

Melanie Böttcher

Genotoxicity and endocrine effects – population relevant impacts of steroids



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Diplom-Biologin Melanie Böttcher
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**Genotoxicity and endocrine effects – population relevant
impacts of steroids**

Gutachter: Prof. Dr. Thomas Braunbeck
Heidelberger Institut für Zoologie, Universität Heidelberg

Prof. Dr. Helmut Segner
Zentrum für Fisch- und Wildtiermedizin FIWI, Universität Bern

Contents

Abstract

Zusammenfassung

Chapter 1: Introduction	1
1.1 General Introduction	2
1.1.1 Endocrine-disrupting chemicals in the aquatic environment	2
1.1.2 Mechanisms of endocrine disruptors	3
1.1.3 The reproductive system of fish	5
1.1.4 Sex steroids	6
1.1.5 Fate of chemicals in the environment and the role of sediments	9
1.2 Concept of own investigations	11
1.3 References	14
Chapter 2: Comparison of <i>in vitro</i> and <i>in situ</i> genotoxicity in the Danube River by means of the comet assay and the micronucleus test	23
2.1 Abstract	24
2.2 Introduction	24
2.3 Material and Methods	26
2.3.1 Samples	26
2.3.2 <i>In vitro</i> tests	27
2.3.3 Field studies	30
2.4 Results	31
2.4.1 Comet assay in RTL cells exposed to sediment extracts <i>in vitro</i>	31
2.4.2 Micronucleus test in RTL-W1 cells exposed <i>in vitro</i>	32
2.5 Discussion	35
2.5.1 <i>In vitro</i> test systems: comet assay versus micronucleus test	35
2.5.2 <i>In situ</i> genotoxicity – the micronucleus test in Danube River fish	37
2.5.3 Correlation between findings in the <i>in vitro</i> and <i>in situ</i> tests	38
2.6 Conclusion	38
2.7 Acknowledgements	39
2.8 References	39
Chapter 3: A novel statistical approach for the evaluation of comet assay data	45
3.1 Abstract	46
3.2 Introduction	46
3.3 Material and Methods	47
3.3.1 Samples	47
3.3.2 RTL-W1 cells	48
3.3.3 Zebrafish (<i>Danio rerio</i>) embryos	49
3.3.4 Cell isolation and comet assay	49
3.3.5 Image analysis and statistics	49

3.3.6	Data presentation	50
3.4	Results and discussion	51
3.4.1	Raw data	51
3.4.2	EC ₅₀	52
3.4.3	LOEC	53
3.4.4	IF _{max}	54
3.4.5	LOEC + IF _{max}	55
3.4.6	3-Step analysis	55
3.4.7	Significant concentration-dependant induction factor (SCDI)	56
3.5	Conclusion	59
3.6	Acknowledgement	59
3.7	References	60
 Chapter 4: Sediment genotoxicity in the Tietê River (São Paulo, Brazil): <i>in vitro</i> comet assay versus <i>in situ</i> micronucleus assay studies		 65
4.1	Abstract	66
4.2	Introduction	66
4.3	Materials and methods	68
4.3.1	Sediment sampling	68
4.3.2	Cell culture	69
4.3.3	Comet assay	69
4.3.4	Fish blood sampling and micronucleus assay	71
4.4	Results	72
4.4.1	Comet assay	72
4.4.2	Micronucleus assay	75
4.5	Discussion	76
4.6	Conclusions	79
4.7	Acknowledgments	80
4.8	References	80
 Chapter 5: New and edited versions of the micronucleus assay with fish		 87
5.1	Abstract	88
5.2	Introduction	88
5.3	Materials and methods	90
5.3.1	Nitroquinoline-N-oxide	90
5.3.2	<i>In situ</i> analysis of micronuclei	91
5.3.3	<i>In vivo</i> laboratory tests: Micronucleus assay with zebrafish embryos	92
5.4	Results	94
5.4.1	Micronucleus test in liver tissues from barbel after <i>in situ</i> exposure to Danube River water	94
5.4.2	Micronucleus test in whole body cell suspensions from zebrafish exposed <i>in vivo</i>	95
5.5	Discussion	96

Contents

5.5.1	The embryonic micronucleus test	96
5.5.2	Micronucleus assays in fixed liver tissues from barbel after <i>in situ</i> exposure	97
5.5.3	Sensitivity of the applied micronucleus assays	97
5.6	Conclusions	99
5.7	References	100
Chapter 6: Low-dose Effects and Biphasic Effect Profiles: Is Trenbolone a Genotoxicant?		107
6.1	Abstract	108
6.2	Introduction	108
6.3	Materials and methods	110
6.3.1	Trenbolone	110
6.3.2	<i>In vitro</i> tests	110
6.3.3	Genotoxicity tests with zebrafish embryos	112
6.4	Results	114
6.4.1	Micronucleus assay	114
6.4.2	Comet assay	116
6.5	Discussion	117
6.5.1	Mutagenicity of trenbolone in the micronucleus assay	117
6.5.2	Genotoxicity of trenbolone in the comet assay	118
6.5.3	Do different biotransformation capacities affect genotoxicity of trenbolone?	119
6.6	Conclusions	121
6.7	References	122
Chapter 7: Uptake and Distribution Pattern of Trenbolone in 72 h – 2 Weeks Old Zebrafish		127
7.1	Abstract	128
7.2	Introduction	128
7.3	Material and methods	129
7.3.1	Maintenance and egg production of zebrafish	129
7.3.2	Trenbolone exposure	130
7.4	Results	131
7.4.1	Not hatched embryo	131
7.4.2	Hatched embryos	132
7.5	Discussion	134
7.6	References	135
Chapter 8: Environmental Effect Assessment for Sexual Endocrine-Disrupting Chemicals: Fish Testing Strategy		137
8.1	Abstract	138
8.2	Introduction	139
8.3	Status of risk assessment schemes with regard to ED issues	140
8.4	The OECD conceptual framework for the testing of EDC	141
8.5	ED-related fish test methods	142

Contents

8.6	Proposal for a testing strategy: Regulatory use of OECD fish-testing tools for SEDCs in ERA schemes	148
8.6.1	First step: Weight-of-evidence approach for identifying potential SEDCs	148
8.6.2	Second step: <i>In vivo</i> confirmation of sexual endocrinedisrupting activity associated with specific mechanisms of action	149
8.6.3	Third step: Characterization of sexual endocrine-mediated adverse effects, including their thresholds for regulatory use	151
8.7	Conclusions	153
8.8	Supplemental data	154
8.9	References	155
	Chapter 8: Supplement A	A-1 - A-37
	Chapter 8: Supplement B	B-1 - B-46
	Chapter 8: Supplement C	C-1 - C-39
	Chapter 9: Conclusion	161
9.1	Investigations on the genotoxic burden of the River Danube in Germany and the Tietê River in Brazil	162
9.1.1	What is the genotoxic burden of two different aquatic systems: The Danube River in Germany and the Tietê River in Brazil?	162
9.1.2	Does the comet assay <i>in vitro</i> correlates with the comet assay <i>in vivo</i> ?	163
9.1.3	Are the comet assay and the micronucleus assay comparable as two alternative genotoxicity / mutagenicity tests in order to characterize the potential genotoxic burden of environmental sediment samples?	164
9.1.4	Are the comet assay and the micronucleus assay with RTL-W1 cells exposed to sediment extracts comparable to results gained in the micronucleus assay with fish caught in the field?	165
9.2	Eligibility of the new/edited protocols for the micronucleus assay	165
9.2.1	What are the most practicable and suitable methods to assess micronuclei in fixed liver tissue?	165
9.2.2	What is a suitable protocol for an application of the micronucleus assay with zebrafish embryos?	166
9.3	Investigations on the model anti-androgen trenbolone	166
9.3.1	Is there a genotoxic potential of trenbolone?	166
9.3.2	Is the genetic damage caused by trenbolone is partly masked as the molecule is linked to a special tissue or organ in zebrafish and does the chorion acts as a barrier in case of trenbolone?	167
9.3.3	Which effects were observed after exposure of different life stages of zebrafish (<i>Danio rerio</i>) to trenbolone?	167
9.4	Suitability of the two generation test	169
9.4.1	Is the two generation test suitable for the detection of effects caused by androgens, anti-androgens and anti-estrogens?	169
9.5	References	170
	List of publications	173
	Acknowledgement	177

List of Figures

1.1	Secondary and metabolic features of estrogens and androgens.	2
1.2	Schematic representation of the hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in teleosts.	5
1.3	Steroidogenic mediators of oocyte growth, oocyte maturation, spermatogenesis and sperm maturation in teleosts.	6
1.4	Structural formula of 17 β -estradiol.	6
1.5	Chemical structure of 11-keto testosterone.	7
1.6	Structural formula of progesterone.	7
1.7	Molecular structures of anabolic sex hormones registered in non European states.	8
1.8	Sources and fate of chemical compounds in the environment.	9
1.9	Information about high production volume chemicals.	10
2.1	Study area and sampling locations.	27
2.2	Genotoxic effects of acetic sediment extracts from Lauchert and Oepfingen respectively, in the comet assay.	31
2.3	Genotoxic activity of sediment extracts in the comet assay with RTL-W1 cells from two independent series of experiments in river flow direction, expressed as concentration-dependent induction factor.	32
2.4	Examples of different dose-response relationships for the mutagenic activity of acetic sediment extracts in the micronucleus assay with RTL-W1 cells.	33
2.5	Mutagenic activity of sediment extracts from the Danube River, tested in the micronucleus assay with RTL-W1 cells.	34
2.6	Micronucleus frequency in barbel (<i>Barbus barbus</i>) erythrocytes from selected sites along the Danube River.	35
3.1	Hydrological system of Germany (inset) and sediment sampling sites along the Upper Danube River.	48
3.2	DNA damage expressed as tail moments and induction factors in primary cells derived from 72 h old zebrafish (<i>Danio rerio</i>) embryos exposed to two selected whole sediment samples from the Danube River in two independent runs of the comet assay.	52
3.3	Genotoxicity ranking of Danube River sediment samples according to LOEC values.	53
3.4	Genotoxicity ranking of Danube River sediment samples according to significant concentration-dependant induction factor (SCDI) values comparing the different exposure paths.	56
3.5	Genotoxicity ranking of Danube River sediment samples according to concentration-dependant induction factor (CDI) values comparing the different exposure paths.	57
3.6	Correlation analysis for two CDI values derived from experiments based on two exposure paths in the comet assay: primary cells from zebrafish (<i>Danio rerio</i>)	

List of Figures

	embryos exposed to whole sediments <i>versus</i> RTL-W1 cells exposed to acetone sediment extracts.	68
4.1	Location of the sampling sites in the Tietê River basin, São Paulo, Brazil	68
4.2	Erythrocytes from <i>Oreochromis niloticus</i> collected in Billings reservoir, stained with Giemsa. Note micronucleus at arrow	72
4.3	Genotoxic effects of acetonetic sediment extracts from Billings reservoir and near to the Tietê River spring, respectively, in the comet assay using RTL-W1 cells	73
4.4	Genotoxicity of sediment samples collected at different locations along the Tietê River in the comet assay, given as maximum induction factors in mg SEQ/ml for the 1:4 dilutions of the highest test concentrations	74
4.5	Concentration-dependant induction factors (CDI; Seitz et al. 2008) of sediment samples collected from the Tietê River basin	75
4.6	Frequency of micronucleated cells (MN) scored from erythrocytes from fish at different sites along the Tietê River	76
5.1	Hepatocytes from the liver of <i>Barbus barbuis</i> from Ehingen	94
5.2	Genotoxic activity of in the micronucleus assay with fixed liver tissue from barbel (<i>Barbus barbuis</i>) caught at Ehingen, expressed as percentage of micronucleus formation (MN [%])	95
5.3	Two cells from the whole body cell suspensions from a 7-day old zebrafish	95
5.4	Dose-response curves for the mutagenic activity of NQO in the micronucleus assay with whole body cell suspensions of zebrafish	95
6.1	Induction of micronucleus formation in RTL-W1 cells by trenbolone	115
6.2	Induction of micronucleus formation in primary cells derived from 168 h old zebrafish (<i>Danio rerio</i>) embryos exposed to trenbolone	115
6.3	DNA damage expressed as tail moments and induction factors in RTL-W1 cells exposed to a concentration range of trenbolone	116
6.4	DNA damage expressed as tail moments and induction factors in primary cells derived from 168 h old zebrafish (<i>Danio rerio</i>) embryos exposed to a concentration range of trenbolone	117
7.1	Longitudinal sections (head to tail) of 72 h old embryos immediately before hatch	131
7.2	Longitudinal sections (dorsal-ventral) of 8 and 14 day old embryos	133
8.1	Schematic presentation of OECD's Test Guidelines Programme to amend established and to develop new test methods, which can be used to identify and quantify effects of endocrine-disrupting chemicals on fish	142
8.2	Schematic presentation of the proposed testing strategy with fish for sexual endocrine-disrupting chemicals	148

List of Tables

3.1	Genotoxicity data and ranking of Danube River sediment samples according to different exposure paths and evaluation methods	54
3.2	Advantages and disadvantages of the concentration-dependent induction factor (CDI)	57
4.1	Highest test concentrations of sediment extracts used in the comet assay, given in sediment equivalents (mg SEQ) per ml medium	76
8.1	Comparison of important features determining the design of <i>in vivo</i> fish screening assays and definitive fish tests	144
8.2	Effect concentrations (EC ₅₀) determined for zebrafish when exposed to different sexual endocrine-disrupting substances	147

Abstract

The first part of the present dissertation assesses genotoxic effects of environmental samples (e.g. sediment, fish blood) from the River Danube and the Tietê River in Brazil. The genotoxic potentials of both rivers were determined by means of the comet assay and the micronucleus test. The results, regarding the ecological status of the Danube River, reveal an overall moderate to severe genotoxic potential with a highly differential localization. Regarding the Tietê River, strong hazard potentials could especially be detected in samples from Billings reservoir, indicating a major impact of discharges of São Paulo. Overall, however, genotoxicity decreases downstream. *In situ* observations (i.e. the micronucleus test with fish erythrocytes) on the River Danube and the Tietê River showed excellent correlation with corresponding *in vitro* tests (i.e. the comet assay and the micronucleus test with RTL-W1 cells) and document the ecological relevance of *in vitro* studies with sediment extracts.

In order to extend the usage of the micronucleus test, modified versions were tested. Results demonstrated that the micronucleus assay with fixed (liver) tissues and the modified micronucleus test with homogenized zebrafish embryos represent valuable tools for the *in vivo* identification of the mutagenic potentials of pure substances, environmental samples and field locations.

As a next step, the genotoxic hazard potential of trenbolone, a model androgen, was tested *in vitro* in the permanent fish cell line RTL-W1 as well as in primary cell cultures derived from zebrafish (*Danio rerio*) embryos after *in vivo* exposure. Results confirm the conclusion that the steroid trenbolone may act as a genotoxic substance. Once the genotoxicity of trenbolone was assessed in the micronucleus test and the comet assay *in vitro* (with RTL-W1 cells) and *in vivo* (with zebrafish embryo cell suspensions), the uptake pattern of trenbolone in zebrafish embryos was investigated in order to assess if the observed genotoxicity is linked to a certain tissue in the zebrafish embryos. The results demonstrate that trenbolone has easy access to all tissues of the embryonic and larval stages of zebrafish and thus no masking of genotoxic effects is expected. Knowing that trenbolone is ubiquitous in the body of the zebrafish embryo, the question emerges if this substance might have further impact on organisms than the genotoxicity observed and the known endocrine effect. Thus, a two generation test with trenbolone was performed, which revealed that the major effect was the masculinisation in the F₁ generation. The significant changes in length were the only effects observed, which could not exclusively be related to an endocrine effect.

The last part of this thesis was a validation of a definite fish test (full life-cycle/two generation) with zebrafish. For this end, beside trenbolone, the anti-androgen flutamide and the anti-estrogen tamoxifen citrate were tested in multi-generation tests. Currently available fish screening assays cover only mechanisms of endocrine action related to sexual endocrine disrupting chemicals. For substances other than sexual endocrine disruptors, there is no possibility to apply a tiered testing strategy. In such cases, a fish life-cycle and/or multigenerational test, as definitive test, is directly required to eliminate reasonable suspicion of an endocrine mode of action. Therefore, it is advisable that future studies focus on harmonization and validation of several elements of the available guidance for fish full life-cycle tests and 2-generation tests.

Zusammenfassung

Der erste Teil der vorliegenden Dissertation beurteilt genotoxische Wirkungen von Umweltproben (wie z.B. Sedimenten und Fischblut) aus der Donau und des Tietê Flusses in Brasilien. Das genotoxische Potential der beiden Flüsse wurde mittels des Comet Assay und des Mikrokerntest bestimmt. Hierbei zeigen die durchgeführten Versuche ein insgesamt moderates bis hohes genotoxische Potential in Abhängigkeit von den Probenahmestellen an der Donau. Hinsichtlich des Tietê, konnte vor allem für die Proben aus dem Billings Reservoir ein stark erhöhtes Gefahrenpotential nachgewiesen werden, was vermutlich in Zusammenhang mit den Abwassereinleitungen von São Paulo steht, insgesamt jedoch nimmt die Genotoxizität flussabwärts ab. *In situ* Beobachtungen an Donau und Tietê (Mikrokerntest mit Fischerythrozyten) zeigten eine sehr gute Korrelation mit den entsprechenden *In vitro*-Tests (Comet Assay und Mikrokerntest mit RTL-W1 Zellen) und unterstützen somit die ökologische Relevanz der *In vitro*-Studien mit Sedimentextrakten.

Um die Nutzung des Mikrokerntest zu erweitern, wurden die bestehenden Methoden modifiziert. Hierbei konnte gezeigt werden, dass der Mikrokerntest mit fixiertem (Leber-) Gewebe und mit homogenisierten Zebrafischembryonen weitere Werkzeuge für die *in vivo* Identifizierung des mutagenen Potentials von Reinstoffen und Umweltproben liefern.

Im nächsten Schritt wurde das genotoxische Gefährdungspotential einer Reinsubstanz ermittelt. Hierfür wurde das Modellandrogen Trenbolon sowohl in dem Mikrokerntest und im Comet-Assay *in vitro* (mit RTL-W1-Zellen) und *in vivo* (mit Zebrafischembryo Zellsuspensionen) getestet. Um eine mögliche Maskierung der Genotoxizität auszuschließen, wurde das Aufnahmemuster von Trenbolon in Zebrafischembryonen untersucht. Die durchgeführten Studien zeigen, dass Trenbolon in keinem bestimmten Gewebe akkumuliert und somit keine Maskierung von genotoxischen Wirkungen zu erwarten ist. In diesem Zusammenhang stellt sich die Frage, ob diese Substanz, neben der bekannten Genotoxizität und den endokrinen Effekten, noch weitere Einflüsse auf die Organismen haben könnte. Um dies heraus zu finden wurde ein Zwei Generationen-Test mit Trenbolon durchgeführt. Die Vermännlichung der F1-Generation erwies sich hierbei als sensitivster Endpunkt. Der einzige signifikante Effekt, der nicht ausschließlich auf eine endokrine Wirkung zurückzuführen ist, sind die konzentrationsabhängigen Längenunterschiede.

