiently into account the appearance of new interactions concurrent with the dramatic organisms-to-environment change after hatching. It is also critised because it is a less practical boundary in studies of fish survival, ecology and fully ignores the aspect of fish culture technology (Peňáz, 2001).

As further reported by Peňáz (2001), it is even more difficult to determinate boundary between the larval and juvenile periods. Boundary between both periods is not characterized by sudden change but rather by lengthy transitional status with sequential, heterochromic transformation of main morphological functions and behaviour, all directing to define status typical of adult phenotype. The main source of bias in assessing the stage when the developing individual becomes juvenile (and when the juvenile period starts) is thus subjective and depends on approach used by researchers to evaluate and time the decisive developmental thresholds. In his study, Peňáz (2001) recommends defining the onset of juvenile period primarily as a stage when all provisional embryonic and larval somatic structures have been already eliminated and when (at a least few and/or rudimentary) adult structures are contemporaneously present, with the stabilisation of relative growth, not necessarily being required principal and obligatory criteria for the completion of the larval period and of metamorphosis similarly as the shifts in type of behaviour and preferred habitat.

Although I cannot evaluate to what extent does the approach of Peňáz to differentiate the early developmental period of fish into embryonic, larval and adult periods of development comply with breeding-, ecological- and other aspects, I assume that the given differentiation fully complies with the requests of toxicity tests. This contention is also justified by the fact that the following standardized methods of assessment of the effects of the tested substances and preparations on early developmental stages of fish come out from this differentiation:

OECD Guideline for Testing of Chemicals 212 Fish, Short Term Toxicity Test on Embryo and Sac-fry Stages (this test starts after fertilisation of eggs and is terminated just before the yolk sac of any larvae in any of the test chambers has been completely absorbed).

OECD Guideline for Testing of Chemicals 210 Fish, Early-life Stage Toxicity Test (this tests starts after fertilisation and it should continue at least until all the control fish have been free-feeding. Test duration depends upon the species used. The recommended duration of test

for *Cyprinus carpio* is 28 days (at temperature of 21–25 °C). At this time and in the given temperature range, fish gain the adult stage described by Peňáz (2001).

In the above-stated tests on early developmental stages of fish, embryos and larvae are exposed to the tested substance in form of a bath containing the tested substance in the requested concentration. Literature (Walker et al., 1996) show description of other two approaches to exposure, the so-called „injection" methods (micro-injection and nano-injection methods) during which the tested substance is applied through injection directly into the fertilized eggs. These methods are recommended for the lipophilic contaminants such as halogenated aromatic hydrocarbons (HAHs, e.g., polychlorinated biphenyls, PCBs) and polycyclic aromatic hydrocarbons (PAHs, e.g., benzo[a]pyrene) that readily bioaccumulate in fish and the accumulation of these lipophilic chemicals by adult fish may have significant consequences on the development and survival of their offspring. Halogenated and polycyclic aromatic hydrocarbons translocate from adult female body stores into eggs during oocyte maturation (Niimi, 1983; Miller, 1993; Walker et al., 1994). Early life stages of fish are often more sensitive than adults to the toxicity of these chemicals (McKim, 1977; Walker and Peterson, 1994). Thus, the presence of persistent, bioaccumulative contaminants in the environment may pose a risk to fish early life stage survival and ultimately reduce recruitment into adult population. These methods have not been tested on our workplace yet but were reported to have a disadvantage in high mortality of embryos.

1.3. Review of the tested substances and preparations

The presented thesis reviews results of embryolarval toxicity tests on common carp (and on tench in one case) with substances and preparations that are (or that have been) applied into the aquatic environment in order to improve water quality, or with a preparation used in toxicity tests on fish. The last one of the substances and preparations tested is sodium nitrite as a source of nitrites. The need for testing the sensitivity of early developmental stages of fish to nitrites came from discussions on the value of immission limit of nitrites in water. It means that this apparently inconsistent group of substances and preparations has a common denominator and this is the aquatic environment and its protection.

The following substances and preparations were tested:

- **Diazinon 60 EC** – was used occasionally as a biocide to suppress extensive propagation of large daphnian zooplankton (at the concentration of 10 $\mu g.l^{-1}$) in fish farming in the Czech Republic and to forestall the risk of oxygen deficit formation. (The applications of this preparation are currently banned in the Czech Republic and given results provide only information about effects of this preparation on early life stages of fish).
- **PAX-18** – is coagulation agent that is mainly used to precipitate phosphates, to decrease the concentration of phosphorus, to prevent surface water eutrophication and incidences of cyanobacteria.
- **Sodium nitrite ($NaNO_2$ p.a**) was tested to check nitrite effects of its sublethal and environmental concentrations on early-life stages of fish and to obtain more detailed information about nitrite effects on fish and to consider the rightfulness of requirement of the EU on reduction of the nitrite immission limit in surface waters.
- **Dimethylsulfoxide (DMSO)** is used among others as auxiliary substance in toxicity tests on aquatic organisms for preparation of solutions of substances insoluble in water, usually in concentrations 0.2–0.5 $ml.l^{-1}$.

More detailed information about test substances and their use are given in the next chapters.

The aims of the study were:

1. To verify the safety of application of low doses of Diazinon 60 EC in the aquatic environment from the point of view of safety for early developmental stages of fish.
2. To assess the sensitivity of early developmental stages of fish to the preparation PAX-18 and to verify the safety of its application into water reservoirs from the point of view of its effects on fish, including their sensitive early life stages.
3. To assess the effect of enhanced concentrations of nitrites for early developmental stages of common carp, and thus the rightfulness of request of the EU to decrease the immission limit for nitrites in surface waters.
4. To verify the toxicity of dimethylsulfoxide solvent for early developmental stages of common carp, and thus the rightfulness of its use as auxiliary substance in embryolarval toxicity tests.
5. To compare the sensitivity of embryonic and embryolarval toxicity tests in use (time-demands and demands for care during these tests are different and therefore it is necessary to check to what extent does the increased demandingness reflect in sensitivity of these tests).

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Chapter 2

Toxicity of Diazinon 60 EC for *Cyprinus carpio* and *Poecilia reticulata*

Máchová, J., Prokeš, M., Svobodová, Z., Žlábek, V., Peňáz, M., Baruš, V., 2007. Toxicity of Diazinon 60 EC for *Cyprinus carpio* and *Poecilia reticulata*. Aquaculture International 15 (3–4), 267–276.

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Záhlaví sudé stránky
Toxicity of Diazinon 60 EC for *Cyprinus carpio* and *Poecilia reticulata*

Záhlaví liché stránky

Chapter 2

Chapter 3

Toxicity of Diazinon 60 EC for embryos and larvae of tench, *Tinca tinca* (L.)

Máchová, J., Prokeš, M., Peňáz, M., Baruš, V., Kroupová, H., 2010. Toxicity of Diazinon 60 EC for embryos and larvae of tench, *Tinca tinca* (L.). Reviews in Fish Biology and Fisheries 20, 409–415.

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Záhlaví liché stránky
Toxicity of Diazinon 60 EC for embryos and larvae of tench,
Tinca tinca (L.)