Der letzte Teil dieser Arbeit beschäftigt sich mit der Validierung eines definitiven Fischtest (Full Life-Cycle/Zwei Generationen-Test) mit Zebrafischen. Aus diesem Zweck wurden neben Trenbolon, das Anti-Androgen Flutamid und das Anti-Östrogen Tamoxifen Citrat in einem Multi-Generationen Test getestet. Die derzeit verfügbaren Screening-Assays decken nur Mechanismen der endokrinen Wirkung in Bezug auf sexuell endokrin wirksame Substanzen ab. Bei Stoffklassen, die nicht zu den sexuellen endokrinen Disruptoren zählen, gibt es keine Möglichkeit, eine gestufte Prüfstrategie anzuwenden. In solchen Fällen, sind ein ‚Life-Cycle‘- und / oder Mehrgenerationen-Test, als definitiver Test unabdingbar. Die im Rahmen dieser Dissertation durchgeführten Zwei Generationen-Tests zeigen noch großes Entwicklungspotential in die zu erfassenden Parameter. Künftige Studien sollten sich daher auf die Harmonisierung und Validierung der verfügbaren Leitlinien für den ‚Fish Full Life-Cycle Test‘ und den 2-Generation-Test konzentrieren.

Chapter 1

Introduction

1.1 General introduction

1.1.1 Endocrine-disrupting chemicals in the aquatic environment

Although regulatory activity is limiting point-source discharges of harmful chemicals to water resources, industrial and urban discharges are still responsible for toxic substances in the aquatic environment (Al-Sabti and Metcalfe, 1995). Discussion of the risks associated with the occurrence of a variety of groups of persistent organic pollutants (polychlorinated biphenyls

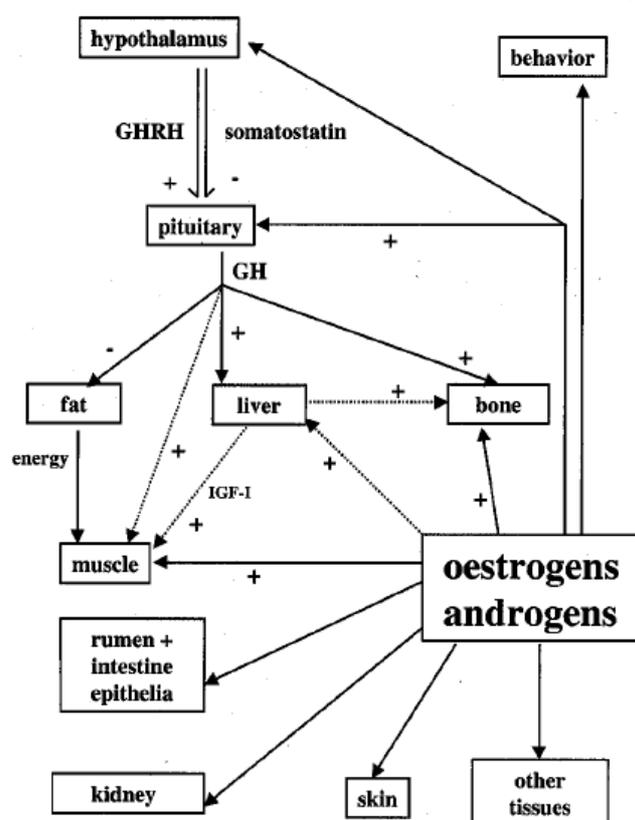


Fig. 1.1: Secondary and metabolic features of estrogens and androgens. The effects of anabolic sex hormones are mediated directly to several tissues and in addition indirectly via the somatotrophic axis involving growth hormone (GH) and insulin-like growth factor (IGF1; according to Meyer 2001).

and related organochlorine compounds, brominated flame retardants, modern pesticides and their metabolites, etc.) in the aquatic environment started to focus on their endocrine-disrupting properties in the early 1990s (Hájková *et al.*, 2007). Several field and laboratory studies have shown that exposure to certain endocrine-disrupting chemicals (EDCs) has contributed to adverse effects in several wildlife species and populations. These effects vary from subtle changes in the physiology and sexual behaviour to permanently altered sexual differentiation (Choi *et al.*, 2004; Colborn, 2002; Galloway and Handy, 2003; Fig. 1.1). Aquatic species at the top of the food web are most affected, but effects have also been observed in terrestrial species.

Over the last decade, scientists have examined and proved the endocrine-disrupting activity of various environmental pollutants including hormones, pharmaceuticals, and surfactants (Al-Sabti *et al.*, 1994; 2000; Orlando *et al.*, 2004). Beside those chemicals for human applications, many veterinary drugs such as growth promoters and antibiotics are extensively used in agriculture and thereby released into the environment.

Consequently, numerous ecotoxicological studies focused on investigating the effects of hormones on (non-target) higher eukaryotes. Ethinylestradiol, the active component of most contraceptive pills, and nonylphenol, a derivative of several industrial chemicals are among the most common studied endocrine-disrupting chemicals ((Radl *et al.*, 2005)). Although very high androgenic activities were detected in sediment samples (Thomas *et al.*, 2002), little research has been conducted on this group of EDCs. However, the potential of androgenic substances to influence animal sexual development already at environmental concentration could be shown by Jegou *et al.* (2001), who reported masculinisation of fathead minnow (*Pimephales promelas*) living in rivers downstream from cattle feedlots. In Europe, the amount of antibiotics used in livestock decreased after 1997, as a result of prohibition of some of the substances and public discussion of their use. In 1997, 5093 tonnes were used, including 1599 tonnes as growth promoters (mostly polyether antibiotics). In 1999, in EU-15 (plus Switzerland) countries, 4688 tonnes of antibiotics were used in livestock production systems. Out of these, 3902 tonnes (83 %) were used for therapeutic reasons (tetracyclines being the most common group), while only 786 tonnes were used as growth promoters. The four feed additives substances left in the EU in 1999 (monensin, avilamycin, flavomycin and salinomycin) were banned in the EU in 2006 (Thorsten *et al.* 2003). However, endocrine disrupting activity can have many origins, since, e.g., a number of environmentally relevant pesticides act as anti-androgens in mammals and probably also in fish (Gray *et al.*, 1994; Gray *et al.*, 1999; Kelce and Wilson, 1997; Kiparissis *et al.*, 2003).

1.1.2 Mechanisms of endocrine disruptors

No data are available on the amounts of hormones used in the different countries. An endocrine disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny or (sub)populations (WHO, 2002). A wide range of substances, both natural and man-made, are thought to cause endocrine disruption both *in vitro* and *in vivo*, including pharmaceuticals, dioxin and dioxin-like compounds, polychlorinated biphenyls, doichlordiphenyltrichlorethan (DDT) and other pesticides, and plasticizers such as bisphenol A. Endocrine disruptors are used for example in feedlots as ear implants or as feed additives (Miller, 2001). The natural hormones commonly used are estradiol (estrogen), progesterone, and testosterone. The synthetic ones include zeranol, melengestrol acetate, and trenbolone acetate. Around 34 countries have approved hormones for use in beef production, among

them Australia, Canada, Chile, Japan, Mexico, New Zealand, South Africa and the United States. No negative direct impact on human health as a result of their correct application has been scientifically proven. However, the relevance of the exposure of endocrine disruptors is dependent upon both the dose and the developmental stage. Numerous studies have demonstrated that exposure to estrogens at critical times during foetal development may irreversibly influence responsive tissues (Newbold, 1995) as well as subsequent growth (Migliaccio *et al.*, 1995). These effects may be manifested as structural, functional, or long-term pathologic changes that are not limited to one sex (McLachlan and Arnold, 1996).

The endocrine system regulates hormone-dependent physiologic functions necessary for survival of the organism and the species (Hontela, 1998). In general, the magnitude of the cellular response to hormones is dependent upon the number of receptors occupied by the hormone which in turn is related to hormone concentration. Therefore, chemicals could potentially alter endocrine function in multiple ways:

- (1) Direct binding to the (estrogen or androgen) receptor (Kime, 1999; McLachlan and Arnold, 1996). These xenoestrogens imitate normally the effect of natural hormones (alkylphenols: (Mueller and Kim, 1978; Shelby *et al.*, 1996; Tabira *et al.*, 1999; White *et al.*, 1994); DDT-isomers (Fry and Toone, 1987; Johnson *et al.*, 1988), some PCB's: (Bitman and Cecil, 1970; Safe, 1995).
- (2) Blocking of the natural hormone binding and function by acting as receptor antagonist (*p,p'*-DDE: Kelce *et al.*, 1995).
- (3) Competitive binding to the plasma proteins leading to an inhibition of the natural plasma protein – estrogen interaction.
- (4) Direct influence to the hormone metabolism, for example by inhibiting the aromatase activity (TCDD: Safe *et al.*, 1991).

Endocrine disruptors can act as oestrogens, anti-oestrogens, androgens or anti-androgens, depending on the mechanism and site of action (Sumpter, 1995). The degree of endocrine disruption depends on a number of varying parameters including species differences in receptor-binding affinity, the presence of multiple receptor subtypes with different binding affinities, differential tissue distributions of the receptor sub-types, reproductive stage-dependent changes in steroid action, and whether the steroid action is genomic or non-genomic (Thomas, 2000).

1.1.3 The reproductive system of fish

The reproductive system of fish is based on the complex interaction between external stimuli and hypothalamic, pituitary and gonad hormones as well as the deactivation of hormones in the liver (Fig. 1.2). External factors such as temperature or photoperiod take action through the hypothalamus, which, after induction, releases gonadotropin releasing hormones (GnRH). GnRH induces the anterior pituitary to release gonadotropins (GtH). A number of fish have two gonadotropins (GtH-1 and GtH-2), which are analogous to mammalian LH and FSH. Gonadotropins are responsible for the production of steroid hormones and gonadal development (Kime, 1999). Concerning the reproductive system there are some differences between mammals and fish. Their most important testicular androgen is 11-keto testosterone instead of testosterone (Kime, 1999). The major target of estradiol is the liver, where it triggers the production of the egg yolk protein vitellogenin, which is taken up by oocytes under control of GtH-1 (Tyler *et al.*, 1991). Subsequently it is converted to yolk proteins such as lipovitellin and phosvitin.

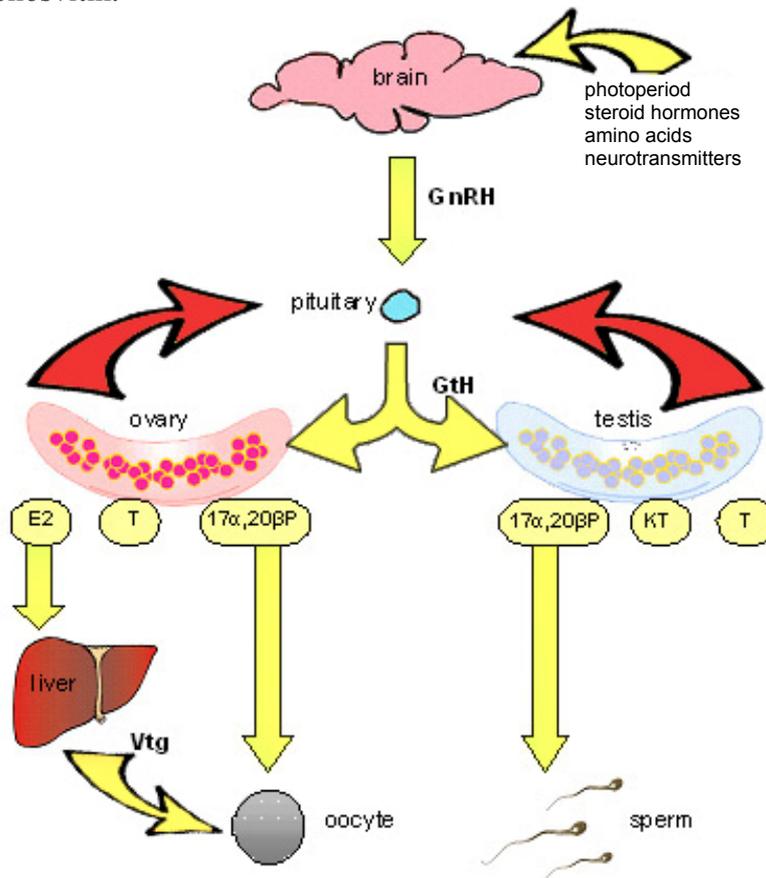


Fig. 1.2: Schematic representation of the hypothalamus-pituitary-gonadal-liver (HPGL) axis during oogenic protein synthesis in teleosts. The HPGL is regulated through the negative feedback mechanism by estradiol, testosterone and 11-ketotestosterone. The hypothalamus, pituitary, gonad and liver are potential targets for endocrine disruptors. GnRH = gonadotropin releasing hormone; GtH = gonadotropin; E2 = 17β-estradiol; T = testosterone; 17,20βP = 17,20β-hydroxy-4-pregnen-3-one; KT = 11-ketotestosterone (redrawn after Kime 1999).

1.1.4 Sex steroids

Natural sex steroids are derivatives of cholesterol (Fig.1.3) and possess a four-ring structure. The specific addition of methyl or ethyl substituents provide the basic structural skeleton for the estrogens, androgens and progestogens, but many variations of substituent groups on the rings are possible (Kime, 1987).

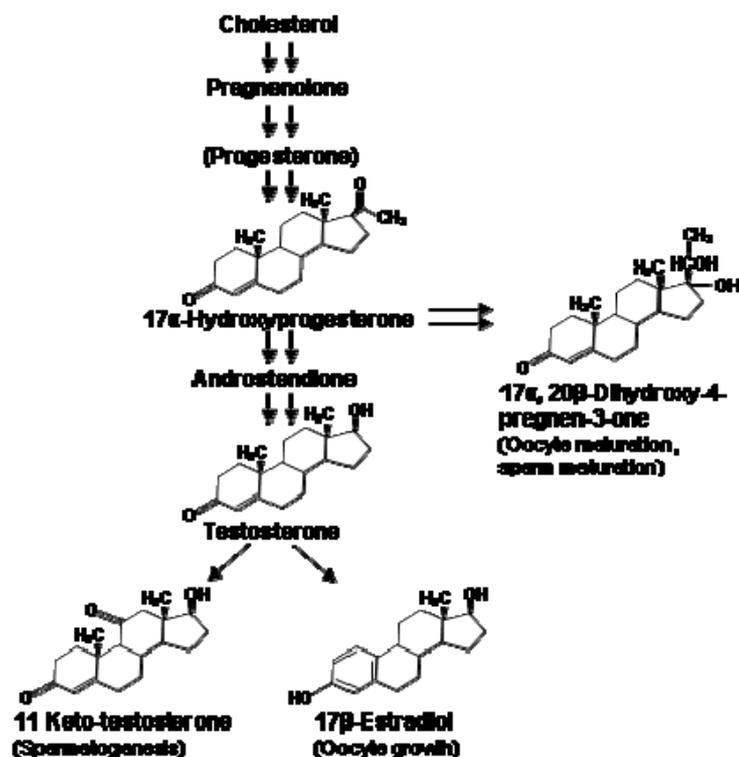


Fig. 1.3: Steroidogenic mediators of oocyte growth, oocyte maturation, spermatogenesis and sperm maturation in teleosts (Nagahama, 1994).

Estrogens are C_{18} steroids, including 17 β -estradiol (Fig. 1.4) and estrone as the most important members of this group. Estrogen is primarily synthesized in the ovary, although enzymes involved in estrogen synthesis are also present in the brain suggesting that small quantities are formed there (Halm *et al.*, 2001). In the ovary, follicle-stimulating hormone (FSH) and luteinising hormone (LH) stimulate the production of 17 β -estradiol in the theca and granulosa cells (Ankley and Johnson, 2004). However, the synthetic pathway of this hormone includes the male hormone testosterone as an intermediate. Apparently, the theca cells, under the stimulatory influence of the LH-like gonadotropin, synthesize testosterone, which diffuses to the granulosa cells. The FSH-like gonadotropin induces the enzyme aromatase in the granulosa cells, which converts testosterone to estrogen (see Fig.

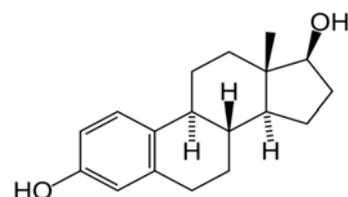


Fig. 1.4: Structural formula of 17 β -estradiol.

1.3). This estrogen has several critical roles, including oocyte development and stimulation of the liver to produce and secrete vitellogenin into the blood stream.

Androgens. The male testis contains of three principal pituitary hormone-responsive cells. The first cell type is the germinal spermatogonia, which differentiate into sperm. Developing sperm can be seen histologically at all stages of development, with groups of sperm, at different stages, maturing within a cyst. The perimeter of each testicular lobule is lined with Sertoli cells that respond to the gonadotropic hormones by fostering the production and maturation of sperm. Finally, there are interstitial or Leydig cells within the interlobular spaces that produce the male 11-ketotestosterone (11-KT). These androgens induce masculine secondary sex characteristics, and provide negative feedback to the hypothalamus and hypo-

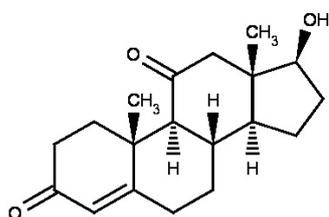


Fig. 1.5: Chemical structure of 11-keto testosterone.

physis. Androgens are C_{19} steroids with the 11-oxygenated derivatives, such as 11-KT (Fig. 1.5) being the most important in male sexual development in fish (Borg, 1994; Fostier *et al.*, 1983). This is in contrast to other vertebrates in which testosterone is the more biologically active androgen. 11-KT is generally present at higher levels than testosterone in males, and found at barely detectable levels

in females. Unlike testosterone, 11-KT cannot be converted to an estrogen. The testis of teleost fish is unique in respect to the high capacity for glucuronide conjugation with androgens (Scott, 1987). In other vertebrate groups such as mammals, steroid conjugation occurs in the liver and is considered a deactivation and elimination pathway (Parkinson, 1996). Thus, the biological significance of testicular glucuronidation in fish is uncertain. However, an interesting function for steroid conjugates may be as a male sex pheromone. For example, experiments using the zebrafish suggest that excreted steroid-glucuronide conjugates from male fish are capable of inducing ovulation in females (Vandenhurk and Resink, 1992).

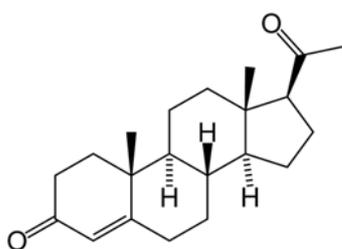


Fig. 1.6: Structural formula of progesterone.

Progesterones are C_{21} steroids (Fig. 1.6) and have received less attention in fish compared to estrogen and androgens. They are likely to be formed in the gonads of most fish species. The most important progesterone in teleost fishes appears to be $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one. This hormone is involved in oocyte maturation and spermiation in males and at time of spawning is present in the gonads at much higher concentrations than estrogen or androgens (Scott, 1987).