Záhlaví sudé stránky
Chapter 3

Chapter 4

Polyaluminium chloride (PAX-18) – acute toxicity and toxicity for early development stages of common carp (*Cyprinus carpio*)

Mácová, S., Máchová, J., Prokeš, M., Plhalová, L., Široká, Z., Dlesková, K., Doleželová, P., Svobodová, Z., 2009. Polyaluminium chloride (PAX-18) – acute toxicity and toxicity for early development stages of common carp (*Cyprinus carpio*). Neuroendocrinology Letters 30 (Suppl. 1), 192–198.

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Machova_Chapter 4

Záhlaví liché stránky
Polyaluminium chloride (PAX-18) – acute toxicity and toxicity for early development stages of common carp (*Cyprinus carpio*)

Záhlaví sudé stránky
Chapter 4

Chapter 5

Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.)

Kroupová, H., Prokeš, M., Mácová, S., Peňáz, M., Baruš, V., Novotný, L., Máchová, J., 2010. Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.). Environmental Toxicology and Chemistry 29 (3), 535–540.

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Machova_Chapter 5

Záhlaví liché stránky
Effect of nitrite on early-life stages of common carp (*Cyprinus carpio* L.)

Záhlaví sudé stránky
Chapter 5

Chapter 6

Early ontogeny, growth and mortality of common carp (*Cyprinus carpio*) at low concentrations of dimethyl sulfoxide

Máchová, J., Prokeš, M., Kroupová, H., Svobodová, Z., Mácová, S., Doleželová, P., Velíšek, J., 2009. Early Ontogeny, Growth and Mortality of Common Carp (*Cyprinus carpio*) at Low Concentrations of Dimethyl Sulfoxide. Acta Veterinaria Brno 78, 505–512.

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Machova_Chapter 6

Zahlavi liché
Early Ontogeny, Growth and Mortality of Common Carp (*Cyprinus carpio*) at Low Concentrations of Dimethyl Sulfoxide

Zahlavi sudé
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Chapter 7

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General Discussion

As already stated, this group of preparations which at the first glance might appear inconsistent, has its common denominator in their use in water management or in aquatic toxicology. It concerns preparations which are (PAX 18) or which were (Diazinon 60 EC) purposefully applied in the aquatic environment, which occur in water in increased concentrations (nitrites) or, which are used in toxicity tests on aquatic organisms as in the case of DMSO (organic polar solvent). In all cases of the substances or preparations concerned, routinely performed acute toxicity tests are not sufficient for their assessment but more demanding and more sensitive tests must be chosen in order to provide more complex information about the substances tested.

Diazinon 60 EC (chemical insecticide, organophosphate, active substance diazinon at a concentration of 600 $g.l^{-1}$) was used in fish-farming in well-founded cases as a biocide to suppress excessive propagation of coarse daphnian zooplankton. As stated in our paper, the concentration of 10 $\mu g.l^{-1}$ (i.e. 100 g per 1 ha at 1 m mean depth of the pond), Diazinon 60 EC eliminated highly selectively daphnian zooplankton and was relatively quickly decomposed in the aquatic environment. This applied concentration caused no acute harm to fish, which supported the acute toxicity tests results: the 96h LC50 is 3 $mg.l^{-1}$ for the guppy (*Poecilia reticulata)* and 10–25 $mg.l^{-1}$ for the common carp (*Cyprinus carpio*). Svoboda et al. (2001) carried out acute toxicity test on common carp (*Cyprinus carpio*) with Basudin 600 EW (pesticide, the active substance of which is diazinon in amount of 600 $g.l^{-1}$) and gained very similar result (96hLC50 = 26.7 $mg.l^{-1}$). This author described effect of Basudin 600 EW (concentration of 32.5 $mg.l^{-1}$) on haematological indices of experimental fish after 96h exposure (significant lower values of erythrocyte count, haemoglobin content and haematocrit, significant decrease in leucocyte count, relative and absolute lymphocyte count and significant increase in relative and absolute count of developmental forms of neutrophile granulocytes). In comparison with control group, significant changes in biochemical parameters (decrease in cholinesterase activity, lactate dehydrogenase activity, lactate and protein concentrations, plasmatic calcium and phosphorus concentrations and increase in glucose concentration, and plasmatic natrium and potassium concentrations) were described in common carp exposed to this preparation for 96 hours in concentration of 32.5 $mg.l^{-1}$ by Lusková et al. (2002).

Effect of sublethal diazinon concentrations on some haematological parameters and blood plasma biochemistry of common carp were studied by Banace et al. (2008) and their results corresponded well to those of Svoboda et al. (2001) and Lusková et al. (2002): fish were exposed to diazinon in the concentrations of 60 and 120 $\mu g.l^{-1}$ for 10, 20 and 30 days. The experimental groups showed significantly lower values of erythrocyte count, haemoglobin content, haematocrit and leucocytes, lymphocyte and monocyte, as well as in alkaline phosphatases and significantly higher values of plasma glucose, alanine aminotransferase, asparate aminotransferase, lactate dehydrogenase compared to the control group. The results of examinations of the biochemical blood plasma profile indicated a marked neurotoxic effect of diazinon in fishes. Changes in values of both erythrocyte and leucocyte profile after exposure to diazinon-based preparation may refer to disruption of haematopoiesis as well as to a decrease on non-specific immunity of the fish.

The olfactory responses of the parr to prostaglandin F_{2a} (PGF_{2a}) were studied after exposure of the epithelium to different concentrations of Diazinon in water. Electrophysiological recordings from the epithelium indicated that the responses to this prostaglandin were significantly reduced at nominal concentrations as low as 1.0 $\mu g.l^{-1}$ and the

threshold of detection was reduced 10-fold at 2.0 $\mu g.l^{-1}$. Mature male salmon parr exposed for a period of 120 h to Diazinon (nominal concentrations from 0.3 to 45 $\mu g.l^{-1}$) also had significantly reduced levels of the reproductive steroids in the blood plasma after priming with ovulated female salmon urine (Moore and Waring, 1996).

These cases document very well that the sensitivity of a toxicity test does not depend on the duration of exposure only but also on the examination methods used for assessing its results. Of course that the assessment of test results only upon the registered mortality of testing organisms does provide the information on the lethal effects of the substance tested, but a harm to the organism is usually caused already during exposure to substantially lower concentrations.

Sensitivity of embryos and larvae of common carp and tench to diazinon was tested in toxicity tests on early developmental stages of these fishes. It was proved in experiment where the fertilized eggs were exposed to concentrations from 10 to 1000 $\mu g.l^{-1}$ Diazinon 60 EC that no statistically significant relationship was found between the above survival rates and Diazinon 60 EC concentrations. Also, no significantly negative effects of Diazinon 60 EC at concentrations tested for hatching and embryo were demonstrated. It was possible to state that common carp embryos that have been exposed to this test since 24 hrs. after fertilization till gaining the larval developmental stage (consumption of yolk sac content), exhibited relatively high resistance.

Markedly higher susceptibility of fish was discovered in the larval toxicity test. In this experiment, larvae after swimming and resorption of the yolk sac (development step L2) were exposed for 10 days to the Diazinon 60 EC in concentrations tested of 10 to 3000 $\mu g \cdot l^{-1}$ and after that they were transferred to water with no admixture of the substance tested for the next 18 days. This test demonstrated that Diazinon 60 EC affected statistically significant difference at cumulative mortality rates, compared with control in larvae exposed to 3000 $\mu g.l^{-1.}$ Markedly higher daily mortality rates compared with controls were found in larvae exposed to concentration of 1000 $\mu g.l^{-1}$. The larvae exposed to concentrations of 100, 1000 and 3000 $\mu g.l^{-1}$ responded by slowing down the growth rate and increasing the weight condition.

Yet more sensitive reaction of fish to Diazinon was demonstrated on tench (*Tinca tinca*) in course of the embryolarval test. In this test, the experimental organisms were exposed for 32 days (since 24 hrs. after fertilization till gaining the first juvenile stage by most fish) to the given preparation in concentrations of 0, 10, 100, 1000 and 3000 $\mu g.l^{-1}$. During the first 15 days of this test, the experimental organisms were totally killed in concentration 3000 $\mu g \cdot l^{-1}$, a concentration of 1000 $\mu g.l^{-1}$ caused high incidence of malformations, decrease in growth rate and ontogenetic development slowed down. A concentration of 100 $\mu g.l^{-1}$ caused mild decrease of growth rate. In any of the tests, any negative effect of Diazinon 60 EC in concentration 10 $\mu g.l^{-1}$ on the experimental organisms was proved.

It is evident from these results that the acute lethal concentration of diazinon for fish ranges by order in $mg.l^{-1}$ units. Sensitivity of the tests increases, if the surviving fish are subjected to haematological, biochemical, histological and other examinations when terminating the test. Sensitivity of embryonal tests is relatively well comparable to test of acute toxicity on juvenile fish; substantially higher sensitivity of fish to diazinon was evidenced when performing the embryolarval test. Yet more sensitive results were obtained from chronic toxicity test, in conclusion of which detailed examinations were made. These can frequently reveal reactions of the fish organism to substantially lower concentration of the tested substance (Banace et al., 2008; Moore and Waring, 1996).

PAX-18 with its active substance polyaluminium chloride is used as a coagulant for the precipitation of phosphorus in water treatment and wastewater treatment plants and also for

the treatment of natural waters. PAX-18 is possible to apply directly into the water (usually in a dose of 5–10 mg Al per one litre) or to the sediment (in tens of gram per 1 m^2 of surface of the bottom sediments) (Pitter, 2009). One possible problem in the water environment could be the presence and the accumulation of aluminium. Risk of acute toxicity for fish during aluminium application to hard water lakes was studied by Wauer and Teien (2010). These authors traced the concentration of reactive Al species in the alkaline water (pH 8) peaked at 2 $mg.l^{-1}$ in parts of the anoxic hypolimnion and was 0.088 in the epilimnion during the five years of treatment. During an Al treatment cycle in summer 2003, perches showed significant Al accumulation on gills, whereas roaches, breams and silver carps remained unaffected. Thus, the Al toxicity towards several fish species seems to be low, although the concentration of reactive Al in the lake water increased by a factor of 2. However, high Al toxicity due to lake treatment with aluminate could not be excluded, as high Al-gill concentration was observed. An Al balance two years after the treatment indicates complete export of added Al into the sediment.

In order to assess risk of use of this preparation for the water environment it is necessary to evaluate its possible danger for water organisms. In order to find out possible negative effects on fish, acute toxicity tests were done on common carp (juvenile fish, age 2–3 months), as well as a test on realry developmental stages of common carp. The mean 96hLC50 value of PAX-18 found in acute toxicity test was 753 $mg.l^{-1}$ (67.8 $mg.l^{-1}$ Al). This value was probably affected by low pH value of water (4.5) in the highest tested concentration of PAX-18. Almost the same value of the 96hLC50 (750 $mg.l^{-1}$of PAX-18) was found by Macova et al. (2010) for juvenile zebrafish (*Danio rerio*). The effect of the interaction between aluminium and low pH in a native fish species *Prochilodus lineatus* was studied by Camargo et al. (2009). Juveniles of this neotropical fish were exposed to 196 $\mu g.l^{-1}$ of dissolved aluminium in acid water, only acid water and to water with neutral pH for 6, 24 and 96 h. Results of this experiment showed that acute exposure to Al causes an ionic unbalance, probably related to the effects of Al on Na^+/K^+-ATPase activity, on the distribution and number of chloride cells in the gills as well as the effect associated with the stress response caused by the presence of metal.

This meant that the acute lethal effect of aluminium on fish revealed in concentrations of units to tens of $mg.l^{-1}$ units but a damage of the fish organism was registered already in by order lower concentrations.

Sensitivity of common carp in its early developmental stages ranged in $mg.l^{-1}$ units as it was showed in results of our experiment and it can be said that the sensitivity of embryolarval test compared to routinely performed acute toxicity test was slightly higher but it did not reach sensitivity of a test attended by other examinations indicating the damage of the organism.

Attention was paid to increased concentrations of **nitrite** in water and to their effects on fish, mainly because of a not unified opinion on the level of immission standard for surface waters. Contemporary legislation (decree of the government No. 269/2005 of the Code of Laws) reports for immission standard for surface waters the concentration 1 $mg.l^{-1}$, while the object value of immission limit is 0.1 $mg.l^{-1}$. These levels greatly exceeded criteria set out in EU Council directive 78/659/EEC on the quality of fresh water necessary to support fish life (0.01 $mg.l^{-1}$ NO_2^- for salmonid waters and 0.03 $mg.l^{-1}$ NO_2^- for cyprinid waters). For this reason, enhanced attention is paid to nitrites and their effect to fish, and its goal is to gain sufficient amount of data for objective and constructive discussion on justifiability of the target limit and vice versa, on its worthless strictness. Another reason is in the fact that concentration of nitrites in surface waters can reach higher concentrations (namely downstream the outfall of pre-purified sewage or communal waters) and even higher

concentrations can accumulate in intensive fish culture systems (Avnimelech et al., 1986) where nitrification is involved in removing ammonia. Elevated nitrite levels during unbalanced nitrification can seriously damage fish health and may lead to mass mortalities (Svobodová et al., 2005). Toxicity of nitrite to adult and juvenile fish is well documented, as well as the fact that the sensitivity of fish to nitrites is decreased with increasing concentration of chlorides in water. Mortality in seawater occurred at nitrite concentrations is 50 to 100 times higher than in fresh water (Crawford and Allen, 1977). The effect of chloride concentrations on the toxicity of nitrite in now known to be so great that experiments in which chloride concentrations are not documented are of very low value because they cannot be meaningfully compared with the results of other studies. The relationship between nitrite toxicity and chloride concentration in water is linear (Russo and Thurston, 1977; Palachek and Tomasso, 1984; Kroupová et al., 2005). According to EIFAC (1984) recommendation, it is very important to monitor the $Cl^-/N\text{-}NO_2^-$. The sensitivity of fish to nitrite depends both on fish species as well as its size and age. Salmonids are among the most sensitive taxa that have been studied, cyprinids are less sensitive (Lewis and Morris, 1986).