The synthesis and, in some cases, interconversion of steroids is quite complex. Many enzymes are involved, and their subcellular location varies between mitochondria, endoplasmic reticulum (e.g. microsomal) and cytoplasm. In general, sex steroid biosynthesis can be divided into seven types of enzyme-catalyzed reactions. These are lyase, hydroxylase, hydroxysteroid dehydrogenase, isomerase, aromatase, reductase and conjugation. Out of these types of reactions, aromatase, hydroxysteroid dehydrogenase and conjugation appear to be the most important ones as possible targets of endocrine disruption, as they are either the final step in 17β -estradiol or 11keto-testosterone synthesis or aid in excretion of steroids. For example, inhibition of aromatase activity can greatly diminish estrogen synthesis and produce anti-estrogenic effects in fish. Steroid conjugation may be important in the production of pheromones as was mentioned previously for androgens but there is also evidence that progesterone conjugates may also function as pheromones (Vermeirssen and Scott, 2001).

Steroid hormones (Fig. 1.7) are poorly soluble in water. To compensate this, steroids bind to specific carrier proteins (termed steroid-binding proteins) and non-specific proteins such as albumin and vitellogenin (Hobby *et al.*, 2000; Monteverdi and Di Giulio, ; Zeginiadou *et al.*, 1997). Since high affinity binding proteins are likely present in all fishes as in other vertebrates, more than 99 % of the circulating hormone is bound (e.g. < 1 % is free in solution; (Fostier and Breton, 1975; Petera, 1991). This may contribute to regulation of hormone activity as it is generally considered that only the unbound or free fraction of the steroid hormone is biologically active. This would imply that modulation of hormone binding and transport in the blood are potential targets for endocrine disruptors.

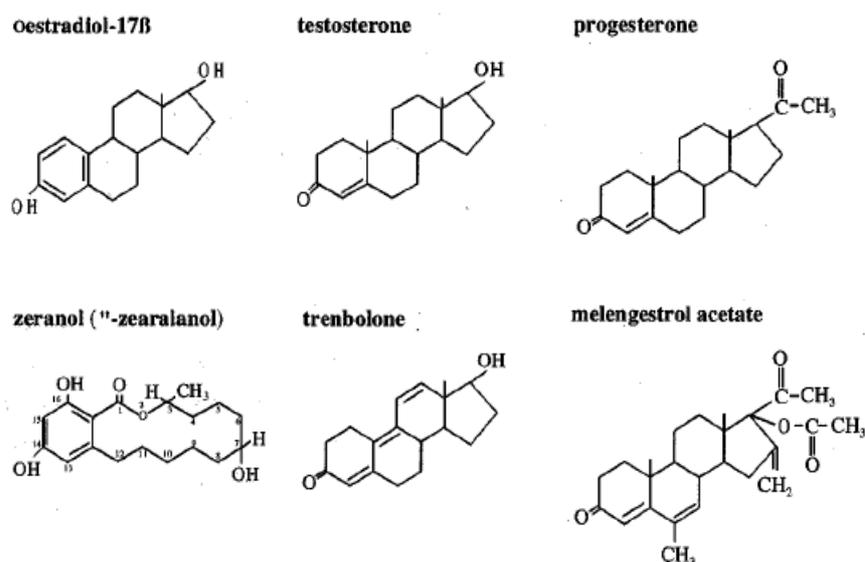


Fig. 1.7: Molecular structures of anabolic sex hormones registered in non European states (estrogens, androgens and progestins; endogenous hormones left and synthetic hormones right; graph according to Meyer 2001).

1.1.5 Fate of chemicals in the environment and the role of sediments

Thousands of synthetic chemicals are currently registered for use in industry, agriculture, commerce and the home, and thousands, perhaps millions of tonnes of them are produced annually all over the world. For instance, the world-wide production of synthetic organic chemicals is estimated at approximately 300-500 million tons per year (McGinn, 2002). In England, Germany, and Austria some pharmaceutical products are used in quantities of more than 100 tons a year (Huschek *et al.*, 2004; Jones *et al.*, 2002; Khan and Ongerth, 2004; Moldovan, 2006). Non-steroidal anti-inflammatory drugs including acetylsalicylic acid (e.g. 836 t in Germany in 2001), paracetamol (e.g. 622 t in Germany in 2001), ibuprofen (e.g. 345 t in Germany in 2001), naproxen (e.g. 35 t in England in 2000), and diclofenac (86 t in Germany in 2001), the oral anti-diabetic metformin (e.g. 517 t in Germany in 2001), and the antiepileptic carbamazepine (e.g. 88 t in Germany in 2001) are some examples of the most commonly used pharmaceuticals (Fent *et al.*, 2006). As illustrated in Fig. 1.8, chemicals and their metabolites can enter the environment mainly *via* excretion and disposal in wastewater.

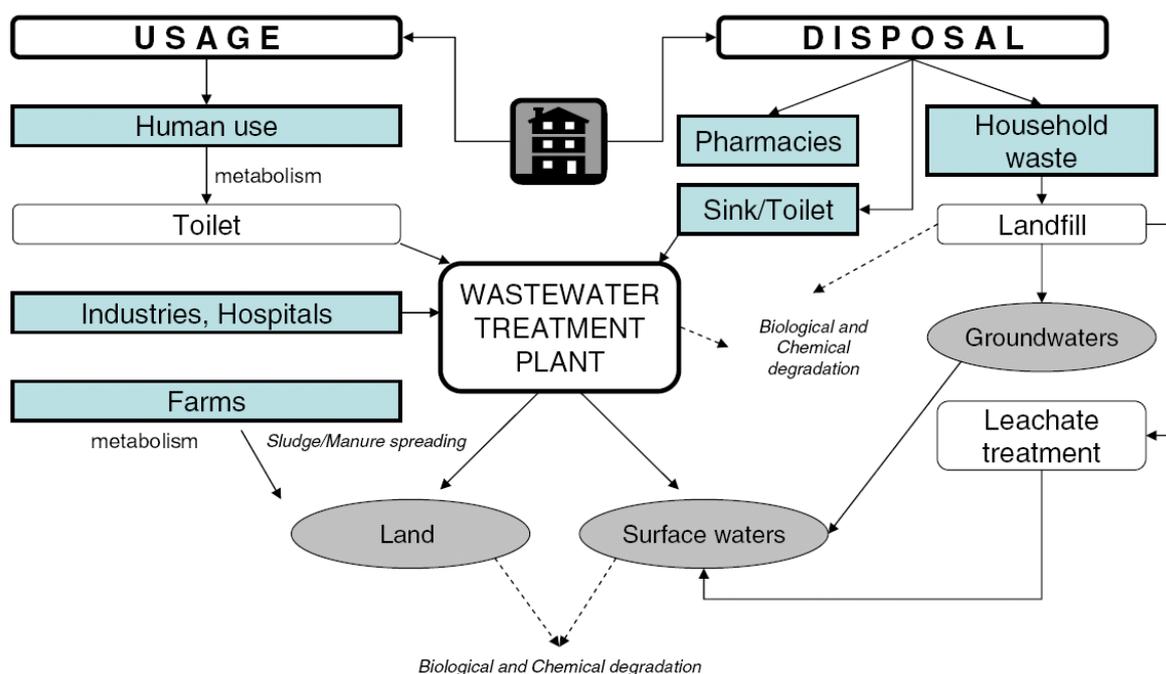


Fig. 1.8: Sources and fate of chemical compounds in the environment (Nikolaou *et al.*, 2007).

Excessive withdrawals and poor water management have resulted in lowered groundwater levels, damaged soils and reduced water quality worldwide. As a direct consequence a lack of appropriate water resources management, a number of countries are facing with ongoing depletion of water resources (Rosegrant *et al.*, 2003). In developing countries, 90 - 95 % of public wastewaters and 70 % of industrial wastes are discharged into surface water without treatment (Bernstein, 2002).

Because of an incomplete elimination in wastewater-treatment plants, residues of many toxic organic compounds are also found in surface waters in well-developed countries (Fent *et al.*, 2006; Giger *et al.*, 2003; Mok *et al.*, 2006; Moldovan, 2006; Pavlogeorgatos *et al.*, 2006; Rizzo *et al.*, 2005). The agricultural sector is the largest user of freshwater resources. In 2000, agriculture accounted for 70 % of water use and 93 % of water depletion worldwide (Turner *et al.*, 2004). Application to fields and subsequent runoff and direct application in aquaculture are the main sources of veterinary pharmaceuticals in the environment (Fent *et al.*, 2006; Johnson *et al.*, 2006; Kay *et al.*, 2005); furthermore, urban runoff carries a wide variety of chemicals including polycyclic aromatic hydrocarbons (PAHs), metals, mixtures of pesticides that could cause deleterious effects on the benthic fauna (Bejarano *et al.*, 2004). Yet, very little is known about the toxic potential or the environmental fate of the vast majority of these chemicals. For 99 % of chemicals (by volume), information on properties, uses and risks are sketchy. Chemicals produced in high volumes (above 1,000 tonnes per year) have been examined more closely (EC, 2001). Even so, there are no data for about 21 % of those, and another 65 % come with insufficient data (Fig 1.9, EC 2001).

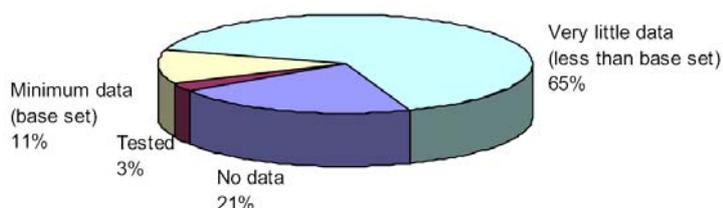


Fig. 1.9: Information about high production volume chemicals (EC, 2001).

As today, the most problematic contaminants for regulatory issues are not primary acutely toxic and water-soluble compounds, but persistent contaminants. They are found to be *widely* distributed within all ecosystems. However, due to their physico-chemical properties, the toxic activity of organic compounds in various types of industrial effluents (e.g., metal refining and founding) or run-off is predominantly associated with particulate materials (White *et al.*, 1996), and, consequently, with sediments. Sediments therefore represent reservoirs of genotoxic and mutagenic hazard, which can continually be re-introduced into the water column *via* resuspension or trophic transfer. Sediments may thus contribute substantially to the exposure of benthic biota as well as subsequent bioaccumulation (Chen and White, 2004; van den Berg *et al.*, 1998).

1.2 Concept of own investigations

This PhD thesis is subdivided into 8 chapters addressing different forms of evaluation of genotoxicity and endocrine disruptors and their relevance for the assessment of the ecological status of aquatic ecosystems. Chapter 1 will give a short introduction into scope and background of this PhD thesis.

The design of strategies for the assessment of endocrine disruptors is a complex process due to the multiple mechanisms by which these compounds may act. Besides the obvious potential for endocrine hazard deriving from androgenic substances, other effects like genotoxicity have to be taken into account as well. Thus, for the assessment of a wider range of the hazard potential of an endocrine compound, a combination of *in vitro* and *in vivo* assays is advisable. *In vivo* assays offer the most straightforward models available to investigate the adverse effects of endocrine disruptors. *In vivo* testing is particularly important for evaluating chronic effects associated with a potential endocrine disrupter. Especially multi-generation tests aim at serving as higher tier tests for collecting definitive concentration-response information on adverse effects suitable for use in ecological risk assessment. Although quite sensitive, these assays are time-consuming, labour-intensive, and costly. In addition, both the European Community and the United States government have directives that propose to minimize the use of animal testing where possible (Gillesby and Zacharewski, 1998). For the screening of genotoxicity, *in vitro* and *in vivo* bioassays proved to be valuable tools in aquatic ecotoxicology to determine the total biological potential of a mixture of chemicals or environmental extracts as well as of individual substances (Chen and White, 2004). Nevertheless, variations between different cell types with respect to co-activators (Cavailles *et al.*, 1994; Cavailles *et al.*, 1995; Halachmi *et al.*, 1994), supplementary proteins (Landel *et al.*, 1995), and growth factors (Ignar-Trowbridge *et al.*, 1992; Ignar-Trowbridge *et al.*, 1995) can result in divergent results. Therefore, a comparison of the test results *in vivo* and *in vitro* will give insight into the mechanisms of actions of trenbolone in a permanent cell line as well as in a living animal.

As freshwater systems serve as drinking reservoirs, their ecological status is of special interest for man. Nevertheless, the resources of accessible renewable freshwater are very limited, especially regarding the fact that only 2.5 % of the earth's surface consists of freshwater. The oceans account for 96.5 %, brackish water for around 1 %. Furthermore, 70 % of all freshwater resources are locked up in glaciers, and permanent snow and the atmosphere {Dompka, 2002 #474; UNESCO, 2005 #475}. More than one-third of the freshwater is used

for agricultural, industrial and domestic purposes, and most of these activities lead to water contamination with numerous synthetic and geogenic compounds (Schwarzenbach *et al.*, 2006). The availability of water has always been a limiting factor to human activities, in particular agriculture, and the increasing level of demand for water is a growing concern. Under a 'Business as usual scenario' (Rosegrant *et al.*, 2002), global water withdrawal is projected to increase by 22 % to 4772 in 2025 km³. This increase will be driven mainly by domestic, industrial and livestock uses; the latter showing an increase of more than 50 %: Water consumption for non-agricultural uses is projected to increase by 62 % between 1995 and 2025. As a direct consequence of the expected increase in demand for water, Rosegrant, *et al.* (2002) projected that by 2025, 64 % of the world's population will live in water-stressed basins (*versus* 38 % today).

The demand on freshwater is increasing rapidly. Therefore, the conservation and or creation of a good ecological status of the existing freshwater systems are mandatory. Hence, the chapters 2 to 4 of this thesis will focus on the ecological status of two river systems important at large scales:

- the Upper Danube River in Germany (Chapters 2 and 3)
- the Tietê River in Brazil (Chapter 4).

For this purpose, a special focus was laid on the comparability of results gained in the field with studies performed *in vitro* and *in situ*. The comet assay and the micronucleus assay with established cell lines are commonly used bioassays for the assessment of genotoxic/mutagenic hazard potential of monosubstances and/or complex environmental samples (Al-Sabti and Metcalfe, 1995; Decordier and Kirsch-Volders, 2006; Fairbairn *et al.*, 1995; Frieauff *et al.*, 1998; Kosmehl *et al.*, 2004; Reifferscheid *et al.*, 2007; Schnurstein and Braunbeck, 2001). Therefore, these results were used as background data for further correlation analyses and interpretation with data of subsequent investigations. The *in situ* genotoxicity was studied by the micronucleus assay with fish erythrocytes. As an intermediate step, in order to improve the ecological relevance of the investigations in the laboratory, the comet assay with *Danio rerio* exposed to whole sediments was conducted as well for sediment extracts from the River Danube.

Thus, the aim of these first three investigations was to characterise and evaluate the genotoxic burden of the rivers Danube and Tietê. We intended to answer the questions whether

- (1) the comet assay with RTL-W1 cells exposed to sediment extracts correlates with the comet assay conducted on zebrafish embryos exposed to whole sediments;
- (2) the comet assay and the micronucleus assay are comparable as two alternative genotoxicity / mutagenicity tests in order to characterize the potential genotoxic burden of environmental sediment samples;
- (3) the comet assay and the micronucleus assay with RTL-W1 cells exposed to sediment extracts are comparable to results gained in the micronucleus assay with fish caught in the field.

In order to obtain a more realistic test scenario of the micronucleus assays applied in the laboratory, several new approaches were developed (Chapter 5). These experiments attempt to elucidate

- (1) the most practicable and suitable methods to assess micronuclei in fixed liver tissue;
- (2) a suitable protocol for an application of the micronucleus assay with zebrafish embryos.

Since the major part of the accessible freshwater today is used for agriculture, the possible pollution resulting out of it is of special interest. Since, pharmaceuticals are used in large quantities in the livestock sector, mainly antimicrobials and hormones, residues of these substances can be found in liquid manure and dung. Hormones are used to increase feed conversion efficiency, particularly in the beef and pig sectors. Their use is not permitted in a series of countries, particularly in Europe {FAO, 2003 #477}. Based on the protocol developed for the micronucleus assay with zebrafish embryos, trenbolone, a potent anabolic steroid used to promote cattle growth, was tested.

Systematic review of the toxicity profiles of a range of EDCs has highlighted the potential for those that exhibit steroid-modulating effects also to possess mutagenic and carcinogenic activity (Choi *et al.*, 2004). Furthermore, as current opinion suggests that pollutants that interfere with steroid action/production may be of most concern to human reproductive health (Fisher, 2004), the model anabolic steroid trenbolone was chosen as test substance. Therefore, the aim of this part of the study was to elucidate the genotoxicity of trenbolone (Chapter 6).

Since very little is known about the mode and sites of action of trenbolone, the next part of this thesis (Chapter 7) attempts to answer the questions

- (1) if the genetic damage caused by trenbolone is partly masked as the molecule is linked to a special tissue or organ in zebrafish;
- (2) if the chorion acts as a barrier in case of trenbolone.

Knowing that trenbolone is ubiquitous in the body of the zebrafish embryo, the question emerges if this hormone might have other influence on organisms than the observed genotoxicity and the known endocrine effect. To answer this question, life-cycle or multi-generation tests serves as appropriate tool. These have been designed to serve as a higher tier for collecting definitive concentration-response information on adverse effects suitable for use in ecological risk assessment.

Since androgens as well as anti-androgens and anti-estrogens have, compared to estrogens, received less attention during the last decades, the androgen trenbolone, the anti-androgen flutamide and the anti-estrogen tamoxifen citrate were tested in a multi-generation test. This part of the thesis forms part of a validation of a definite fish test (full life-cycle/two generation) with zebrafish. Within this scope, it deals with the questions if the two generation test is suitable for the detection of effects caused by androgens anti-androgens and anti-estrogens (Chapter 8). As the two-generation-test with trenbolone was conducted within the scope of this PhD thesis, an in-depth evaluation of the results will be presented in chapter 9 of this thesis. Since the studies on the effects of flutamide and tamoxifen citrate were carried out in collaboration with the Fraunhofer Institute at Schmallenberg, these results will not be discussed in detail.

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The ecological status of the River Danube

The Danube is, with 2850 km, the second longest river in Europe. From its origin in Donaueschingen, it passes ten Central and Eastern European countries, before opening into the Black Sea in Romania and the Ukraine. As some 70 million people depend on the Danube as drinking water reservoir, the ecological status of this ecosystem is of extreme importance.

Records of the upper Danube River document improvements of water quality over the last three decades. The reasons for the improvement of major water quality parameters are more restrictive waste water legislation, implementation of water protection programs as well as modernization of sewage plants ². This improvement, however, stands in contrast to only minor recoveries or even further declines of fish stock in the Danube River ⁷. Similar trends have been observed for many other streams in Europe, USA and Canada since the mid 1980s ^{3,6}. It may be concluded that common health and quality parameters apparently fail to assess the health of river systems in particular, at least with respect to fish populations. As a consequence, more subtle parameters are required for assessing the status of aquatic ecosystems.

¹ <http://www.wikipedia.org>

² WWA (2004). Download from <http://www.bayern.de/wwa-in/Technik/Guete/Biologie/Qualitaet/qualitaet.htm>

³ Burkhardt-Holm, P., W. Giger, H. Guttinger, U. Ochsenbein, A. Peter, K. Scheurer, H. Segner, E. Staub and M. J. Suter (2005). "Where have all the fish gone? The reasons why fish catches in Swiss rivers are declining." *Environ. Sci. Technol.* 39: 441A-447A.