Tolerance of fish to nitrites depending on the size and age of fish was studied by Perrone and Meade (1977) and they indicated that Coho salmon fry had a greater tolerance to nitrite than coho yearlings. Similar phenomenon was observed by Barlett and Neumann (1998), Russo et al. (1974).

Comparison of the effects of nitrites on fish as to the duration of exposure time was enabled using results of our tests. These were acute and subchronic toxicity tests on rainbow trout performed with the use of diluting water with chloride concentration 10 $mg.l^{-1}$ Cl^-. Results of acute toxicity test showed the acute lethal concentration of nitrites at the given concentration of chlorides ranging in tens of $mg.l^{-1}$: 24hLC50 = 31.9 $mg.l^{-1}$, 48hLC50 = 25.1 $mg.l^{-1}$, 72hLC50 = 11.9 $mg.l^{-1}$ and 96hLC50 = 11.2 $mg.l^{-1}$ NO_2^-. Results of subchronic toxicity test, where the rainbow trout of the same origin was exposed to enhanced nitrite concentrations for 28 days, showed that survival was not affected by exposures up to 1 $mg.l^{-1}$ NO_2^-. On the basis of growth rate inhibition data, the values of NOEC resp. LOEC were estimated at 0.01 $mg.l^{-1}$ NO_2^- resp. 0.2 $mg.l^{-1}$ NO_2^-. At 0.01 $mg.l^{-1}$ NO_2^- (the lowest concentration tested) there was observed segmental hyperplasia of the respiratory epithelium of secondary lamellae and elevated glucose and decreased potassium in the plasma of experimental fish (Kroupova et al., 2008). The results revealed that prolongation of exposure duration increased the sensitivity of the test by three orders and the examinations of experimental fish attending the termination of the test yet increased its sensitivity.

In the embryo-larval test on common carp no significant negative effects of nitrite at the concentrations tested (0.7–330 $mg.l^{-1}$ NO_2^- at 10 $mg.l^{-1}$ Cl^-) on hatching or embryo viability were demonstrated, but significant differences in early ontogeny among groups were noted in the following days of the test. The computed LOEC and NOEC concentrations upon the lethal effects were 28 and 7 $mg.l^{-1}$ NO_2^-, respectively. Fish from all the concentrations showed a dose-related delay in development compared with the controls. In this case it could be stated that the sensitivity of embryolarval test compared to acute toxicity test was by two orders higher, but lower than that of a chronic toxicity test.

DMSO (dimethyl sulphoxide) is an important polar aprotic solvent and is used as auxiliary substance in toxicity tests on aquatic organisms, because it is less toxic than other members of this group of solvents. In the toxicity tests DMSO is usually used in the concentrations of 0.2 to 0.5 $mg.l^{-1}$. The suitability of this solvent for toxicity test on early life stage of fish was check out by embryo-larval test on common carp. Results of this test showed that mortality of embryos an larvae, intensity of development and growth, weight and occurrences of abnormalities did not result in any lethal effect or abnormalities compared to the control over

the entire test period (29 days) nor in the highest concentration of DMSO (5 $ml.l^{-1}$). Growth indicators were similar in DMSO treated (at concentrations of 0.2; 0.5 and 1.0 $mg.l^{-1}$) and control groups. However mean body weight of larvae exposed to concentration of 5 $mg.l^{-1}$ was significantly lower compared to the control. Similar low toxicity of DMSO for early stage of fish another authors found (Hallare et al., 2006). Also the acute toxicity of DMSO for juvenile fish is low and for example, Huthinson et al. (2006) states the acute toxicity (values of 48 and 96hLC50) for juvenile fish reach tens grams per litre.

Benvile et al. (1968) exposed coho salmon to low concentrations of DMSO for periods up to 100 days, to estimate survival time (LET50) and investigate chronic effects. Exposure in the range 0.01–2.0% (v/v) for 100 days did not induce lethality, and proximate analysis did not indicate any changes in body composition. Furthermore, histological analysis showed no evidence of pathological changes, for example in gill and kidney structure. On the other hand, Mortensen and Arukwe (2006) and other authors call attention to significant effect of DMSO (in concentrations ten times less than OECD recommended concentration) on endocrine responses in fish system. Mortensen and Arukwe (2006) recommend re-evaluating the use of DMSO as carrier solvent in fish endocrine disruption studies.

Several investigators have assessed the toxicity of DMSO to fish cell lines in vitro, either alone or in combination with other potential cytotoxic agents (Mori and Wakabayashi, 2000; Parkinson and Agius, 1987). However, the solvent concentrations used were generally in excess of those commonly accepted in chronic ecotoxicity testing. Furthermore, these cell lines are derived from a variety of tissues and can offer little information about the potential for physiological responses to solvent exposure at the level of the whole organisms.

Nevertheless, Hutchinson et al. (2006) warn us about solvents use in toxicity test because solvents might evert effects on test organisms through direct or indirect, specific or non-specific mechanisms. For example, presence of organic solvents in an exposure system over long periods of time may provide an additional carbon source for microbial growth. This in turn may exert positive or negative effects on certain test species (e.g. filter feeding invertebrates) while microbial interaction with test substances (e.g. biodegradation or transformation), or test conditions (e.g. reduction in dissolved oxygen concentrations) may have deleterious effects.

It is evident from the above that due to low toxicity of DMSO it is difficult to compare results (and thus also the sensitivity) of different toxicity tests. According to the results of Bresh (1991), sensitivity of a test depends on the test methodology, as well as on the texture of the substance tested. This author compared toxic threshold concentrations of three substances (4-chloroaniline, 3,4-dichloraniline and diazinon) on the base of results of early life-stage test in zebrafish versus a growth test in rainbow trout. Results of these tests showed in case of 4-chloroaniline the negative effect of this substance in both types of tests only in the highest concentration tested (1 $mg.l^{-1}$) and it could be said that in case of this substance the sensitivity of these tests was identical. In case of 3,4 dichloranilin, the test on zebrafish showed negative effect of this substance in concentration of 0.2 $mg.l^{-1}$, while the test on rainbow trout with the same concentrations did not show any negative effect. In case of diazinon, no any negative effect of this substance on the tested organism was proved in any test and thus, the sensitivity of the tests could not be compared.

In our case, results of subchronic test on juvenile rainbow trout were compared to those of embryolarval test on common carp with similar goal as did the above-cited author. Both tests were performed with comparable quality of the diluting water (the same concentration of chlorides, 10 $mg.l^{-1}$). Results of Kroupova et al. (2008) revealed that the subchronic toxicity test appeared to be more sensitive. It showed negative effect of nitrites on the tested organisms already in concentration of 0.01 $mg.l^{-1}$ $N0_2^-$ while in the embryolarval test it was in concentration of 0.7 $mg.l^{-1}$ $N0_2^-$. However, higher sensitivity of this test was achieved by wide

spectrum of examinations performed in conclusion of the subchronic test, on the bases of which the effects of the tested substance revealed already in very low concentrations. This fact provided evidence that the sensitivity of the test used is given not only by choice of the testing method itself, but also by choice of parameters to be examined and assessed in the course of and in conclusion of the test. According to the fact that the haematological, biochemical and other methods of assessment are still developing and improving, the diagnostics of damage of the fish can be expected to be still more precise. Size of the testing organism will be of greater importance, in order to allow sampling of enough blood and tissues for further investigations.

The following part of discussion is focused on the results of the embryonic and embryo-larval tests in order to compare sensitivity of these two types of toxicity tests, because the time and labour intensity of these two tests differs. The embryonic test is relatively easy to perform. Moreover, this test on cyprinid fish at 20 °C is conducted within ca. 4–6 days, while the embryolarval test at this temperature lasts for ca. 30 days. The embryolarval test also requires much more care. When the fish reach larval period in course of this test, they must be provided with exogenous (optimally live) food and to exchange the bath two- to three times a day, in order not to alter their development by their own produced metabolites. It is therefore necessary to evaluate to what extent the higher work-demandingness of the embryolarval test is reflected by its higher sensitivity or, if the sensitivity of the embryolarval test is adequate to its higher demandingness.

In case of the preparation Diazinon 60 EC, fertilized eggs of tench (*Tinca tinca*) were exposed to this preparation in concentrations of 10, 100, 1000 and 3000 $\mu g.l^{-1}$ and no significantly negative effects of this substance on hatching time, duration on rate and embryo vitality were observed. It could be thus stated that the results of the embryonic test were negative at the above concentrations of Diazinon 60 EC. Evaluating the results of the embryolarval test it was evident that mass mortality in the group exposed to the highest Diazinon 60 EC concentration (3000 $\mu g.l^{-1}$) occurred at days 8–14 and total mortality was observed in this group within ensuing 24 h. In these days the fish of this group reached the stages E9–L1. However, fish in other groups including control achieved L1–L2 at that time. In the subsequent period, fish exposed to 1000 $\mu g.l^{-1}$ started to fall behind. At the end of the trial, only fish exposed to 1000 $\mu g.l^{-1}$ were significantly delayed in development compared to the control group. Also, malformations and other abnormities were observed most frequently in this group. Slightly lower specific growth rates were measured in fish exposed Diazinon 60 EC at the concentrations of 100 and 1000 $\mu g.l^{-1}$ compared to controls. When compared with the embryonic test, the embryolarval test appeared expressively (even by order) more sensitive. Total mortality of the experimental organisms in concentration of 3000 $\mu g \cdot l^{-1}$ and enhanced mortality in concentrations of 1000 and 100 $\mu g.l^{-1}$ registered during the first 13 days of the test indicated this period to be critical for the fish. It was the period of the first exogenous nutrition, mixed nutrition, and completely exogenous nutrition. However, there was also a possibility that toxic effects of diazinon appeared late, since fish during this time complete the development of the cytochrome P450 system which was involved in bioactivation of diazinon to its more toxic metabolite diazooxon (Hogan and Knowles, 1972).

Yet more sensitive reaction of fish to Diazinon was demonstrated during the embryolarval test on tench (*Tinca tinca*). In this test, the experimental organisms were exposed for 32 days (since 24 hours after fertilization till gaining the first juvenile stage by most fish) to the given preparation in concentrations of 0, 10, 100, 1000 and 3000 $\mu g.l^{-1}$. Total mortality of the experimental organisms in concentration of 3000 $\mu g.l^{-1}$ occurred during the first 15 days, a concentration of 1000 $\mu g.l^{-1}$ caused high incidence of malformations, decrease in growth rate and ontogenetic development slowed down. A concentration of 100 $\mu g.l^{-1}$ caused mild

decrease of growth rate. Although common carp and tench are frequently considered to be equally sensitive fish, the above cases showed more expressive changes for tench in response to exposure to Diazinon 60 EC. Duration of the exposure was undoubtedly reflected by sensitivity of the test in this case, as for tench it last for the whole test period (32 days), while in case of common carp the duration of exposure was 7 days only. Results similar at the same order were described by Bresh (1991) who performed an experiment on the early stages of zebrafish (*Danio rerio*). Also Aydin and Koprucu (2005) found out that hatching success of carp in concentrations of 250 to 8000 $\mu g.l^{-1}$ decreased from 60 to 6% respectively with increasing concentration of diazinon. Above data show that our results are consistent with those found in literature.

In case of PAX-18 preparation, the fertilized eggs of common carp were exposed to concentrations 5, 10, 50 and 75 $mg.l^{-1}$. Hatching of embryos was not affected in concentrations of 5, 10 and 50 $mg.l^{-1}$, but the highest tested concentration (75 $mg.l^{-1}$) caused the mortality of organisms before the hatching. It meant that the embryonic toxicity test on common carp demonstrated the negative effect of PAX-18 preparation in concentration of 75 $mg.l^{-1}$. The following lower concentration tested (50 $mg.l^{-1}$) revealed enhanced mortality in the next course of the test (day 14 to 18). No other changes (effect on growth rate, ontogenic development, etc.) were proved in this test. Despite of that, the embryolarval test appeared to be more sensitive that the embryonic one in this case also, though the differences in sensitivity were not as expressive as in the tests with Diazinon 60 EC.

In case of sodium nitrite, fertilized fish eggs were exposed to nitrites in concentrations of 0.7; 2.7; 33; 67 a 330 $mg.l^{-1}$ NO_2^- (chloride concentration in water was 10 $mg.l^{-1}$ Cl^-). No significantly negative effects of nitrite on hatching and embryos viability were observed. In the following days of the experiment total mortality in the group exposed to the highest nitrite concentration and in the groups exposed to 67 and 33 $mg.l^{-1}$ NO_2^- increased mortality was observed. Moreover, fish from the all concentrations showed a dose-related delay in development and higher occurrences of abnormalities compared with the controls. This meant that also in the case of nitrites, the sensitivity of embryolarval test was about 3 orders higher than that of the embryonic test.

The presented results confirm that the sensitivity of toxicity test when the fish organism is exposed to the tested substance already from the fertilized eggs stage till reaching the juvenile period, is unequivocally higher and can provide substantially more information on the effect of the tested substance on growth and development of the organism during its early developmental period. It is necessary to say that this concussion is not a surprise and it follows from longer duration of exposure of the organism to the substance tested, as well as from the period which the fish undergo (mainly the critical period of transfer from endogenous to exogenous nutrition, when fish begin to actively utilize gills to bring oxygen in the organism while during the previous short period they accepted oxygen through the whole body surface). We also observed the enhanced mortality of test organisms in our tests during this period. A very important point in embryo larval test is the possibility to observe substantially higher number of indicators of negative effect of the tested substance on the testing organism, than in the case of embryonic test. Results of our tests show that decreased growth rate of the experimental organisms, slow of oncogenic development or enhanced number of abnormalities often decides about the sensitivity of the test.