⁴ Cook, P.M., J.A. Robbins, D.D. Endicott, K.B. Lodge, P.D. Guiney, M.K. Walkero, E.W. Zabelo and R.E. Peterson (2003). "Effects of aryl hydrocarbon receptor-mediated early life stage toxicity on lake trout populations in Lake Ontario during the 20th Century." *Environ. Sci. Technol.* 37 (17): 3864-3877.

⁵ Faller, P., B. Kobler, A. Peter, J., Sumpter and P. Burkhardt-Holm (2003). "Stress status of gudgeon (*Gobio gobio*) from rivers in Switzerland with and without input of sewage treatment plant influent." *Environ. Toxicol. Chem.* 22 (9): 2063-2072.

⁶ Lafontaine, Y. de, N.L. Gilbert, F. Dumouchel, C. Brochub, S. Moore, E. Pelletier, P. Dumont and A. Branchaude (2002). "Is chemical contamination responsible for the decline of the copper redhorse (*Moxostoma hubbsi*), an endangered fish species, in Canada?" *Sci. Total Environ.* 298: 25-44.

⁷ Keiter, S., Rastall A., Kosmehl T, Wurm K., Erdinger L., Braunbeck T. and H. Hollert (2006). "Ecotoxicological assessment of sediment, suspended matter and water samples in the Upper Danube river." *Environ. Sci. Pollut. Res.* 13 (5).

For decades, researchers have tried to identify a causal link between chemical contamination and genotoxicity in fish populations⁸. Nonetheless, to the best of our knowledge, studies comparing results of *in situ* assays, reflecting real exposure conditions (actual ecological status), and results from *in vitro* approaches (e.g., using samples such as sediments) from the same ecosystem for exposure are scant. This is even more surprising, since, in order to elucidate the comparability and adequacy of *in vitro* tests to replace field studies, combination and correlation of both approaches are indispensable.

The major focus of the first two chapters is the assessment of genotoxicity of Danube River basin sediments using the comet assay and the micronucleus assay with RTL-W1 cells, the comet assay with embryos of zebrafish (*Danio rerio*) and the micronucleus test with barbel (*Barbus barbus*) erythrocytes.



River flow of the Danube from its spring in Donaueschingen to the Delta in the Black Sea (<http://www.wikipedia.org>).

⁸ Macek, K. J. (1980). "Aquatic toxicology: fact or fiction?" *Environ. Health Perspect.* 34: 159-62.

Chapter 2

Comparison of *in vitro* and *in situ* genotoxicity in the Danube River by means of the comet assay and the micronucleus test

Authors: Melanie Boettcher, Stefanie Grund, Steffen Keiter, Thomas Kosmehl, Georg Reifferscheid, Nadja Seitz, Paula Soares Rocha, Henner Hollert, Thomas Braunbeck

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2.1 Abstract

Genotoxicity can be correlated with adverse reproductive effects or may even result in elevated extinction risk for particular species of an ecosystem. It may thus be a valuable tool for screening of pollution and potential environmental harm. Since many genotoxicants tend to adsorb to particulate matter, sediments and suspended materials are of particular interest for genotoxicity screening under field conditions. In order to relate the genotoxic potential of sediments to genetic damage of fish, RTL-W1 cells were exposed *in vitro* to acetic sediments extracts collected at 10 selected sites along the upper Danube River and analyzed in the comet and micronucleus assays, and these *in vitro* results were compared to micronucleus formation in erythrocytes of European barbel (*Barbus barbus*) caught in the field. The two *in vitro* bioassays showed excellent correlation indicating comparability of genotoxic potentials *in vitro*. Sampling sites could be clearly differentiated with respect to severity of effects, with Rottenacker as the most heavily contaminated site, with Ehingen and Schwarzach as moderately genotoxic, and with least effects in the tributary Lauchert. All other sediment extracts showed intermediate genotoxic or mutagenic effects. *In situ*, micronucleus formation in barbel erythrocytes indicated severe genotoxicity at Rottenacker, moderate effects at Ehingen, but minor contamination at Riedlingen and Sigmaringen. *In situ* observations thus showed excellent correlation with corresponding *in vitro* tests and document the ecological relevance of *in vitro* studies with sediment extracts. With respect to the ecological status of the Danube River, results overall indicate a moderate to severe genotoxic potential with a highly differential localization.

Keywords: Genotoxicity, micronucleus assay, comet assay, *in situ*, *in vitro*

2.2 Introduction

Records of the upper Danube River document improvements of water quality over the last three decades. The reasons for the improvement of major water quality parameters are more restrictive waste water legislation, implementation of water protection programs as well as modernization of sewage plants. This improvement, however, is in contradiction to only minor recoveries or even further declines of fish stocks in the Danube River [1]; similar trends have been observed for many other streams in Europe, USA and Canada since the mid 1980s [2-5]. It may be concluded that common health and quality parameters apparently fail in

particular river systems at least with respect to fish populations. As a consequence, more subtle parameters are required for assessing the status of aquatic ecosystems.

Biotests, for example, give signals of pollution and potential damage in the environment [6]. Among other parameters, genotoxicity is of special interest, since it may directly be correlated with adverse reproductive effects [7] or even lead to an elevated risk of extinction [8]. Previous studies on fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and fern (*Onoclea sensibilis*) have shown that exposure to mutagens in water may well enhance the frequency of heritable recessive lethal mutations [9-11], and the accumulation of such deleterious mutations can directly contribute to the decline of small populations *via* a phenomenon known as mutational meltdown [12].

Genotoxic activity of organic compounds in various types of industrial effluents (e.g., metal refining and founding) or run-off is predominantly associated with particulate materials [13], and, consequently, with sediments. Sediments therefore represent reservoirs of genotoxic and mutagenic hazard, which can continually be re-introduced into the water column *via* resuspension or trophic transfer. Sediments may thus contribute substantially to the exposure of benthic biota as well as subsequent bioaccumulation [14,15].

For the screening of sediment genotoxicity, *in vitro* bioassays proved to be valuable tools in aquatic ecotoxicology to determine the total biological potential of a mixture of chemicals or environmental extracts as well as individual substances [14]. In the present study, the comet assay was selected as an *in vitro* genotoxicity assay, since it represents a rapid, sensitive and inexpensive method for measuring genotoxic effects in individual cells [16,17]. It allows the detection of DNA strand breaks and alkali-labile sites by measuring the migration of DNA fragments from immobilized nuclear DNA [18]. Additionally, it can be conducted on virtually any eukaryotic cell type, *in vivo* as well as *in vitro* [17]. In this study, the comet assay was performed *in vitro* following exposure of the cell line RTL-W1 [19] to organic sediment extracts from the upper Danube River.

In contrast, the micronucleus assay is based on the loss of chromosomes or chromosome fragments during meiosis, which are not reincorporated into the nucleus after cell division and, therefore, are transformed into a smaller nucleus or micronucleus [20,21]. The micronucleus test was also used for RTL-W1 cells previously exposed to the organic sediment extracts. Surprisingly, although this fish-derived permanent cell line has repeatedly proved to be a useful tool for the detection of genotoxic pollutants in sediments, it has not been used previously in the micronucleus assay.

However, even though organic extracts of sediments have frequently been used to assess the potential ecotoxicological hazard of sediments [14,22-24], the relevance of such studies for the field situation is difficult to assess [25]. In contrast, bioassays with animals collected in the field represent a more realistic exposure scenario, which better reflects ecosystem health status and the fate of introduced contaminants [26]. Therefore, European barbel was selected for assessing the genotoxic potential *in situ* by means of the micronucleus test in erythrocytes. Since fish occupy a top position in the food web, they can act as amplifier for genotoxic substances which might be already present in some smaller organisms. Especially European barbel (*Barbus barbus*), as benthic fish, may accumulate toxicants from sediments and should, thus, be particularly more at risk to environmental pollution [27]. Furthermore, in common fish zonation along river systems [28], a 'barbel zone' has been defined with the barbel as the dominant species. The ecological significance of this species thus further corroborates its value as an indicator species, and the wide zoogeographic distribution of *Barbus* sp. in Europe allows the future comparison between different rivers within *in situ* monitoring programs.

For decades, researchers have tried to identify a causal link between chemical contamination and genotoxicity in fish populations [29]. Nonetheless, to the best of our knowledge, studies comparing results of *in situ* assays, reflecting real exposure conditions (actual ecological status), and results from *in vitro* approaches (e.g., using samples such as sediments) from the same ecosystem for exposure are scant. This is even more surprising, since, in order to elucidate the comparability and adequacy of *in vitro* tests for replacing field studies, combination and correlation of both approaches are indispensable.

Thus, the objectives of this study were (1) to compile data from different *in vitro* test systems to identify the hazard potential of acetone-extractable material of sediments from the upper Danube River, and (2) to compare this to the *in situ* genotoxic potential in order to elucidate the ecological relevance of *in vitro* results and their suitability as possible bioassay systems.

2.3 Materials and methods

2.3.1 Samples

In May and July, near-surface sediment samples were taken by means of a van Veen-gripper or a stainless steel shovel at eight locations along the upper Danube River (Sigmaringen, Riedlingen, Rottenacker, Ehingen, Oepfingen, Ingolstadt, Bad Abbach, Jochenstein, all Germany) as well as at two tributaries (Lauchert and Schwarzach; Fig. 2.1). Cooled samples

were transferred to the laboratory, freeze-dried and sieved at 2 mm. Sub-samples were used for preparation of organic extracts in a Soxhlet apparatus (Keiter et al. [1]). The resulting extract concentration was adjusted to an equivalent of 20 g sediment equivalents dry weight per ml DMSO (Serva, Heidelberg, Germany). Extract concentrations used in the comet assay with RTL-W1 cells are given in mg sediment equivalent per ml (SEQ/ml).

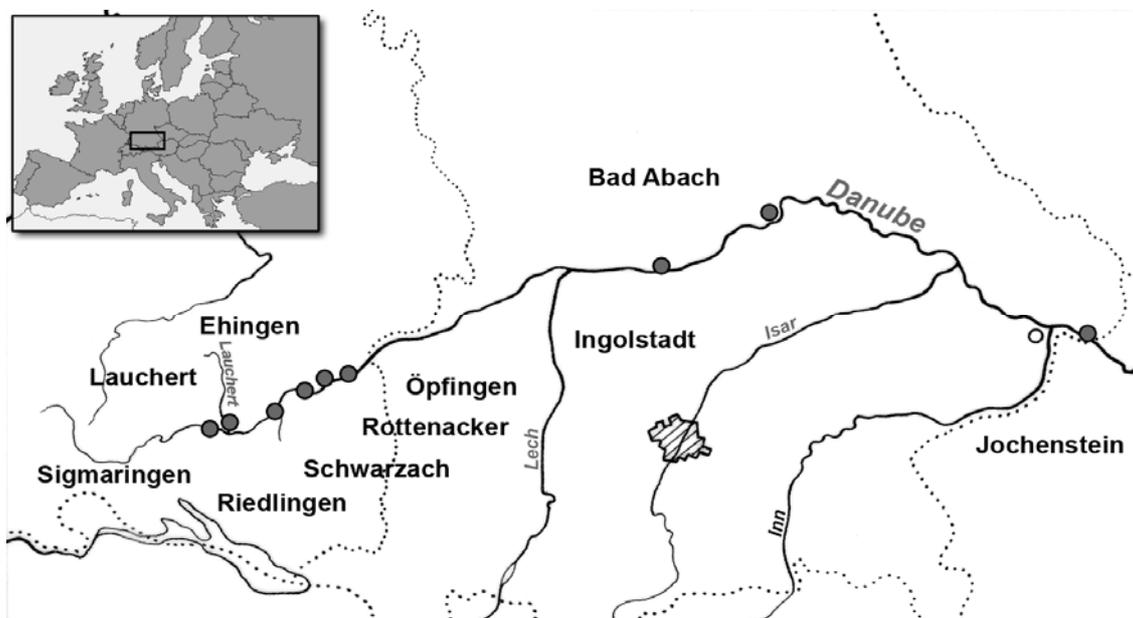


Fig. 2.1: Study area and sampling locations.

2.3.2 *In vitro* tests

Cell culture conditions

RTL-W1 cells derived from rainbow trout liver (*Oncorhynchus mykiss*); were cultured in Leibovitz L15 medium (Sigma, Deisenhofen, Germany) according to Klee et al. [30]. Prior to use in the *in vitro* assays, RTL-W1 cells were washed twice with phosphate-buffered saline (PBS; Sigma) and trypsinized for 2 min according to Kosmehl et al. [31], using 0.05 % trypsin (Sigma) and 0.02 % ethylenediaminetetraacetic acid (EDTA; Sigma-).

Negative controls

For the *in vitro* tests, three different types of negative control were used: (1) a medium control (test medium only), (2) a solvent control with a concentration range between 2.5 and 40 mg DMSO/ml in the test medium, and (3) a process control for Soxhlet extraction with empty extraction thimbles. The resulting Soxhlet control extract was concentrated and resolved in DMSO for testing. Since no significant differences could be observed between all three controls, they will collectively be referred to as ‘negative controls’.

Comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh et al. [18] with modifications according to Schnurstein and Braunbeck [32] as well as Kosmehl et al. [31] using acetonic sediment extracts and RTL-W1 cells. In order to guarantee optimal adhesion, fully frosted slides (Langenbrink, Emmendingen, FRG) were used; they were cleaned in 99 % ethanol and coated with 1 % normal melting agarose (NMA), which was hardened for 5 min at 37 °C and scraped off afterwards. This procedure increases the adhesion of the following 0.5 % NMA layer. Cells were embedded in 0.7 % low melting agarose (LMA) layers on the precoated slide and again coated with an additional layer of 0.7 % NMA. Slides were cooled on ice for 3 min and dried at 37 °C for 5 min. Following lysis in 100mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10 % DMSO (pH 13.0) for 1.5 h in the dark at 4 °C. After electrophoresis in the same buffer at 25 V and 310 mA for 20 min, samples were neutralized by incubation in 400 mM Tris at pH 7.4 for 2 min. Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide and covered with a cover slip. In the neutral red assay [33], the different tested extract concentrations had been shown to not induce more than 20 % lethality. DMSO was used as solvent and never exceeded a concentration of 0.1 %. Exposure was carried out for 24 h at 20 °C. After incubation, cells were washed with PBS, trypsinized and processed for the comet assay.

All slides were examined at a magnification of 320x using a fluorescent microscope (Aristoplan, Leica, FRG) equipped with an excitation filter of 518 nm and an image analysis system (Optilas, Munich, Germany) with a grey-scale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, DK) and the Comet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, the tail moments of 100 randomly selected cells were analyzed.

For statistical analysis, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 3.5; SPSS-Jandel, Erkrath, Germany). In cases of significant differences, a post-hoc test according to Dunn was used to identify groups differing significantly. The induction factor (IF) was calculated by dividing the median of each concentration by the median of the corresponding control group.

To simplify the comparisons, data were converted into the “concentration-dependant induction factor” CDI according to Seitz et al. [34]. The CDI is a simple index value that integrates all important information, providing a basis for a general comparison of the genotoxic potential in the comet assay.

The CDI is calculated as follows:

$$CDI = \sum_{i=1}^n \frac{IF_i}{c_i}$$

where IF_i = induction factor of the concentration i
 c_i = concentration i
 n = n concentrations

Micronucleus test

For the *in vitro* version of the micronucleus test, RTL-W1 cells were exposed to sediment extracts and to nitroquinoline-*N*-oxide (NQO, Sigma) for 20 h. For exposure, cells were transferred to 6-well plates with ethanol-cleaned cover slips (Assistent, Sondheim, Germany) and incubated for 12 h in pure medium to allow for complete cell attachment.

Afterwards, exposure and fixation were conducted as described by Gauthier et al. [35]. After treatment, cells were incubated with pure medium for another 72 h. Cells were fixed for 10 min with methanol/acetic acid (4/1) (v/v) diluted in PBS (1/1). A second fixation (2 x 10 min) was performed with methanol/acid acetic (4/1) (v/v). Subsequently to fixation and air drying, the slides were stained for one minute with undiluted Giemsa (Gurr, BDH Laboratory Supplies, Poole, UK) and covered with DePeX (Serva, Heidelberg, Germany). After 24 hours of solidification, 2000 cells per slide were scored. Micronuclei were counted under the light microscope equipped with an oil immersion lens at 1200× magnification. Criteria for micronuclei in RTL-W1 cells were set according to ISO 21427-2 [36].

Results were recorded as percentage of cells containing micronuclei compared to the total number of counted cells. The induction factor for each site was calculated by computing the percentage of micronuclei from exposed cells to the percentage of micronuclei in the negative controls. Statistical significances were assessed by using the Chi-square-test with Yates correction [36,37]. Therefore, for each concentration and sediment extract, the number of cells with micronuclei was compared to those without micronuclei and with the corresponding negative control (SigmaStat 3.5). Values differing significantly from the negative control were marked in the graph with a probability value from $p \leq 0.05$ with one asterisk (*), $p \leq 0.01$ with two asterisks (**) and for $p \leq 0.001$ with three asterisks (***)

To further evaluate the induction assessed in the micronucleus test, genotoxicity was related to NQO mutagenicity by calculating NQO equivalents. Since NQO is a strong, well-known mutagenic substance, it has widely been used as a reference substance in various biotests [8,9,38-40]. For this purpose, an NQO calibration curve was generated. Cells were exposed to NQO at concentrations between 11.9 and 190 µg/L. By relating the induction factors of sediment extracts to an NQO calibration curve, an equivalent to the NQO concentration was

obtained. The value of the corresponding NQO concentration will be expressed as induction per gram sediment (see formula). This conversion of mutagenic potentials from sediment extracts allows a direct comparison of the sampling sites under consideration of each tested concentration. NQO concentrations obtained from the calibration curve were translated by the following formula:

$$\text{NEQ } [\mu\text{g NQO equivalent/g sediment}] = \frac{\text{NQO } [\mu\text{g/L}]}{\text{extract concentration [g/L]}}$$

The average NQO equivalents ($\text{NEQ}_{\text{average}}$) for all sites were calculated from the NQO equivalents of each tested concentration.

2.3.3 Field studies

Blood samples

Blood samples were collected from wild European barbel (*Barbus barbus*) immediately after capture at the sites Riedlingen, Sigmaringen, Rottenacker and Ehingen. At each sampling site, five mature fish were collected by electro fishing. All electro-fishing models rely on two electrodes which deliver current into the water to stun fish. The current runs from the anode (which is attached to the landing net) to the cathode (swimming freely in the water), creating a high-voltage potential. When a fish encounters a large enough potential gradient, it becomes affected by the electricity. After catch, fish were anaesthetized with a saturated benzocaine (Sigma) solution and opened ventrally. Cardiovascular blood samples were obtained by puncture of the heart with heparinized syringes (Hawksley and Sons Limited, Lancing, UK). The blood was smeared immediately at ethanol-cleaned slides. After drying, the slides were fixed in methanol for at least 1 min and stained with undiluted Giemsa (Gurr). The slides were then covered with DePeX (Serva). Sex of the fish was not determined, the length ranged between 12 and 33 cm, the weight between 14.5 and 41 g.