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English Summary

The role of toxicity tests on early life stages of fish in assessing the toxicity of substances and preparations

Jana Máchová

Aquatic environment is the final deposition of substances and preparations used by man during his life or, which arise as intermediate products or waste during their production. It is therefore necessary to know the rate of risks which the individual components represent for the aquatic environment. Risk assessment for a given substance or preparation for the aquatic environment cannot be performed only upon the knowledge of its chemical and physical properties but above all upon the results of biological assays on fish and other aquatic organisms. Fish, which represent the highest positioned organisms in the aquatic environment and which are contemporarily the last link in the food chain, are the most frequently used organisms in toxicity tests.

Performance of toxicity tests on fish is standardized at the international level. Above all, the OECD and ISO standards are concerned. Individual tests and methodical approaches differ mutually with their time- and financial demandingness, requests for laboratory equipment and erudition of the staff, as well as with their sensitivity and reporting value. A choice of a proper test usually represents a certain trade-off between the criteria given above.

From the point of view of their duration, toxicity tests can be differed into short-term ones (acute toxicity tests) and long-term ones (subchronic and chronic toxicity tests). In a special position between these two types, there are tests on early developmental stages of fish (embryonal and embryolarval toxicity tests). The presented study is aimed to assess the sensitivity of these tests and their importance for aquatic toxicology, using examples of chosen (and seemingly inconsistent) substances and preparations tested, which are of specific importance for water management or for aquatic toxicology.

Diazinon 60 EC (organophosphate, active substance diazinon at a concentration of 600 $g.l^{-1}$) was used as biocide to suppress extensive propagation of coarse daphnian zooplankton and also as pesticide in agriculture. Presented results give information about acute toxicity of Diazinon 60 EC for guppy (*Poecilia reticulata*) and common carp (*Cyprinus carpio*) and about its effect on early life stages of common carp. The 96hLC50 for *Poecilia reticulata* is 3 $mg.l^{-1}$, for common carp 10–25 $mg.l^{-1}$. As demonstrated by results of embryonic toxicity test for the common carp, Diazinon 60 EC at the concentration of 1000 $\mu g.l^{-1}$ (the highest tested centration) has no negative effect for hatching and embryo viability. During the larval test, larvae after swimming and resorption of the sac viteline were exposed to Diazinon 60 EC at concentrations 10, 100, 1000 and 3000 $\mu g.l^{-1}$ for 10 days. Thereafter larvae were transferred to water with no admixture of the substance tested and were observed for next 18 days, when the experiment was terminated. The results of larval test shown, that, Diazinon 60 EC at the concentration of 1000 and 3000 $\mu g.l^{-1}$ caused higher mortality rates of larvae compared to control. A significant retardation of ontogenesis was observed in the concentration of 3000 $\mu g.l^{-1}$. Diazinon 60 EC in concentration of 10 $\mu g.l^{-1}$ (e.g. concentration used in special cases in pond management to suppress extensive propagation of coarse daphnian zooplankton) didn´t damage growth not ontogeny development of larvae and we can suppose, that this concentration of Diazinon 60 EC is not dangerous to carp not in these sensitive (critical) stages of ontogeny.

Toxicity of Diazinon 60 EC for early life stages of tench (*Tinca tinca* L.) was assessed in one month lasting embyo-larval toxicity test. The testing organisms in the fertilized eggs stage were exposed to Diazinon 60 EC concentrations of 10, 100, 1000, and 3000 $\mu g.l^{-1}$ (0, 6, 60,

600, 1800 $\mu g.l^{-1}$ active substance of diazinon, respectively). Exposure to tested substance was terminated after 29 days, when fish came of juvenile stage. At the highest tested concentration (3000 $\mu g.l^{-1}$), total mortality of fish was observed within the first 13 days of exposure. Concentration of 1000 $\mu g.l^{-1}$ caused larvae damage, decrease in growth rate and slowed down ontogenetic development. Concentration of 100 $\mu g.l^{-1}$ mildly decreased growth rate, but 10 $\mu g.l^{-1}$ exhibited no changes compared to the control. Values of lowest (LOEC) and no (NOEC) observed effect concentrations of Diazinon 60 EC were 205 $\mu g.l^{-1}$ and 31 $\mu g.l^{-1}$, respectively.

The preparation **PAX-18**, with its active ingredient polyaluminium chloride (9% of Al), is a coagulation agent that is used mainly to precipitate phosphates, to prevent surface water eutrophication and incidences of cyanobacteria. It is applied to the water environment and thus could present a potential risk to fish. Order to toxicity test on fish was carried out. The results of tests shown, that the acute toxicity for common carp (96hLC50) is 753 ± 24.3 $mg.l^{-1}$. This value is 7–17 times higher than the concentration which is usually applied to water. Effect on early development stage expressed as the no observed effect concentration (NOEC) was 10 $mg.l^{-1}$, the lowest observed effect concentration (LOEC) was 50 $mg.l^{-1}$. These values were calculated on the bases of mortality of embryos and larvae. No significant effect of this preparation were found on hatching, length and weight parameters morphology and histopathology was detected in concentrations of 50 $mg.l^{-1}$ and lower. Moreover, fish in eutrophicated water sources are exposed to PAX-18 concentrations corresponding with the lowest observed effect concentration. But length of exposure is only for a short time, therefore the effect on them can be considered as minimal.

Nitrite is an intermediate stage in conversion of a toxic fish metabolite, ammonia to less harmful nitrate. During imbalance in this process, (mainly in the intensive fish farming) nitrite can accumulate to relatively high concentrations in the water. The results of toxicity test on early life stage of carp document effects of nitrite on these organisms: The tested concentrations (0.7–330 $mg.l^{-1}$ NO_2^- at 10 $mg.l^{-1}$ Cl^-) had no significant effect on hatching and viability of embryos, but there were found significant changes in total accumulated mortality in fish exposed to concentrations 33, 67 a 330 $mg.l^{-1}$ NO_2^- during next days of the experiment. Total mortality was observed in the concentration of 330 $mg.l^{-1}$ NO_2^- during days 7 and 8. On the basis of accumulated mortality of fish in the experimental groups, main lethal concentrations of nitrite were estimate at 29 d LC50 = 88 $mg.l^{-1}$ NO_2^-; lowest observed concentration (LOEC) = 28 $mg.l^{-1}$ NO_2^-; and no observed effect concentration (NOEC) = 7 $mg.l^{-1}$ NO_2^- Also significant differences in early ontogeny among groups were noted. Fish from all the concentrations showed a dose related delay in development compared with controls. Lordosis, kyphosis, scoliosis and body shortening were observed at all concentrations and in controls, as was yolk sac deformation and oedema, eye deformation, and cardiac oedema. The incidence of these malformations was positively correlated with nitrite concentration. Histopathology revealed epidermal spongiosis; oedema and hyperplasia of the gill epithelium, including hypertrophy and hyperplasia of eosinophilic granular cells (chloride cells); and interstitial oedema of skeletal muscle in fish exposed to 67 $mg.l^{-1}$ NO_2^-. Similar, but milder, changes were observed at lower nitrite concentrations.