As a reference, fish from Riedlingen were caught and maintained in Heidelberg University facilities for 60 days under flow-through conditions (4 L/h) in a 400 L basin, with continuous dechlorinated tap water and aeration. The photoperiod was adjusted to a 12 h light/12 h dark cycle, water temperature averaged 14 ± 1 °C. Once a week, barbel were fed with bloodworms, otherwise with trout flake food (Trouvit pro aqua, Milkivit, Burgheim, Germany)

Micronucleus test

The frequency of micronuclei in erythrocytes was assessed from fish sampled along the River Danube. Per slide, 2000 erythrocytes were examined. Since the micronucleus frequency of at least four individuals (8000 erythrocytes) per sampling site was assessed, a statistical analysis could be carried out [41]. An all pair-wise comparison according to Dunnett's test ($p \leq 0.05$) was used to identify groups that differed significantly.

2.4 Results

2.4.1 Comet assay in RTL cells exposed to sediment extracts *in vitro*

Acetonic sediment extracts from the Danube River were tested in the comet assay with RTL-W1 cells. 8 of 10 sediment extracts displayed genotoxic activity in terms of DNA fragmentation (tail moment), revealing clear dose-response relationships. Sediment extracts from Rottenacker, Oepfingen and Schwarzach differed significantly from the negative control at all concentrations tested. In order to demonstrate the relation between sediment test concentration and the resulting tail moment and induction factor, one example illustrated in detail (Fig. 2.2). For the sediment extract from the Lauchert tributary (Fig. 2.2a), only one concentration in each of the two replicates showed significant genotoxicity compared to the negative control (5 mg SEQ/ml and 40 mg SEQ/ml, respectively). In contrast, for sediment extracts from Oepfingen (Fig. 2.2b), all extract concentrations in either replicate displayed elevated genotoxic effects compared to the negative controls. Furthermore, the induction factor increased with concentration.

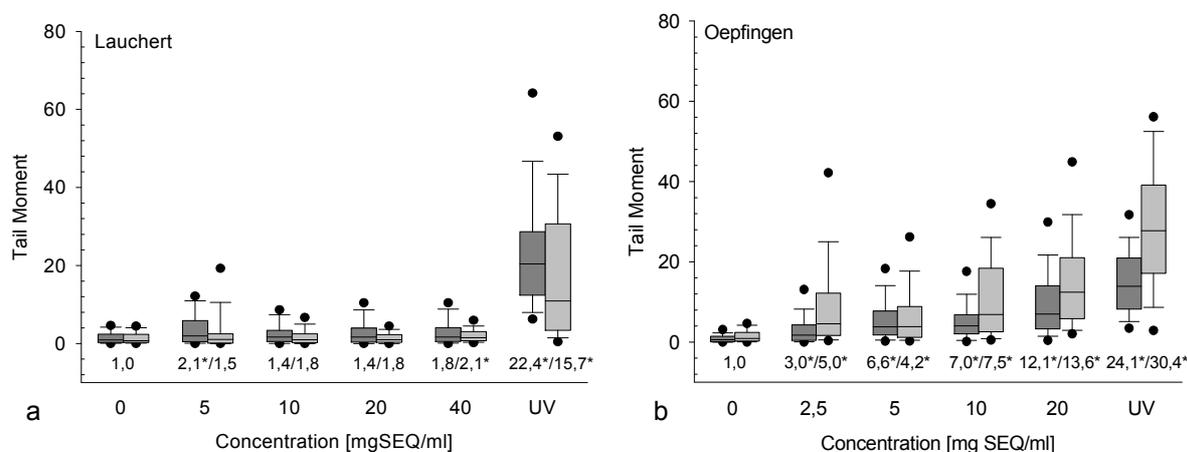


Fig. 2.2: Genotoxic effects of acetonic sediment extracts from Lauchert (a) and Oepfingen (b) respectively, in the comet assay. Numbers below the box plots identify the induction factor for each concentration. Asterisks (*): Values differ significantly from the negative control (post hoc test according to Dunn's; $p \leq 0.05$).

Results for the comet assay with RTL-W1 cells were computed as concentration-dependent induction factors (CDIs) to enable a better comparison of the results from different sampling sites (Fig. 2.2). The sediment extract from Rottenacker induced the highest CDI of 11.4. The CDIs for the extracts from Ehingen, Schwarzach and Oepfingen ranged between 2 and 4, the sediment extracts from Bad Abbach, Jochenstein, Ingolstadt and Sigmaringen caused CDIs between 1 and 1.6. The CDIs for the other extracts from Riedlingen and the tributary Lauchert were below 1.

2.4.2 Micronucleus test in RTL-W1 cells exposed *in vitro*

NQO in the micronucleus test with RTL-W1 cells

4-Nitroquinoline-*N*-oxid was tested in 5 independent replicates (Fig. 2.3). The induction of micronuclei in RTL-W1 cells increased with concentration, finally reaching a maximum induction factor of 5. All concentrations tested differed significantly from the control ($p \leq 0.01$). Based on the curve progression of each replicate (single data/graphs not shown), it is unlikely that the maximum induction factor of 5 will be exceeded by higher NQO concentrations.

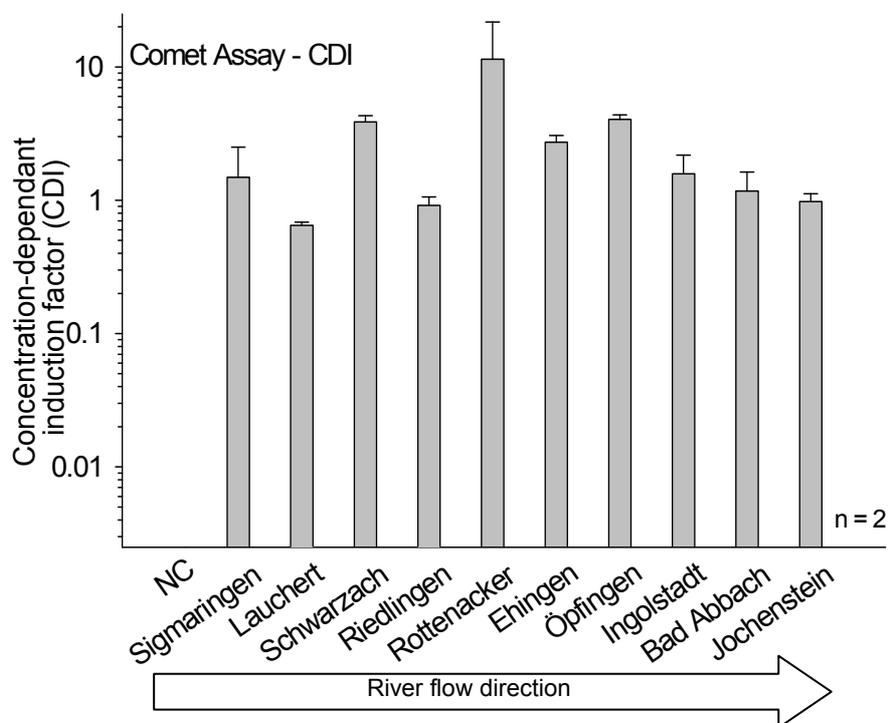


Fig. 2.3: Genotoxic activity of sediment extracts in the comet assay with RTL-W1 cells from two independent series of experiments in river flow direction, expressed as concentration-dependent induction factor (CDI; Seitz et al. 2007). NC = negative control.

Induction of micronuclei in RTL-W1 cells by sediment extracts

All tested sediment samples produced a significant induction of micronuclei, if compared to the negative control. The extracts from Schwarzach, Rottenacker and Ehingen showed a strong increase of the induction factor. Dose-related effects of the extracts showed scarce similarities: At low concentrations, micronucleus induction for all extracts increased with concentration. However, this initial increase stagnated for higher concentrations of sediments from Schwarzach, Rottenacker and Ehingen, induction (Ehingen: Fig. 2.4a) or even decreased to negative control levels (Sigmaringen, Schwarzach and Rottenacker; Fig. 2.4b). The micronucleus rate at the highest concentration tested in this assay (40 mg SEQ/ml) could not be assessed due to cytotoxic effects of the sites Rottenacker, Ehingen, Schwarzach, Ingolstadt, Jochenstadt and Bad Abbach. In contrast, the induction rate of sediment extracts from Lauchert (Fig. 2.4c), Riedlingen and Oepfingen showed a linear dose-response relationship.

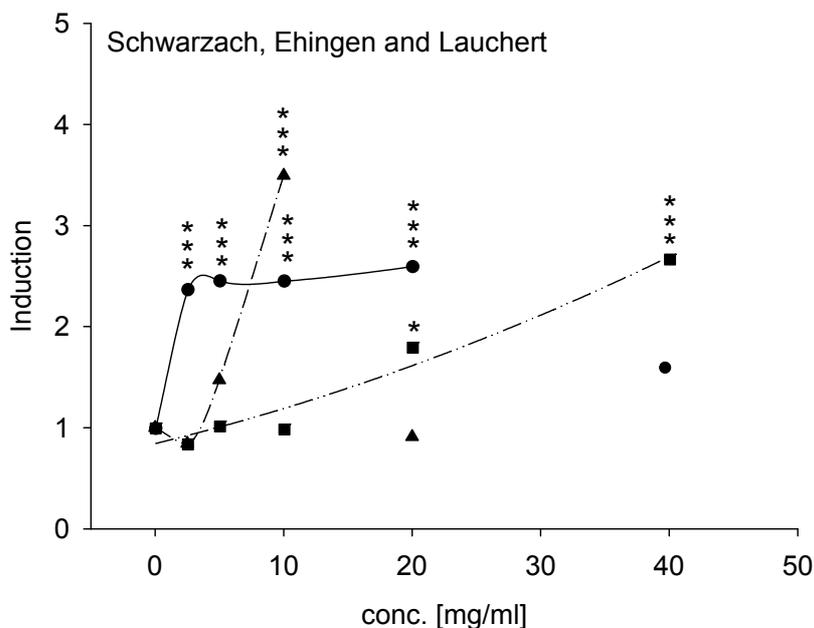


Fig. 2.4: Examples of different dose-response relationships for the mutagenic activity of acetonic sediment extracts in the micronucleus assay with RTL-W1 cells. Data are given as induction factors over controls. Asterisks: Values differ significantly from the negative control (Chi-square test with Yates correction; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

The results of the micronucleus test with RTL-W1 cells were analyzed by means of NQO equivalents (NEQ; Fig. 2.5). The genotoxic effects in RTL-W1 cells of 1 g sediment from Rottenacker were identical to those caused by 3.1 $\mu\text{g/L}$ NQO. Elevated NEQs were also calculated for sediment extracts from Ehingen (1.3 $\mu\text{g/L}$) and the Schwarzach tributary (1.4 $\mu\text{g/L}$). In contrast, NEQs from the Lauchert tributary was not elevated (NEQ = 0.1 $\mu\text{g/L}$).

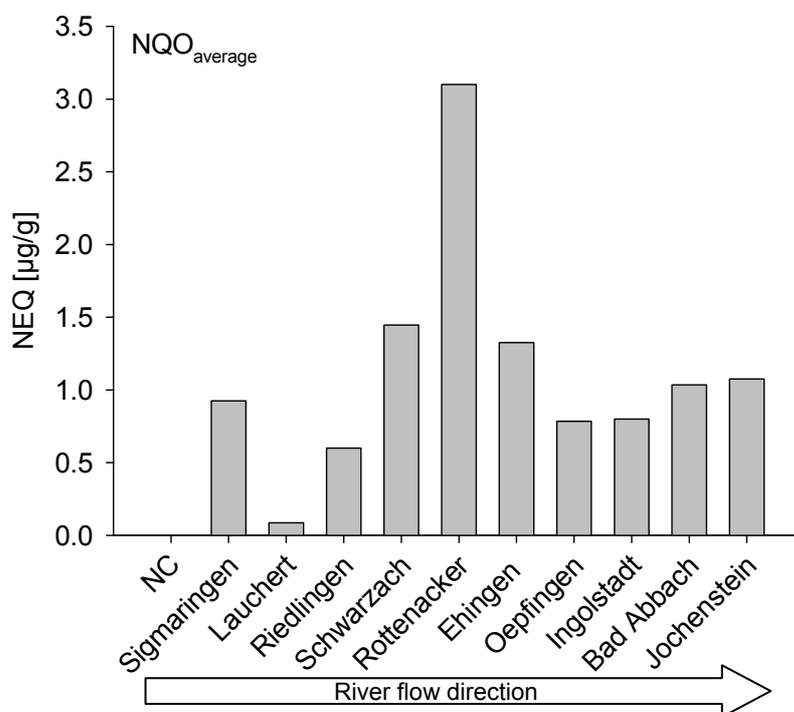


Fig. 2.5: Mutagenic activity of sediment extracts from the Danube River, tested in the micronucleus assay with RTL-W1 cells. Mutagenicity is displayed *via* the NEQ value in $\mu\text{g/g}$. Extracts are arranged in river flow direction.

Micronucleus test in erythrocytes from barbel after in situ exposure in the Danube River

The frequency of micronuclei in erythrocytes was assessed from barbel sampled along the River Danube (Sigmaringen, Riedlingen, Rottenacker and Ehingen; Fig. 2.6); the micronucleus frequency is given as percentage (MN [%]). The highest micronucleus rate was determined for Rottenacker (MN [%] 0.31); however, only slightly lower rates were recorded for Ehingen (MN [%] 0.27) and Riedlingen (MN [%] 0.24). The micronucleus frequencies for these three sites were significantly higher, if compared to negative controls (MN [%] 0.06). Only the micronucleus rates from erythrocytes from barbel caught at Sigmaringen (MN [%] 0.14) were not significantly elevated.

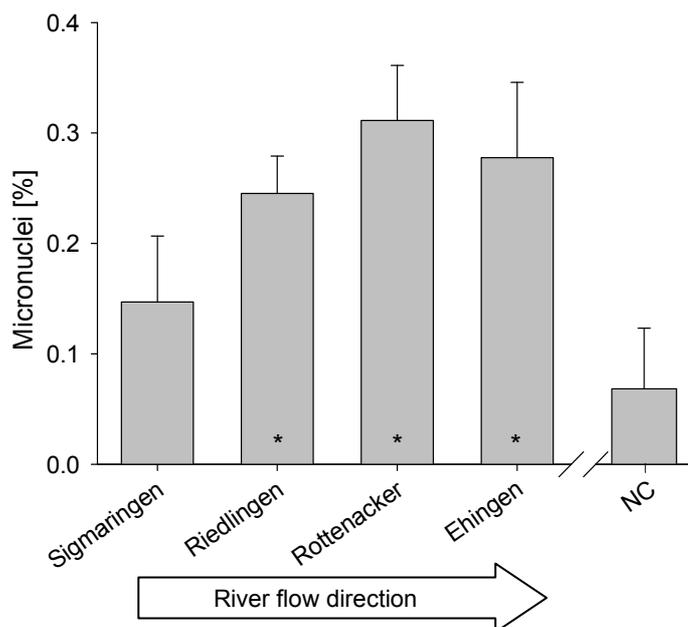


Fig. 2.6: Micronucleus frequency (MN [%]) in barbel (*Barbus barbus*) erythrocytes from selected sites along the Danube River. Each box contains data from five barbels with 2000 erythrocytes assessed per barbel. NC: Negative control; Asterisk (*): Values differ significantly from the negative control (post hoc test according to Dunnett; $p \leq 0.05$).

2.5 Discussion

2.5.1 *In vitro* test systems: comet assay versus micronucleus test

Rocha et al. [42] exposed RTL-W1 cells to sediment extracts from the Tiête River in Sao Paulo State (Brazil) and analyzed these using the comet assay. The upper part of this river includes the origin as well as the metropolitan region of Sao Paulo City. The CDI for sediments from the spring was below 1 (0.37) and, thus, comparable to sediment extracts from the Danube tributary Lauchert (0.5). Approaching Sao Paul City, the CDI increased, finally reaching a maximum of 3.3 for sediment extracts from Billings's reservoir located in Sao Paulo City. The sediment extract from Rottenacker exceeds this value by a factor of 3.5. The sediment extracts from Ehingen, Schwarzach and Oepfingen are comparable with those from Billings. Downstream Sao Paulo City the CDIs decreased below 1 again. Preliminary studies revealed Billings's reservoir as a water body extremely polluted by domestic sewage as well as by industrial effluents [43,44]. As a consequence, the results obtained for sediment extracts from the Danube Rive are even more striking.

The sediments studied in the present investigation have also been tested in the sediment-contact version of the comet assay with zebrafish (*Danio rerio*) embryos [34], i.e. in an assay

that applies the comet assay to cells from full organisms with intact cell-to-cell interactions [45]. The results obtained by Seitz et al. [34] provided clear evidence of genotoxic contamination of sediments from the upper Danube River. At some localities, there was significantly increased genotoxicity in native sediments even at dilutions of up to 32-fold (Rottenacker). Again, sediments from the Lauchert tributary were free of genotoxicity. A correlation analysis confirmed a high correlation between comet assays with *Danio rerio* exposed to native sediments [34] and comet assays with RTL-W1 cells exposed to acetonitrile sediment extracts (this study; $r_{\text{Pearson}} = 0.75$ with $p < 0.05$; $R^2 = 0.56$).

Diekmann [10]) showed that NQO at concentrations as low as 0.1 µg/L may significantly affect egg production in zebrafish. Since NQO only causes genotoxicity and mutagenicity, but no general and cellular toxicity, effects reported by Diekmann [10] can be related to DNA damage or DNA loss. Even though the genetic damage was assessed in a somatic cell line, it cannot be excluded that similar genotoxic effects might as well appear in the somatic cells and consequently might be transmitted to future generations. Therefore, an NEQ from Rottenacker of 3.1 µg/L might well have consequences on fish populations. Furthermore, an NQO concentration of 14.6 µg/L proved to be lethal in a full life-cycle test with *Danio rerio*; unfortunately concentrations between 2.9 µg/L and 14.6 µg/L had not been tested (Diekmann [10]). The sediment extract from Rottenacker would reach this lethal NEQ concentration with approximately 5 g sediment equivalent/L water, the extracts from Schwarzach and Ehingen with approximately 10 g sediment equivalent/L water. The fact that during a flood event one litre of water from the Elbe River can contain up to one gram suspended matter [46], this potential for genetic damage is of more concern.

By comparing the CDIs and NEQs along the river flow direction, 'hot spots', i.e. sampling sites with evaluated genotoxic potential could be determined. In both tests, the highest genotoxicity was induced by sediment extracts from Rottenacker. The genotoxic activity measured in the comet assay for sediment extracts from Ehingen, Schwarzach and Oepfingen could be classified as also genotoxic in the micronucleus test with exception of Oepfingen, which only caused minor genotoxicity.

In conclusion, based on these two *in vitro* tests, sediment extracts from the sampling site Rottenacker would be categorized as severely genotoxic, those from Ehingen and Schwarzach as moderately genotoxic. Little to no genotoxic activity was shown by the tributary Lauchert. The remaining sediment extracts induced low genotoxic effects.

A correlation coefficient of 0.97 ($p < 0.001$; $R^2 = 0.81$), was calculated for the tested sediment extracts in the two *in vitro* test systems indicating genotoxic potential of analyzed sediment extracts were highly correlated.

2.5.2 *In situ* genotoxicity – the micronucleus test in Danube River fish

For the assessment of genotoxicity *in situ*, sampling sites were selected based on their results in the *in vitro* assays. Rottenacker was ranked as a severely genotoxic/mutagenic site, Ehingen as a moderately contaminated site, and Riedlingen as well as Sigmaringen as mildly to minimally polluted sites. Consistent with the previous *in vitro* test results, the mutagenic potentials of the sampling sites did not change with river flow direction.

Minissi [47] conducted the micronucleus rates in European barbel sampled from an uncontaminated river in Italy (Mignone; low to mild contamination) with those from a contaminated river in Italy (Tiber; heavily contaminated). Barbel erythrocytes from the Mignone River showed a micronucleus induction rate of 0.015 %, those from Tiber a rate of 0.032 %. The control fish induced micronucleus formation in 0.005 % of the cells.

Rocha and colleagues [42] also determined the micronucleus frequency in erythrocytes from fish (*Oreochromis niloticus*) collected in the Tiête River in Sao Paulo State, Brazil. The average micronucleus induction rates for the two reference sites (Bariri and Promissao) were 0.1 %. In comparison, along the highly contaminated river sections around Sao Paulo City (Billings's reservoir; MN [%] 0.6 %) there is a 6-fold higher induction of micronuclei in erythrocytes. Comparing results from the present study with those from Minissi (1996) and Rocha et al. [42], micronuclei in erythrocytes from barbel caught at Rottenacker (induction factor = 4.6) were induced almost as strong as for the Tiber (induction factor = 6.4) and for the Tiête River in Sao Paulo City (induction factor = 6.0). Therefore, the mutagenic contamination of the Danube River in Rottenacker is comparable to two other highly contaminated rivers in Italy and Brazil. Similar induction rates of micronuclei were found for barbel from Ehingen (induction factor = 4.1). In contrast, micronuclei induction from barbel of the Danube River near Sigmaringen (induction factor = 2.1) is comparable to the induction of micronuclei in Mignone, Italy (induction factor = 3), and, thus, the mutagenic potential corresponds to a non-contaminated river in Italy.

2.5.3. Correlation between findings in the *in vitro* and *in situ* tests

Since only four sites along the Danube River were sampled for *in situ* micronucleus tests, the correlation analysis is restricted to these four sites and the negative controls. To the best of our knowledge, this is the first study comparing results of the *in situ* micronucleus test with those of *in vitro* micronucleus tests. Correlation analysis between these two test systems revealed a correlation coefficient of $r_{\text{Spearman}} = 0.90$ (with $p < 0.1$; $R^2 = 0.61$), indicating high correlation. Likewise, the same correlation coefficient was calculated for the analysis between the comet assay and the micronucleus assay with barbel erythrocytes ($r_{\text{Spearman}} = 0.90$ with $p < 0.1$; $R^2 = 0.61$). Results of the *in situ* tests confirm Rottenacker and Ehingen as ‘hot spots’ for genotoxic potency. In the present study, findings obtained in *in vitro* test correlate well with field studies, and are, therefore, consistent. Similar studies conducted by Rocha and colleagues [42] in the Tiête River with the micronucleus test *in situ* and the comet assay *in vitro* with RTL-W1 cells, also confirmed a high correlation between these two test systems.

Furthermore, studies applied on four sites (i.e., Black Rock Harbour, Connecticut; Puget Sound, Washington; Hamilton Harbour, Lake Ontario, Canada; the Black River, Ohio, USA), i.e. highly contaminated with known mutagens (e.g., PAHs), high levels of sediment genotoxicity, and an abnormally high incidence of genotoxic effects, neoplasms and preneoplastic lesions in indigenous biota, demonstrated a causal relationship between sediment genotoxicity and *in situ* effects (e.g., cancer, DNA damage, etc.) in selected species of aquatic biota [15]. These tests, based on bacterial mutations [48-55] or aberrations in fish and mouse cell lines [54,56], corroborate the proposed link between genotoxicity *in vitro* and *in situ*.

2.6 Conclusion

Conventional water quality criteria do not include assessments of important parameters such as genotoxicity and, thus, fail to account for effects by genotoxic compounds. The present study documents that fish collected from certain localities in the Danube River display marked genotoxicity, whereas fish sampled at other sites do not. This differential distribution of the genotoxic potential could be confirmed by *in vitro* studies into the effects of organic sediment extracts in permanent fish cell cultures. However, the good correlation between *in vitro* and *in situ* genotoxicity bioassays found for the upper Danube River needs to be confirmed in additional studies.

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Chapter 3

A novel statistical approach for the evaluation of comet assay data

**Authors: Nadja Seitz, Melanie Boettcher, Steffen Keiter, Thomas Kosmehl,
Werner Manz, Henner Hollert, Thomas Braunbeck**

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3.1 Abstract

The present study forms part of a weight-of-evidence framework including genotoxicological studies into the upper Danube River basin, which aims at elucidating the reasons for the decline in fish catch. The major focus of this paper is the assessment of genotoxicity of Danube River basin sediments using the comet assay with RTL-W1 cells and embryos of zebrafish (*Danio rerio*). A frequently discussed question in such approaches is how to aggregate and to compare the data obtained from genotoxicity testing. There is a need to develop mathematical methods combining the information from dose-response curves and level of effectiveness (maximum genotoxic effect). For comparison and ranking of the genotoxic potential of different sampling locations along the Danube River, several methods based on EC₅₀, LOEC, maximum induction factor were compared with respect to their validity. An evaluation system termed the “3-step analysis” was developed to facilitate consideration of a maximum of aspects of the raw data. The so-called “concentration-dependent induction factor” (CDI) introduces an index for a straightforward, precise and realistic assessment of the genotoxic potential of any kind of field sample or genotoxic agents.

Keywords: Comet assay – Danube basin – evaluation – genotoxicity – sediment contact assay – zebrafish (*Danio rerio*)

3.2 Introduction

Water quality in Central European streams has improved remarkably during the last two decades [1-8]. This improvement in water quality is in contrast to the little recovery or even decline in fish stocks observed in many European streams since the mid 80s and has to be discussed in context with alterations in fish community structures over the last 100 years [9-16]. The present study forms part of a large-scale weight-of-evidence project into the reasons for decline in fish catches observed in the Danube River since the late 80s [13,17]. The study has been designed on the basis of the Sediment Quality Triad approach by Chapman and colleagues [18,19]. Part of the results has already been published ([14]); further publications are in preparation [20-22] or are planned, respectively (e.g. community structure data).

Discharge of toxic wastewater into surface water systems often contains carcinogens and mutagens which can induce DNA and chromosome damage. Such DNA lesions may cause consequences at different biological levels up to the population level, and thus take influence on the stability of ecosystems. Thus, various authors have classified the assessment of DNA

damage as an important marker of exposure to the complex anthropogenic pollutant mixture in sediments [23-26]. Especially the comet assay (e.g. [27-34] has been used by many working groups for environmental biomonitoring studies as a simple, sensitive and broad-specificity tool [35-41].

Despite the wide application and increasing popularity of the comet assay, further work is needed for its standardization [41,42]. For instance, there is no standard statistical method for the evaluation of comet data, which would allow direct comparison of data from different studies [43]. A frequently discussed question is how to aggregate and to compare the data obtained from genotoxicity testing. There is a need to develop mathematical methods combining the information from dose-response curves and level of effectiveness (maximum genotoxic effect).

For this reason, the present study compared different standard evaluations including the EC_{50} , the Lowest Observed Effect Concentration (LOEC) and the maximum induction factor (IF_{max}) approaches to newly developed methods for the assessment of the genotoxic potential. Thus, from a more statistical point of view, the aims of the present study were to first create an evaluation system very close to raw data and taking into account as many information as possible, and then to reduce this system to a single index value allowing a simple assessment of genotoxicity of both pure substances and complex environmental samples. By means of this index, it should be possible to compare both different sites along rivers with respect to their genotoxic potential, and different exposure paths.

In the present study, two exposure paths for the comet assay were compared: (1) Cells from the permanent fish cell line RTL-W1 were exposed to acetone sediment extracts. (2) Alternatively, zebrafish (*Danio rerio*) embryos have been recently used after whole sediment exposure to determine the actual bioavailable embryo toxicity [44] and genotoxicity [45]. Therefore, the comet assay was combined with the prolonged version of a sediment fish embryo assay designed for the examination of teratogenic effects [46,47].

3.3 Materials and methods

3.3.1 Samples

In 2004, near-surface whole sediment samples were taken by means of a van-Veen-gripper or a stainless steel shovel at eight locations along the Upper Danube River in Germany (Fig. 3.1) as well as at two small tributaries (Lauchert and Schwarzach). Cooled samples were

transferred to the laboratory, freeze-dried and sieved to 1.25 mm within at maximum 48 h. Subsamples of the freeze-dried sediments were used for preparation of organic extracts in a Soxhlet apparatus (for further details see Hollert et al. [48]). The resulting extract concentration was 20 g sediment dry weight per ml DMSO. Extract concentrations used in the comet assay with RTL-W1 cells were given in mg sediment equivalent (SEQ)/ml.



Fig. 3.1: Hydrological system of Germany (inset) and sediment sampling sites along the Upper Danube River. Samples were also taken at the smaller tributaries Lauchert and Schwarzach nearby the confluence with the Danube. Sampling sites: 1 = Sigmaringen, 2 = Lauchert (Sigmaringendorf), 3 = Schwarzach (Riedlingen), 4 = Riedlingen, 5 = Rottenacker, 6 = Ehingen, 7 = Öpfingen, 8 = Ingolstadt, 9 = Bad Abbach, 10 = Jochenstein.

3.3.2 RTL-W1 cells

RTL-W1 cell lines derived from rainbow trout (*Oncorhynchus mykiss*) liver primary cultures [49] were cultured in Leibovitz L15 medium according to Klee et al. [50]. The comet assay with RTL-W1 cells was conducted according to the protocol developed by Singh et al. [51] and modified by Kosmehl et al. [52]. A cytotoxicity test was performed prior to the comet assay to avoid an overlap of acute toxicity and genotoxicity. The No Observed Effect Concentrations (NOEC; 10 % lethality in the cytotoxicity assays) were used as highest concentrations in the comet assays, resulting in different maximum extract concentrations. The tested extract concentration ranges were as follows: Sigmaringen, Lauchert, Riedlingen, Ingolstadt, Bad Abbach, Jochenstein: 5 - 40 mg sediment equivalent (SEQ)/ml, Schwarzach, Rottenacker, Ehingen, Öpfingen: 2.5 - 20 mg SEQ/ml. UV light (240 - 280 nm for 5 min) was used as positive control.

3.3.3 Zebrafish (*Danio rerio*) embryos

Sexually mature zebrafish (*Danio rerio*) were kept and eggs were produced according to Kosmehl et al. [45]. A fertilization rate of $\geq 80\%$ served as a quality control for adequate test materials. Eggs were exposed to native sediment samples diluted with artificial sediment (quartz powder, grain size W4, Quarzwerke, Frechen, Germany) and artificial water (according to ISO 7346/3) at 27.0 ± 0.1 °C in the dark for 72 h in 6-well plates (3 g sediment mixture, 5 ml artificial water and 5 eggs per cavity) according to the protocol of Hollert et al. [44] for the fish embryo assay. Pure quartz powder was used as a negative control. As a positive control, unexposed embryos were incubated in 0.1 % (34 mM) H₂O₂ for 1 h [45].

3.3.4 Cell isolation and comet assay

After 72 h, 7 - 8 embryos per concentration were processed for cell isolation and subsequently for the comet assay. Cell isolation was carried out mechanically according to the protocol by Kosmehl et al. [45]. Subsequent the exposure of embryos to native sediments in the fish egg assay, the No Observed Effect Concentrations (NOEC; 10 % lethality in the fish embryo assays) were used as highest concentrations in the comet assays, resulting in different maximum extract concentrations. In any case, overlapping of acute toxicity and genotoxicity had to be excluded. The tested sediment dilution and concentration ranges were as follows: Sigmaringen, Lauchert, Riedlingen: 1:1 to 1:8 or 600 - 75 mg dried sediment/ml, respectively, Jochenstein: 1:2 to 1:16 or 300 - 37.5 mg/ml, Öpfingen, Ingolstadt, Bad Abbach: 1:4 to 1:32 or 150 - 18.75 mg/ml, Schwarzach, Rottenacker, Ehingen: 1:16 to 1:128 or 37.5 - 4.69 mg/ml. The comet assay (embedding, lysis and electrophoresis) was carried out under alkaline conditions following the procedure of Singh et al. [51] with some modifications [45,53].

3.3.5 Image analysis and statistics

All slides were examined at 340 x magnification using a fluorescence microscope (Zeiss Axioskop 50, Jena, FRG) equipped with an excitation filter of 518 nm and an image analysis system (Optilas, Munich, FRG) with a grey-scale CCD camera (JAI Pulnix TM-765E Kinetic, Glostrup, DK) and the Komet 3.0 software (Kinetic Images, Liverpool, UK). For each concentration, the tail moments (i.e. the product of the fluorescence intensity in the tail and the tail length) of 100 randomly selected nucleoids were measured on two replicate slides [53].

For statistical interpretation, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 3.1; Systat, Erkrath, FRG). In cases of significant differences, a *post-hoc* test according to Dunnett ($p < 0.05$) was employed to identify groups that differed significantly. The induction factor (IF) was used to compensate for the variability between different runs and was calculated by dividing the median of each concentration by the median of the corresponding negative control [52].

3.3.6 Data presentation

For reasons of comparison, data are presented in different units:

50 % effect concentration (EC₅₀): The EC₅₀ is the (calculated) agonist concentration, which produces 50 % of the maximum effect.

Lowest Observed Effect Concentration (LOEC): The LOEC was determined as the lowest test concentration, which significantly induced DNA damage if compared to the corresponding control.

Maximum induction factor IF_{max}: The induction factor (IF) is the relative increase of the tail moment of a given agonist concentration over the respective control. The IF_{max} is the highest IF score of a particular sample obtained from the dose-response curve (irrespective of the concentration). IF_{max} values were determined for each sampling site and compared one to another.

LOEC + IF_{max}: Ranking of samples according to (1) their LOEC values and (if several LOECs were equivalent) (2) to their IF_{max} values.

“3-Step analysis”: Ranking of samples according to a 3-step procedure, taking into account all relevant information of the dose-response curve (i.e., concentrations and respective IF): (1) LOEC, (2) maximum IF in significantly genotoxic concentrations, (3) maximum IF in non-significantly genotoxic concentrations. Concerning steps (2) and (3), it is important to compare only IF values determined at identical concentrations. The result of the 3-step analysis is visualized in a three-dimensional diagram with the x-axis showing sampling sites, the y-axis giving the induction factors (IF), and the z-axis indicating agonist concentrations. This system was used as the baseline for the comparison with all other evaluation methods.

Significant concentration-dependent induction factor (SCDI): The SCDI is calculated integrating all concentrations with significantly increased genotoxicity (labeled with *) and respective induction factors according to the following term:

$$SCDI = \sum_{i=1}^{n^*} \frac{IF^*_i}{c^*_i}$$

IF^*_i induction factor of the concentration i
 c^*_i concentration i (here: 1 - 4)
 n^* number of concentrations
 $*$ significantly increased genotoxicity

Concentration-dependent induction factor (CDI): The CDI is calculated integrating all concentrations (not only with significantly increased genotoxicity) and respective induction factors according to the following term:

$$CDI = \sum_{i=1}^n \frac{IF_i}{c_i}$$

IF_i induction factor of the concentration i
 c_i concentration i (here: 1 - 4)
here: $n = 4$ concentrations

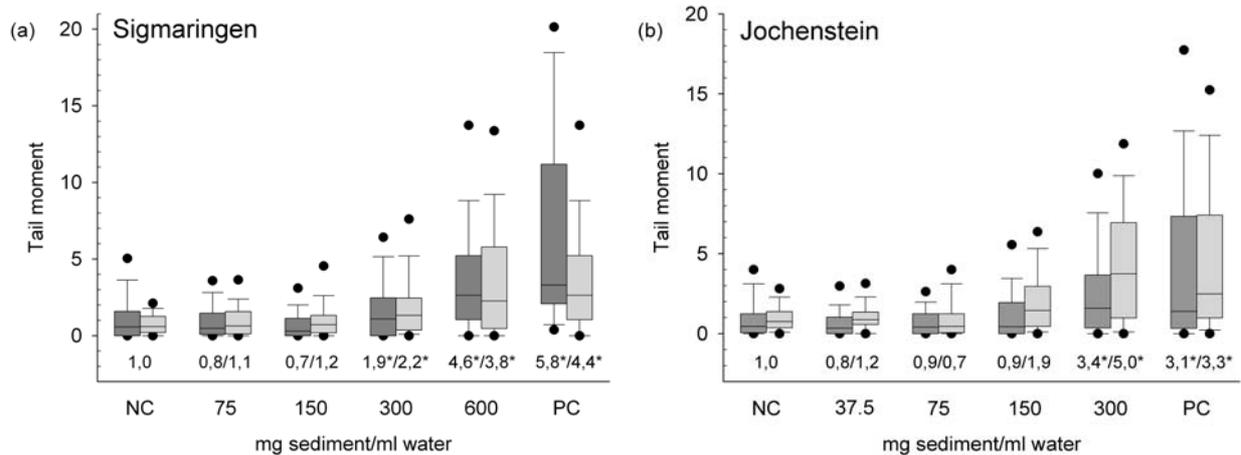
Within a given series of experiments, the number of concentrations tested (n) needs to be standardized. SCDI and CDI are index values, which represent the genotoxic potential in their heights, but they do not allow an absolute quantification of genotoxicity, since neither a maximum nor a minimum genotoxicity level can be established as a reference. For an easier comparison of data, SCDI and CDI are given at logarithmic scales.

3.4 Results and discussion

In the following sections, different methods for the evaluation of genotoxicity based on data of the comet assay with embryos from *Danio rerio* and RTL-W1 cells are presented in a comparative way.

3.4.1 Raw data

The results from comet assays both with whole sediments and zebrafish embryos and with acetone sediment extracts and the cell line RTL-W1 showed a good reproducibility and low variability between positive and negative controls in different runs (Fig. 3.2 for the zebrafish embryo comet assay).



Figs. 3.2 a, b: DNA damage expressed as tail moments (bars) and induction factors (digits) in primary cells derived from 72 h old zebrafish (*Danio rerio*) embryos exposed to two selected whole sediment samples from the Danube River in two independent runs of the comet assay (different grey shades). Genotoxicity is given as box plots of 100 cells per concentration from two replicate slides (50 cells each). The box plots show the medians, standard deviations, 25 % and 75 % percentiles (upper and lower limit of the bar), 5 % and 95 % percentiles (dots). * Significantly different from negative control (NC; Dunnett's test, $p < 0.05$). PC = positive control.