Dimethyl sulfoxide (DMSO) is an important polar aprotic solvent, which is used for many chemical reactions. Furthermore DMSO is also used as auxiliary substance in toxicity tests on aquatic organisms. In order to was necessary to check out its toxicity for the experimental organisms. The aim of the present study was to evaluate an effect of DMSO in concentrations of 0.2, 0.5, 1.0 and 5.0 $ml.l^{-1}$ on early development, growth and mortality of early life stages in common carp. Results of provided test shown, that 29-days continuous exposure of experimental organisms (from fertilized egg to the end of the larval period) had no effect on mortality embryos and larvae, intensity of development and growth, weight and occurrence of

abnormalities. Thus confirms the possibility of using of this substance for toxicity tests on early life stages of carp, because the recommended concentrations of them (of 0.2 a 0.5 $ml.l^{-1}$) don't damage the testing organisms.

Czech Summary

Úloha testů toxicity na raných vývojových stadiích ryb při posuzování toxicity látek a přípravků

Jana Máchová

Vodní prostřední je konečnou deponií látek a přípravků, které člověk ve svém životě užívá, nebo které vznikají jako meziprodukty či odpad při jejich výrobě. Proto je nutné znát míru rizik, kterou jednotlivé komponenty pro vodní prostředí představují. Odhad rizika konkrétní látky či přípravku pro vodní prostředí není možné provádět jen na základě znalostí jejich chemických a fyzikálních vlastností, ale především na základě výsledků biologických zkoušek na rybách a dalších vodních organismech. Ryby, které představují ve vodním prostředí nejvýše postavené organismy a současně jsou posledním článkem potravního řetězce, jsou v testech toxicity nejčastěji užívanými organismy.

Provádění testů toxicity na rybách je standardizováno na mezinárodní úrovni. Jedná se především o normy řady OECD a ISO. Jednotlivé testy a metodiky se vzájemně liší svou časovou i finanční náročností, požadavky na vybavení laboratoře a erudici pracovníků, a samozřejmě také svou citlivostí a vypovídací schopností. Volba vhodného testu je obvykle určitým kompromisem mezi výše uvedenými kritérii.

Z hlediska délky trvání rozdělujeme testy toxicity na krátkodobé (testy akutní toxicity) a dlouhodobé (testy subchronické a chronické toxicity). Své speciální postavení mezi těmito dvěma typy testů mají testy na raných vývojových stadiích ryb (embryonální testy a embryolarvální testy toxicity). Citlivost těchto testů a jejich význam pro vodní toxikologii se snaží hodnotit předložená práce na příkladech vybraných (zdánlivě nesourodých) testovaných látek a přípravků, které však mají ve vodním hospodářství nebo ve vodní toxikologii svůj specifický význam.

Diazinon 60 EC (organofosfát, účinná látka diazinon v koncentraci 600 $g.l^{-1}$) byl používán jako biocid k potlačování nadměrného rozvoje hrubého dafniového zooplanktonu a také jako pesticid v zemědělství. Předložené výsledky poskytují informace o akutní toxicitě Diazinonu 60 EC pro akvarijní rybu *Poecilia reticulata* a kapra obecného (*Cyprinus carpio*) a pro raná vývojová stadia kapra obecného. Hodnota 96hLC50 pro *Poecilia reticulata* je $3.mg \cdot l^{-1}$, pro kapra obecného 10–25 $mg.l^{-1}$. Jak ukázaly výsledky embryonálního testu na kapru obecném, Diazinon 60 EC v koncentraci 1 000 $mg.l^{-1}$ (nejvyšší testovaná koncentrace) neměl žádný negativní vliv na líhnutí a životaschopnost embryí. V průběhu larválního testu byly larvy po rozplavání a spotřebování žloutkového váčku vystaveny po dobu 10 dnů Diazinonu 60 EC v koncentracích 10, 100, 1 000 a 3 000 $\mu g.l^{-1}$ a poté převedeny do vody bez testované látky a dále sledovány po dobu 18 dnů, kdy byl test ukončen. Výsledky larválního testu ukázaly, že expozice larev Diazinonu 60 EC v koncentracích 1 000 a 3 000 $\mu g.l^{-1}$ zvýšila mortalitu larev ve srovnání s kontrolou, larvy vystavené koncentraci 3 000 $\mu g.l^{-1}$ vykazovaly významné zpomalení ontogenetického vývoje. Koncentrace 10 $\mu g.l^{-1}$ Diazinonu 60 EC (tj. koncentrace doporučovaná pro tlumení nadměrného rozvoje hrubého dafniového zooplanktonu) neovlivnila růst ani ontogenetický vývoj larev kapra obecného a lze předpokládat, že tato koncentrace nepředstavuje nebezpečí pro kapra obecného ani v průběhu tohoto citlivého (kritického) období vývoje.

Toxicita Diazinonu 60 EC pro raná vývojová stadia lína obecného (*Tinca tinca* L.) byla hodnocena na základě embryo-larválního testu toxicity. Testovací organismy ve stadiu oplozené jikry byly vystaveny Diazinonu 60 EC v koncentracích 10, 100, 1 000, and 3 000 $\mu g.l^{-1}$, což odpovídá koncentracím aktivní látky – diazinonu 0,6; 60; 600; 1 800 $\mu g.l^{-1}$.

Expozice testované látce byla ukončena po 29 dnech, kdy ryby dosáhly juvenilního vývojového stadia. V nejvyšší testované koncentraci (3 000 $\mu g.l^{-1}$) došlo v průběhu prvních 13 dnů expozice k totálnímu úhynu larev, v koncentraci 1 000 $\mu g.l^{-1}$ bylo zaznamenáno snížení růstové rychlosti a zpomalení ontogenetického vývoje larev. U organismů vystavených koncentraci 100 $\mu g.l^{-1}$ bylo zaznamenáno mírné zpomalení růstu, a v koncentraci 10 $\mu g.l^{-1}$ již nebyly zaznamenány žádné změny ve vývoji ani růstu larev ve srovnání s kontrolou. Na základě výsledků embryo-larválního testu byly stanoveny koncentrace Diazinonu 60 EC LOEC – 205 $\mu g.l^{-1}$ (nejnižší koncentrace, při které byl zaznamenán vliv na testovací organismy) a NOEC – 31 $\mu g.l^{-1}$ (nejvyšší koncentrace, při které nebyl zaznamenán žádný vliv na testovací organismy).

Přípravek **PAX-18** s aktivní látkou polyaluminium chlorid (9% Al) je koagulační činidlo, které se užívá hlavně na ochranu povrchových vod před eutrofizací a rozvojem sinic. Vzhledem k tomu, že uvedený přípravek je aplikován do vodního prostředí, přestavuje potenciální riziko pro ryby. Proto byly provedeny testy toxicity na rybách. Akutní toxicita přípravku pro kapra obecného vyjádřená jako 96hLC50 byla 753 ± 24,3 $mg.l^{-1}$. Tato hodnota je 7–17krát vyšší než koncentrace, ve které je přípravek obvykle aplikován do vody. Vliv na raná vývojová stadia kapra obecného vyjádřený hodnotou NOEC (nejvyšší koncentrace, při které nebyl zaznamenán žádný vliv na testovací organismy) byla 10 $mg.l^{-1}$ a hodnota LOEC (nejnižší koncentrace, při které byl zaznamenán vliv na testovací organismy) 50 $mg.l^{-1}$. Tyto hodnoty byly stanoveny na základě zjištěné mortality embryí a larev. Výsledky histopatologického a morfologického vyšetření embryí a larev z koncentrace přípravku 50 $mg.l^{-1}$ byly negativní. Nicméně, koncentrace přípravku užívaná ke srážení fosfátů koresponduje s hodnotou LOEC. Nutno však vzít v úvahu, že při aplikaci přípravku do vodního prostředí jsou ryby vystaveny jeho působení pouze krátkou dobu, a proto lze předpokládat, že aplikace přípravku PAX-18 má na raná vývojová stadia ryb minimální vliv.