3.4.2 EC₅₀

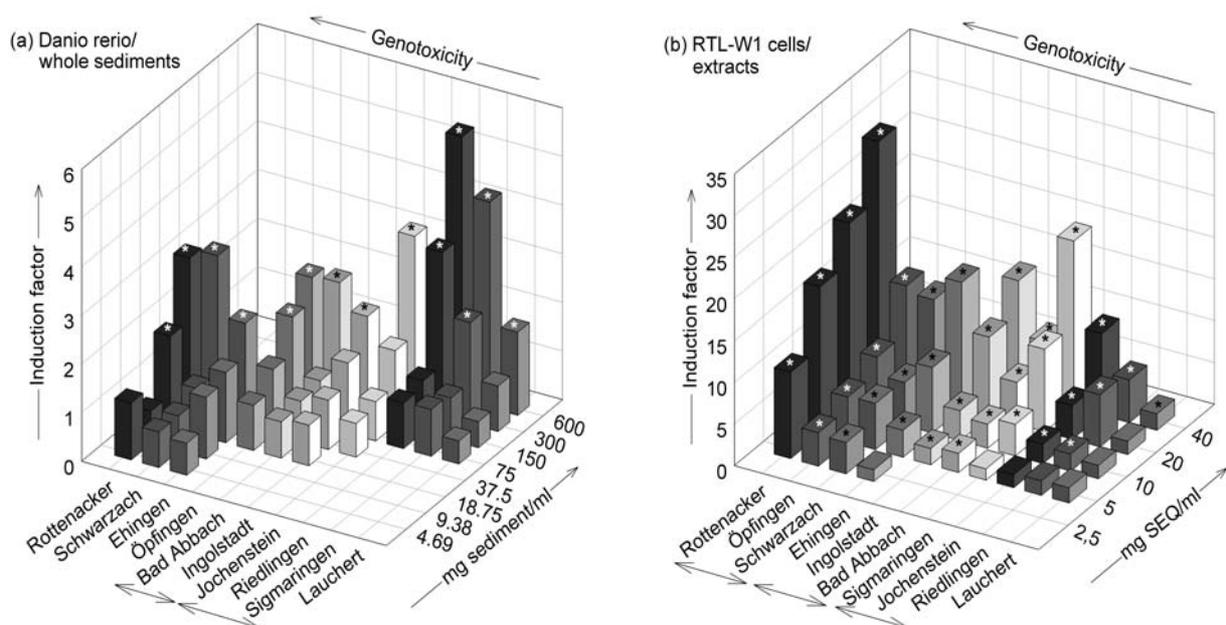
The EC₅₀ is difficult to determine in the comet assay, since it requires clear dose-response curves. Although some authors were able to show definite dose-response relationships [32,36,54], others failed to do so [55-57]. For instance, Devaux et al. [58] found that DNA single strand-breaks were less pronounced after benzo[a]pyrene (B[a]P) exposure of hepatocytes for 24 and 48 h than for 4 h. The decrease in DNA strand breaks with increasing exposure time and higher B[a]P concentration could have been due to a high degree of fragmentation of DNA and the loss of these fragments during electrophoresis. In addition, activation of the DNA repair system and modulation of the enzyme reactions producing DNA-reactive metabolites and oxidative products could have accounted for a decrease in liver DNA strand breaks after prolonged exposure [58]. Kim & Hyun [41] found similar results for longer-time exposure to B[a]P; likewise, Mitchelmore & Chipman [57] observed a decrease in the measurable DNA damage after exposure to higher concentrations of B[a]P.

Even more complicated, for EC₅₀ calculation in the comet assay, it would be necessary to establish a definite maximum effect level. Above a given concentration, the observable genotoxic effect should not increase any more. However, taking into account that the maximum concentrations applied in the comet assay depend on results of previous cytotoxicity assays (in case cells are exposed) or, as in the present study, on the fish embryo toxicity assay, it is obvious that not necessarily a full dose-response relationship with a peak

level of genotoxicity can be established, since there might be an overlap of genotoxicity with cytotoxicity or embryo toxicity, respectively. For these reasons, it appears inadequate to use pure EC_{50} values as a method of evaluation in the comet assay.

3.4.3 LOEC

The Danube sediment samples could easily be ranked according to their LOECs as a measure of genotoxicity in the two exposure scenarios (Fig. 3.3; for exact data, see Tab. 3.1). The ranking appears reasonable, but it remains incomplete, in cases where there are groups of samples with identical LOECs (indicated by double-headed arrows in Fig. 3.3). Another problem are substances with a very narrow genotoxicity window and substantial overlap with cytotoxicity (e.g. 4-nitroquinoline). Therefore, further parameters are required for adequate ranking of genotoxicity.



Figs. 3.3 a, b: Genotoxicity ranking of Danube River sediment samples according to LOEC values. (a) Whole sediments tested in the comet assay with zebrafish (*Danio rerio*) embryos. (b) Acetone sediment extracts tested in the comet assay with RTL-W1 cells. Column heights represent the induction factors (mean values of two independent runs); concentrations with significantly increased genotoxicity (post hoc test according to Dunnet; $p < 0.05$) are marked by asterisks (*). Groups of samples with identical LOECs (indicated by two-headed arrows) are arranged according to the 3-step analysis as described in the box.

Tab. 3.1: Genotoxicity data and ranking of Danube River sediment samples according to different exposure paths and evaluation methods. Sites with a grey background differ from the 3-step analysis ranking. * Two different LOEC values were measured in the two test runs (Ehingen: 37.5 and 18.75 mg/ml, Lauchert: 600 and > 600 mg/ml).

<i>Danio rerio</i> /whole sediments						RTL-W1 cells/sediment extracts	
3-step analysis	LOEC (mg sed./ml)	IF _{max}	LOEC +IF _{max}	SCDI (x 1000)	CDI (x 100)	3-step analysis	CDI (x 10)
(1) Rottenacker	(1) 18.75	(5) 3.2	(1)	(1) 183.7	(1) 50.4	(1) Rottenacker	(1) 114.0
(2) Schwarzach	(2) 37.5	(4) 3.4	(2)	(2) 89.4	(3) 37.3	(2) Öpfingen	(2) 40.6
(3) Ehingen	(3) 37.5*	(8) 2.1	(3)	(3) 56.6	(2) 40.8	(3) Schwarzach	(3) 37.3
(4) Öpfingen	(4) 75	(7) 2.6	(4)	(4) 44.6	(4) 12.9	(4) Ehingen	(4) 27.5
(5) Bad Abbach	(5) 150	(6) 2.6	(5)	(6) 17.4	(6) 9.2	(5) Ingolstadt	(6) 15.6
(6) Ingolstadt	(5) 150	(9) 2.1	(6)	(8) 13.8	(5) 10.4	(6) Bad Abbach	(7) 11.7
(7) Jochenstein	(7) 300	(3) 3.5	(9)	(9) 11.8	(7) 5.1	(7) Sigmaringen	(5) 16.4
(8) Riedlingen	(7) 300	(1) 5.1	(7)	(5) 20.2	(8) 4.0	(8) Jochenstein	(8) 9.9
(9) Sigmaringen	(7) 300	(2) 4.2	(8)	(7) 14.0	(9) 3.3	(9) Riedlingen	(9) 9.9
(10) Lauchert	(10) 600*	(10) 1.7	(10)	(10) 2.8	(10) 1.6	(10) Lauchert	(10) 6.6

3.4.4 IF_{max}

Fig. 3 provides information not only about the LOEC of the different sediment samples, but also about the induction factors relative to concentrations. It is obvious that ranking the sampling sites according to their maximum induction factors (Tab. 3.1) is not appropriate, since it ignores the concentration the IF_{max} has been calculated for. For example, the highest IF_{max} values were determined for samples collected at Riedlingen, Sigmaringen and Jochenstein, three sediments with a high LOEC and therefore probably low genotoxicity. However, these IF_{max} values were measured at relatively high sediment concentrations. In other sediment samples, intermediate IF_{max} values (e.g. Schwarzach, Rottenacker, Öpfingen) were measured at up to 16-fold higher dilutions; in other words, samples with relatively low IF_{max} may be more toxic than samples with lower IF_{max}.

Higher genotoxicity, i.e. higher tail moments and higher induction factors, can be obtained when lower dilutions are tested. However, as already mentioned, test concentrations applied in the genotoxicity assay depend on previously performed cytotoxicity or fish egg assays. Therefore, acute mortalities in cell tests or embryo toxicities indirectly influence the values of IF_{max}.

3.4.5 LOEC + IF_{max}

In order to take into consideration the concentration-dependency of sediment toxicity, sediments were ranked according (1) the LOEC *and* (2) the IF_{max}. Thus, a refinement of the relatively imprecise approach based on the LOEC only can be achieved. Nevertheless, methods based on the IF_{max} ranking are always questionable, as they also include information about toxicity other than genotoxicity, if the values compared are obtained from different concentrations.

3.4.6 3-Step analysis

As a descriptive method, the “3-step analysis” takes into account all bits of information that can be drawn from the dose-response curves of all samples. Therefore, it allows a comprehensive comparison of the genotoxic potential. The crucial point of this ranking is that only induction factors measured at the same sediment concentration are compared to each other.

3-Step analysis:

- (1) LOEC
- (2) Maximum IF in significantly genotoxic concentrations
- (3) Maximum IF in not significantly genotoxic concentrations

Given its optimal representation of the genotoxic potential, the 3-step analysis was selected as a baseline for the comparison with the other methods (Tab. 1). Ranking according to the LOEC shows a good correlation with the 3-step analysis, but samples cannot be arranged definitely within certain groups. In contrast, IF_{max} ranking hardly shows any congruence with the 3-step analysis. Sediments with relatively low toxicity may be scored top due to high IF_{max} values at low dilutions. Combining LOEC and IF_{max}, the correlation with the 3-step analysis increases for the sediments tested in this study. However, since different maximum concentrations may have been tested for samples with the same LOEC (e.g. Riedlingen and Jochenstein), the validity of this method may be limited.

The only problem of the 3-step analysis consists in the relatively complicated three-dimensional representation and bad comparability of different samples due to the fact that there is no possibility to quantify genotoxicity. To overcome these disadvantages, the “significant concentration-dependant induction factor” (SCDI) and the “concentration-dependant induction factor” (CDI) were developed as two novel approaches of analysis and compared to the logical, but more complicate 3-step analysis.

3.4.7 Significant concentration-dependant induction factor (SCDI)

The SCDI ranking (Fig. 3.4) correlates quite well with the 3-step analysis (Tab. 3.1). However, since it only takes into account significantly genotoxic concentrations, it is possible that a different number of concentrations are included in the calculation of the SCDI, thus potentially leading to misjudgment of genotoxicity in certain cases. This is why in the present study the genotoxic potential of the Riedlingen sediment (two significant concentrations) is overestimated, if compared to other mid-ranking sediments, e.g. Bad Abbach, Ingolstadt and Jochenstein (only one significant concentration). By defining artificial boundaries between concentrations with significantly increased genotoxicity and such without, the former are overvalued, and the continuous progression of the dose-response curve is not reflected.

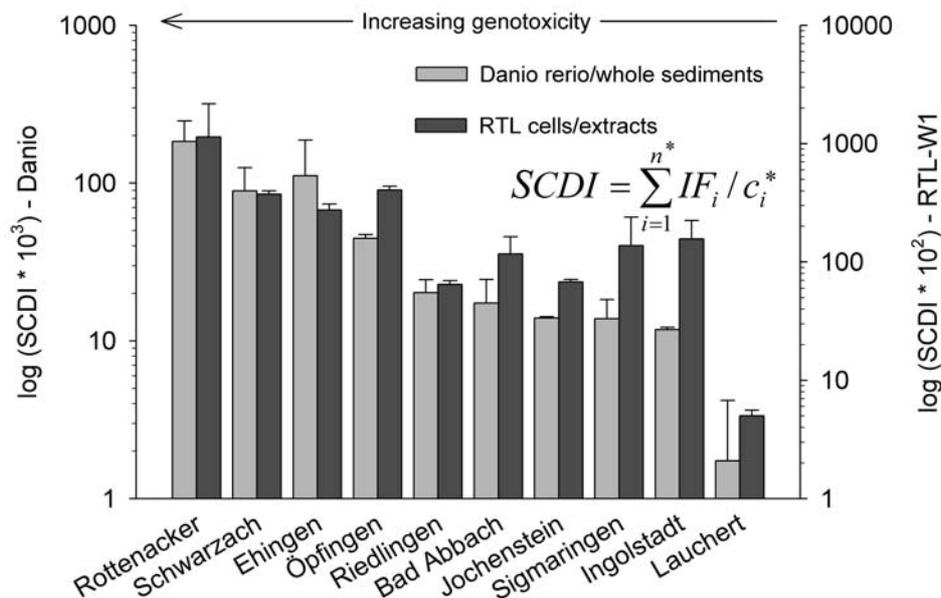


Fig. 3.4: Genotoxicity ranking of Danube River sediment samples according to significant concentration-dependant induction factor (SCDI) values (mean values \pm S.D. of two independent runs) comparing the different exposure paths. Basis of the ranking is the genotoxicity in the whole sediment testing with zebrafish (*Danio rerio*) embryos (light bars); the ranking of the sediment extract testing with RTL cells (dark bars) was adjusted.

Concentration-dependant induction factor (CDI)

The CDI (Fig. 3.5, Tab. 3.2) was developed as a simple index value which integrates all important information, providing a basis for a general comparison of the genotoxic potential in the comet assay.

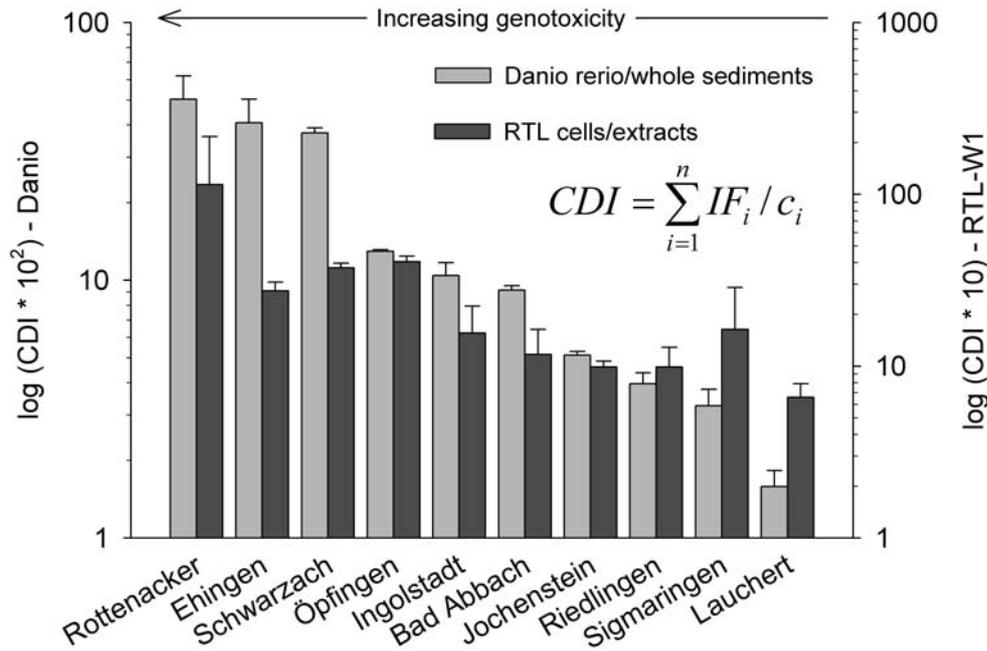


Fig. 3.5: Genotoxicity ranking of Danube River sediment samples according to concentration-dependant induction factor (CDI) values (mean values \pm S.D. of two independent runs) comparing the different exposure paths. Basis of the ranking is the genotoxicity in the whole sediment testing with zebrafish (*Danio rerio*) embryos (light bars); the ranking of the sediment extract testing with RTL-W1 cells (dark bars) was adjusted.

Tab. 3.2: Advantages and disadvantages of the concentration-dependent induction factor (CDI).

Advantages	Disadvantages
<ul style="list-style-type: none"> • More realistic than IF_{max} ranking • More comprehensive and precise than LOEC ranking • Can be calculated easily using data from dose-response curves • Considers induction factors and concentration-dependency • Considers whole dose-response curve (not only concentrations significantly different from controls) • Simple acquisition as an index value • Good comparability and interpretation 	<ul style="list-style-type: none"> • Application of same number of test concentrations required • Selection of test concentrations according to cytotoxicity or embryo toxicity tests required • No distinction between significantly and not significantly genotoxic concentrations • No absolute quantification of genotoxicity • Potential overestimation of lower concentrations

The CDI ranking correlates even better than the SCDI with the 3-step analysis (Tab. 3.1); in only two cases, two consecutive sediments are interchanged (Ehingen and Schwarzach, Ingolstadt and Bad Abbach). Upon a closer inspection of Fig. 3.3, however, it becomes evident that the samples in question do not differ much with respect to their genotoxicity. In the case of the Bad Abbach and Schwarzach sediments, the induction factor in the maximum (and significantly genotoxic!) concentration is higher than in the corresponding sediment dilution from Ingolstadt and Ehingen, respectively. In the rest of the (not significantly genotoxic!) concentrations, the induction factors in the latter samples are higher than in the former ones. Whereas the 3-step analysis overemphasizes the significantly genotoxic concentrations, the CDI considers all concentrations equally. Thus, an exchange of these sites in the ranking list seems tolerable.

As in the sediment contact test (zebrafish embryos), the CDI ranking reflects well the 3-step analysis in the extract exposure test (RTL-W1 cells; Tab. 1). Furthermore, a good correlation could be demonstrated between the two exposure paths. In both test systems, a very similar genotoxicity pattern along the river with highly genotoxic local hotspots could be observed (Fig. 3.5). Within groups of similar toxicity, ranking does not vary much between the two exposure paths. The correlation analysis (Fig. 3.6) also shows a good correlation ($r_{\text{Spearman}} = 0.81$ with $p < 0.05$ and $r_{\text{Pearson}} = 0.79$ with $p < 0.05$).

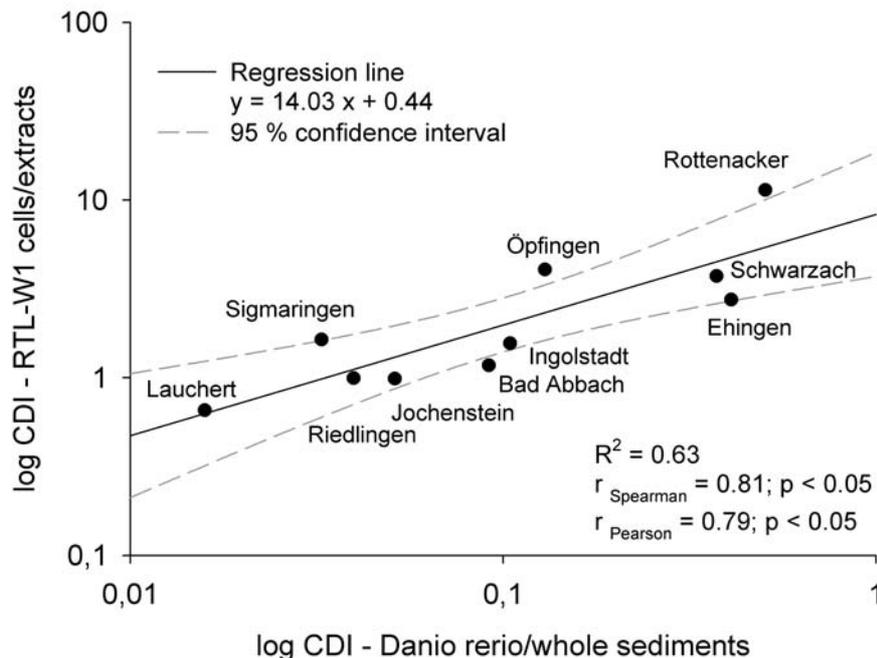


Fig. 3.6: Correlation analysis for two CDI values derived from experiments based on two exposure paths in the comet assay: primary cells from zebrafish (*Danio rerio*) embryos exposed to whole sediments *versus* RTL-W1 cells exposed to acetone sediment extracts.