Dusitany jsou meziproduktem přeměny toxického amoniaku – (metabolického produktu ryb) na méně toxické dusičnany. Pokud dojde k narušení průběhu tohoto procesu (děje se tak zejména v recirkulačních systémech chovů ryb), mohou se dusitany ve vodě vyskytovat i v relativně vysokých koncentracích. Výsledky provedeného testu toxicity dokumentují vliv dusitanů na raná vývojová stadia kapra obecného: testované koncentrace (0,7–330 $mg.l^{-1}$ NO_2^- při 10 $mg.l^{-1}$ Cl^-) neměly žádný významný vliv na přežívání jiker a kulení embryí, ale v dalším průběhu testu byly zaznamenány v koncentracích 33, 67 a 330 $mg.l^{-1}$ NO_2 významné změny v kumulativní mortalitě embryí a larev. Totální úhyn organismů byl zaznamenán v nejvyšší testované koncentraci v průběhu 7. až 8. dne pokusu. Na základě kumulativní mortality pokusných organismů byly vypočteny hodnoty 29 d LC50 = 88 $mg.l^{-1}$ NO_2^- a hodnoty LOEC (nejnižší koncentrace, kde byly zaznamenány změny) – 28 $mg.l^{-1}$ NO_2^- a NOEC (nejvyšší koncentrace, kde nebyly zaznamenány změny) – 7 $mg.l^{-1}$ NO_2^-. Ve všech testovaných koncentracích byly u pokusných organismů také zaznamenány významné změny v ontogenetickém vývoji ve srovnání s kontrolou. U pokusných i kontrolních ryb byl zaznamenán výskyt skoliózy, kyfózy, zkrácení těla a dále deformace žloutkového váčku a otoky, deformace očí a otoky srdce. Četnost výskytu těchto malformací vykazovala přímou souvislost se zvyšující se koncentraci dusitanů ve vodě. Histopatologická vyšetření odhalila u ryb exponovaných koncentraci 67 $mg.l^{-1}$ NO_2^- epidermální spongiózu, edém a hyperplasii žaberního epitelu včetně hypertrofie a hyperplazie chloridových buněk a intersticiální edém kosterního svalstva. Podobné, ale mírnější změny byly pozorovány u ryb vystavených nižším koncentracím dusitanů.

Dimethyl sulfoxid (DMSO) je důležité polární rozpouštědlo, které se užívá při mnohých chemických reakcích. Navíc se DMSO využívá jako pomocná látka (rozpouštědlo) v testech toxicity na vodních organismech. Proto bylo nutné ověřit toxicitu tohoto přípravku pro pokusné organismy. Cílem prezentované práce bylo posoudit vliv DMSO v koncentracích

0,2; 0,5; 1,0 a 5,0 $ml.l^{-1}$ na vývoj, růst a mortalitu kapra obecného v období jeho embryolarválního vývoje. Výsledky provedeného testu ukázaly, že kontinuální 29denní expozice přípravku DMSO v uvedených koncentracích nemá negativní vliv na mortalitu embryí a larev, intenzitu růstu a ontogenetického vývoje, ani na výskyt abnormalit. Tím bylo potvrzeno, že použití DMSO v doporučovaných koncentracích (0,2 a 0,5 $ml.l^{-1}$) pro testy toxicity na raných vývojových stadiích kapra obecného nepoškozuje testovací organismy a neovlivňuje výsledky testu.

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Training and Supervision Plan during Study

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Research department	2008–2009 – Department of Water Toxicology and Fish Diseases of RIFCH 2009–2011 – Laboratory of Aquatic Toxicology and Ichthyopathology of FFPW
Daily supervisor (non officially)	Dipl.-Ing. Hana Kroupová, Ph.D.
Supervisor	Prof. Zdeňka Svobodová, DVM, DSc.
Period	October 2008 until September 2011

Ph.D. courses	**Year**
Toxicology and fish diseases	2008
Pond aquaculture	2008
Applied hydrobiology	2008
Ichthyology	2010
English language	2009

International Conferences	**Year**
13th EAFP International conference on diseases of fish and shellfish, Grado, Italy	2007
1st International Workshop of Aquatic Toxicology and Biomonitoring, Vodňany, Czech Republic	2008
Hygiena Alimentorum XXX, Slovak Republic	2009
46th Congress of the European Society of Toxicology, EUROTOX 2009, Dresden, Germany	2009
12th International Congress of Toxicology, IUTOX 2010, Barcelona, Spain	2010

National Conferences	
13. Toxikologická konference Toxicita odpadů a látek významných ve vodním prostředí	2008
14. Toxikologická konference Toxicita odpadů a látek významných ve vodním prostředí	2009
Odborná konference Intenzita chovu ryb a ekologické aspekty v rybářství, Vodňany	2010

Curriculum Vitae

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Education

1973–1978	Dipl.-Ing. (MSc.) Institute of Chemical Technology, Prague, specialization: Technology of water
2007–2011	Ph.D. student (combined form of study) at the University of South Bohemia, Faculty of Fisheries and Protection of Waters, Laboratory of Aquatic Toxicology and Ichthyopathology, Vodňany, Czech Republic, *Ph.D. thesis: The role of toxicity tests on early life stages of fish in assessing the toxicity of substances and preparations*.

Professional experience

1978–1979	Study stay at University of chemical technology Prague
1979–1995	Research worker, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czechoslovakia
1995–1997	Teacher – Secondary school Netolice, Czech Republic
1997–2000	Head of the laboratory in Agriculture Cooperative Vodňany
2000 – Present	Research worker, USB, Research Institute of Fish Culture and Hydrobiology (RIFCH), Vodňany, Czech Republic
2006–2010	Head of accredited Aquatic toxicology laboratory at USB RIFCH, Vodňany, Czech Republic
2008–Present	Head of the Laboratory of Aquatic Toxicology and Ichthyopathology, USB RIFCH, Vodňany, Czech Republic

Current Research Program

- Toxic effects of substances and preparations for fishes and other water organisms, accumulation of pollutants in water environment
- Influence of Pond management intensity on water quality

Teaching/Advising:

2010 – Present	Guarantee (together with MSc. R. Grabic, Ph.D.) of the subject Hydrochemistry for students of the 1st year at the Faculty of Fisheries and Protection of Waters, USB České Budějovice

Special experience

- Analyses of water, hydrochemistry
- Certificate for handling of laboratory animals according to the Law on Protection Animals against Cruelty

Ph.D. courses

Pond aquaculture, Applied hydrobiology, Ichthyology and systematics of fish, Toxicology and fish diseases, English language