3.5 Conclusions

The use of the comet assay in this field study provided clear evidence of genotoxic contamination in sediments from the upper Danube River, yet with a wide variability between the different sampling sites. The genotoxic potentials of the sediments tested potentially explain at least part of the total ecotoxicological burden. The genotoxic effects may serve as one explanation for the observed fish declines. However, further reasons need to be explored.

To the best of our knowledge, the present study is one of the first comprehensive field applications of the sediment contact comet assay with *Danio rerio* embryos in river sediment testing. It corroborates the conclusion by other authors that the comet assay is an appropriate bioassay for the assessment of the genotoxic potential of aquatic samples. If possible, the comet assay with whole sediments should be performed as one line of evidence in the context of an integrated weight-of-evidence approach [14,59] contributing important data to understand the interacting and overlapping effects in the contaminated ecosystem.

The present study suggests the 3-step analysis as an adequate tool for the comprehensive analysis of comet data. This descriptive method takes into account all information of the dose-response curves of the different samples. With the concentration-dependent induction factor (CDI), an index value could be introduced which is adequate for a straightforward, precise and realistic assessment of the genotoxic potential integrating level of effectiveness and information from the dose-response curves. It can be used for an easy comparison of different environmental samples or genotoxic agents. Nevertheless, further research and a broader data base are needed for a better adaptation of the CDI.

3.6 Acknowledgements

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The Tietê River in Brazil

The Tietê River is located within the Brazilian state of São Paulo. Even though its spring in Salesópolis (Serra do Mar) is located only 22 km from the ocean, the geographical conditions are forcing the river to run for 1130 km towards the centre of the continent before joining the Paraná River.

The Tietê River, crossing São Paulo city, is one of the most polluted rivers in the world, due the insufficiency of treatment plants and many industrial sources for a multitude of anthropogenic pollutants. The Tietê River is inserted in the Tietê River Basin, the largest hydrographical basin of São Paulo State ¹. This basin is subdivided into three sub-basins, because of its distinct geomorphologic characteristics: Upper Tietê, Middle Tietê and Lower Tietê ². The Middle Tietê River basin is then subdivided in Superior Middle Tietê and Inferior Middle Tietê. The water supply system provides about 60 m³ s⁻¹ for this area, about 80 % of which is returned untreated to the main water courses ³. Therefore, one of the largest environmental projects in Latin America, the Tietê Project, was launched in 1991. It has aimed at expanding the wastewater collection and treatment in the 8000 km² metropolitan region of São Paulo with a view to reducing the disposal of pollutants into rivers and creeks and to improving water quality ³. With intense participation from non-governmental organisations, the level of wastewater treatment increased from 24 % in 1992 to 65 % in 1998 ⁴. Despite this heavy pollution, little is known about the biological hazard potential of the river or the substance and sites of concern.

¹ Rocha et al. 2009

² Brocanelli, P., 1998. A incorporação da água no meio ambiente da cidade de São Paulo. Dissertação de mestrado, Universidade Presbiteriana Mackenzie, Faculdade de Arquitetura e Urbanismo, São Paulo.

³ WHO, 1997. Case Study VI* - The Upper Tietê Basin, Brazil in Water pollution control: a guide to the use of water quality management principles. Editors R. Helmer and I. Hespanhol

⁴ Davey, Tom, Ed. (2004). River Tietê Project improving water quality for over 17 million Brazilians. Aurora, Ontario, Canada Steve Davey.

Chapter 4

Sediment genotoxicity in the Tietê River (São Paulo, Brazil): *in vitro* comet assay versus *in situ* micronucleus assay studies

Authors: Paula Soares Rocha, George Luiz Luvizotto, Thomas Kosmehl, Melanie Boettcher, Volker Storch, Thomas Braunbeck, Henner Hollert

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4.1 Abstract

The *in vitro* comet assay with the permanent fish cell line RTL-W1 and the *in situ* micronucleus assay using erythrocytes from indigenous tilapia (*Oreochromis niloticus*) were used to detect genotoxicity in Tietê River sediments (São Paulo, Brazil). Either test was successful in identifying site-specific differences in genotoxicity, with a high correlation between *in situ* and *in vitro* results indicating the relevance of the latter even for environmental studies. Discharges from São Paulo city have major impact on genotoxic effects by sediment-bound contaminants; however, overall genotoxicity decreases downstream. The high genotoxic burden of the Tietê River warrants measures to reduce the input of toxic effluents.

Keywords: comet assay, fish erythrocytes, genotoxicity, micronucleus assay, sediments, RTL-W1 cell line, Tietê River, Brazil, effluent

4.2 Introduction

Reservoirs are complex aquatic systems mediating between rivers and lakes; they usually reflect multiple impacts generated by a variety of anthropogenic activities (Tundisi et al. 1999). The sediment compartment is the intermediate or final receptor of insoluble (or slightly water soluble) pollutants and can act as a sink for various substances. Sediments accumulate chemicals up to concentrations many times higher than free water column (Ahlf et al. 2002a, Baudo and Muntau, 1990, Burton 1991, Fracácio et al. 2003, Hollert et al. 2002). As pollutants may be made available under certain environmental conditions (such as dredging or flood events), sediments can also become a source of diffuse contamination to the free water space (Ahlf et al. 2002b, Hollert et al. 2003). Sediment pollutants are not only linked to organisms in aquatic ecosystems, but also to human health *via* water and fish consumption (Chen and White 2004, Hollert et al. 2005, Keiter et al. 2006, Maier et al. 2006).

Since fish represent vertebrates, which are top predators in the food web, considerable efforts have been undertaken to develop fish-based test systems for the assessment of sediment-bound substances (Hollert et al. 2003, 2005, Kosmehl et al. 2004). Due to their ability to metabolize xenobiotics and accumulate pollutants, fish represent important monitoring systems within aquatic genotoxicity assessment (Balch et al. 1995, Grisolia and Cordeiro 2000, Metcalfe et al. 1990, Minissi et al. 1996). Recently a number of laboratory investigations and field studies have documented a correlation between genotoxic pollutants

and heritable reproduction effects on individuals as well as a potential link to the declines of fish populations (for reviews, see Chen and White 2004 as well as Keiter et al. 2006).

Passing through São Paulo city and São Paulo state in Brazil, the Tietê River is one of the most polluted rivers in the world due to the insufficiency of effluent treatment and numerous direct industrial sources for a multitude of anthropogenic pollutants. Despite this heavy pollution, little is known about the biological hazard potential of the mixture of chemicals in its waters and sediments. The Tietê River is embedded in the Tietê River Basin, the largest hydrographical basin of São Paulo State. This basin is subdivided into three sub-basins due to distinct geomorphologic characteristics: Upper, Middle and Lower Tietê (Brocanelli 1998). The river comprises several reservoirs along its course, which are widely used for providing drinking water, as a water source for agricultural irrigation, as receptors of domestic and industrial effluents, and as recreation sites. Given the extent of the pollution and the importance of the river, research into the origin and effects of pollutants and subsequent suggestions for the ecological improvement of the river basin quality might become an important model for further research and biological risk assessment of highly contaminated river systems not only in Brazil, but throughout the world.

In this study, two bioassays were applied to detect the genotoxic potential of the Tietê River basin: the *in vitro* version of the comet assay with a permanent cell line, and the *in situ (in vivo)* version of the micronucleus assay with erythrocytes from fish collected in the field. The *in vitro* approach serves as an efficient, fast and cost-effective screening for the evaluation of the potential biological activities of the complex mixtures (Hilscherova et al. 2002). However, since various factors (chemical, physical and biological) affect environment conditions, the transfer of results obtained by *in vitro* techniques to the field is a complex task, and the establishment of extrapolation parameters is a crucial issue. For this end, a combination of *in vitro* and *in situ (in vivo)* bioassays represents a promising approach, since *in situ* bioassays are more likely to reflect the real exposure situation in the environment; applied in combination with *in vitro* bioassays, the *in situ* assays may be used to confirm or falsify the potential toxicity revealed by the *in vitro* bioassays. In fact, the combination of comet and micronucleus assays has been applied successfully to detect genotoxic and mutagenic potentials in several environmental monitoring studies (Böttcher et al. in press, Bolognesi et al. 2004, Buschini et al. 2003).

Thus, the aims of this study were (1) to find out possible interactive genotoxic and mutagenic effects from multiple contaminants, since the Tietê River receives a highly complex load of

organic pollutants from domestic sewers, industrial residues, agricultural and agroindustrial activities, as well as numerous inorganic substances of industrial sources, and (2) to elucidate, by comparison of *in vitro* and *in situ (in vivo)* approaches, the ecological relevance of *in vitro* results and their ability as possible bioindicator systems. In contrast, this study did not attempt to determine which contaminants were responsible for the genetic damage.

4.3 Materials and methods

4.3.1 Sediment sampling

The study area comprised a location in Salesópolis near the Tietê River's spring and the reservoirs Ponte Nova and Billings (Upper Tietê); Barra Bonita (Superior Middle Tietê); Bariri and Promissão (Inferior Middle Tietê) and Três Irmãos (Lower Tietê; Fig. 4.1). The Upper Tietê River basin corresponds to the drained area of Tietê River from its spring across the metropolitan area of São Paulo city (> 19,000,000 inhabitants) characterized by high densities of anthropogenic population and dramatic deterioration subsequent to intensive urbanization. The Superior Middle Tietê River basin is dominated by a high density of urban, industrial and agricultural areas. The Inferior Middle Tietê River basin is characterized predominantly by agricultural areas and the Low Tietê river basin by pastures and sugar cane culture (CETESB 1997).

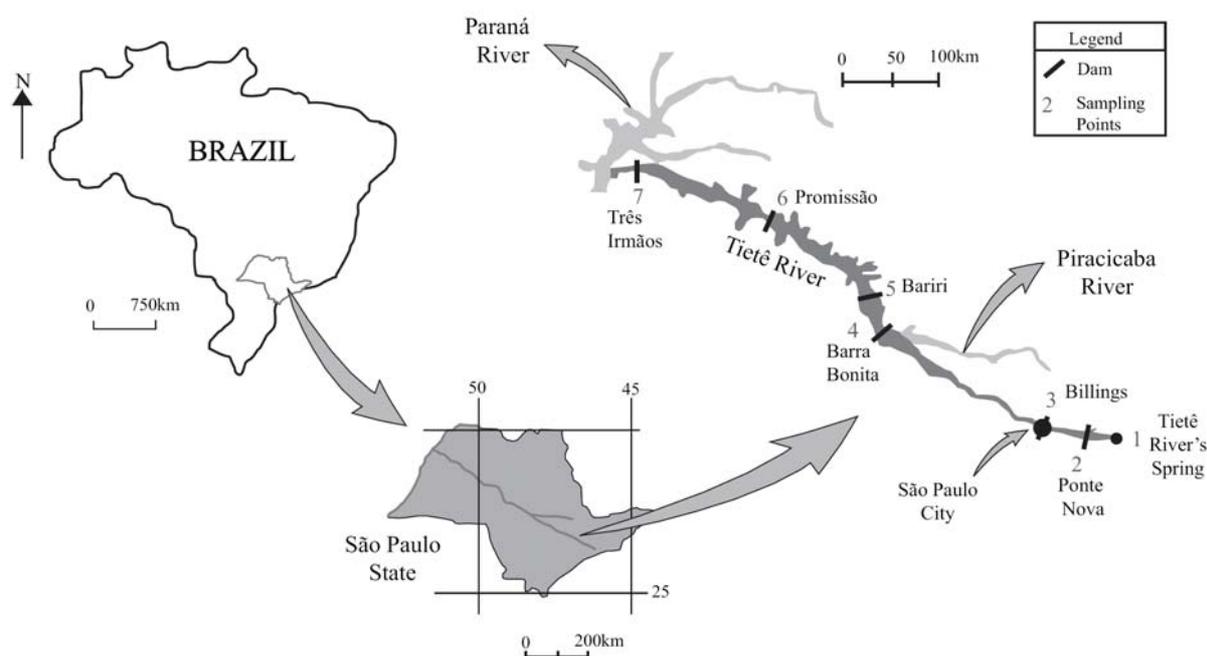


Fig. 4.1: Location of the sampling sites in the Tietê River basin, São Paulo, Brazil.

Surface sediments were collected in May and December 2005 by means of an Eckman-Birge dredge, with ten replicates at each site (with a distance of 10m from sample to sample). Replicates were homogenized, and 1.5 kg of each sediment sample were frozen immediately, stored at -10°C and transported to Germany. Transfer of the samples to Germany was permitted by the Brazilian National Department of Mineral Production (DNPM). Samples were freeze-dried, and extracts were prepared by Soxhlet extraction using acetone p.a. as solvent and re-dissolved with dimethyl sulfoxide (DMSO; Sigma-Aldrich, Deisenhofen, Germany) as described by Hollert et al. (2000). The resulting concentration of extracts was 20 g dry sediment equivalent per 1 ml solvent.

4.3.2 Cell culture

The fibroblast-like permanent cell line RTL-W1 (Lee et al. 1993) derived from rainbow trout liver (*Oncorhynchus mykiss*) was used to perform the comet assay. According to Kosmehl et al. (2004), RTL108 lls are able to detect the genotoxic potential of acetonic sediment extracts. Furthermore, RTL-W1 cells have relatively high biotransformation capacities, if compared to other fish cell lines such as RTG-2 cells (Kosmehl et al. 2004).

The cells were maintained in 75 cm² culture flasks (TPP, Trasadingen, Switzerland) in Leibowitz (L15) medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1 % penicillin/streptomycin solution (10,000 U/10,000 □m) in 0.9 % NaCl (Sigma-Aldrich) at 20 °C (Seiler et al., 2006). They were trypsinized using 0.05 % trypsin/ 0.02 % EDTA and washed twice with PBS before being used in experiments (Kosmehl et al. 2004).

4.3.3 Comet assay

The comet assay was performed under alkaline conditions following the procedure of Singh et al. (1988) in the modification by Schnurstein and Braunbeck (2001) as well as Kosmehl et al. (2004), using acetonic sediment extracts.

In order to determine the highest test concentrations to be used for *in vitro* exposure of RTL-W1 cells, the cytotoxic potential of individual extracts was determined using the neutral red assay as detailed by Babich and Borenfreund (1992) with modifications described by Klee et al. (2004). The highest test concentrations were defined as the extract concentrations inducing less than 20 % mortality after 48 h of exposure. Cells were exposed to four sequential

concentrations of each sediment extract, in serial dilutions from 1:1 to 1:8 of the highest concentrations in supplemented L15 medium (Sigma-Aldrich; Table 4.1). Each extract was tested in 6-well plates (TTP Renner) after 24 h settlement of the cells (Kosmehl et al. 2004) for 48 h at 21 °C in three independent replicates. Supplemented L15 medium served as a negative control; exposure to UV light at 240 - 280 nm for 5 min was used as a positive control.

Tab. 4.1: Highest test concentrations of sediment extracts used in the comet assay, given in sediment equivalents (mg SEQ) per ml medium.

Sampling area	(mg SEQ/ml)
Spring	200
Ponte Nova	22
Billings	12
Barra Bonita	152.6
Bariri	31
Promissão	19.7
Três Irmãos	24.5

After the incubation, cells were rinsed with PBS, trypsinized and embedded in an agarose layer on fully frosted microscope slides (Langenbrink, Emmendingen, Germany) as detailed by Kosmehl et al. (2004). Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide (Sigma- Aldrich) and covered-slipped. Slides were examined using a fluorescent microscope with 340 x magnification (Axioplan, Zeiss, Germany) equipped with an excitation filter of 518 nm and an image analysis system (Optilas, Munich, Germany), and cell images were recorded with a high sensitivity CCD camera (Pulnix TM-765E Kinetic; Germany). For each concentration, 100 cells were scored and a computerized image-analysis system (Comet Version 5.5, Kinetic Images, Liverpool, UK) was used to determine DNA tail moments (tail length x fluorescence intensity in the tails). Induction factors were calculated by the comparison of median values of tail moments from exposed cells to median values of tail moments from corresponding negative controls. ANOVA-on-ranks followed by a post-hoc test according to Dunn's ($p < 0.05$) was used to calculate significant statistical differences between groups. After this, maximum induction factors per mg SEQ/ml (IF/(mg SEQ/ml)) were computed. Since the final sediment concentrations (SEQ) differed between samples according to the corresponding LOECs for cytotoxicity, the maximum induction factor induced by the sample was divided by the concentration inducing this effect. Thus, maximum

induction factors (IF) per mg SEQ/ml were calculated for each sample in order to allow direct comparison of genotoxic potentials of samples from different sites.

In order to take into account the concentration-dependency of the sediment genotoxicity, we also applied the Concentration-dependent induction factor (CDI), developed by Seitz and co-workers (Seitz et al. 2008). The CDI is a simple index that integrates all important information, providing a basis for a general comparison of the genotoxic potential in the comet assay. The CDI integrates all concentrations and respective induction factors and is calculated according to the following equation:

$$CDI = \sum_{i=1}^n \frac{IF_i}{c_i} \quad \begin{array}{l} \text{where } IF_i = \text{induction factor of the concentration } i \\ c_i = \text{concentration } i \\ n = n \text{ concentrations} \end{array}$$

4.3.4 Fish blood sampling and micronucleus assay

The present study did not involve any animal experiments, since all experimental work was carried out *in vitro* (cell cultures). Sampling at the Tietê River was carried out in compliance with Brazilian conservation regulations. Field fish were sacrificed immediately after catch and never subjected to experimental manipulation.

Fish blood samples were collected from mature Nile tilapia (*Oreochromis niloticus*), an African fish introduced into Brazil in the 1970s. According to Ueng and Ueng (1995), different species of tilapia may serve as biological indicators for environmental pollution partially because the fish survive in highly polluted habitats. This species was selected due to its abundance in the Tietê River and the fact that it has repeatedly been used as a source of cells for the micronucleus test (Bücker and Conceição 2004, Cavas and Ergene-Gözükara 2005, Grisolia and Cordeiro 2000, Palhares and Grisolia 2002, Ventura et al. 2008). Moreover, tilapia is intensively used for human consumption in Brazil. At two sites (Spring and Três Irmãos reservoirs), no fish blood could be collected, because it was not possible to catch any *Oreochromis niloticus*. Since no fish blood could be collected from the original reference site, based on previous studies (Mozeto et al. 2004), Promissão reservoir was selected as a complementary reference site in order to allow the comparison between sites. Peripheral blood samples were obtained with heparinized syringes from the gills of ten fish per site. The blood was immediately smeared onto microscope slides previously cleaned with 99% ethanol. After drying, samples were fixed in methanol for at least 1 minute and subsequently stained with pure Giemsa. For conservation, slides were covered with cover slips by DEPEX (Serva, Heidelberg, Germany). Two thousand erythrocytes were examined

per fish on coded slides (Fig. 4.2). Micronuclei were recorded using a light microscope (Axioplan, Zeiss) equipped with an oil-immersion lens at 1,200x magnification. For the identification of micronuclei, the following scoring criteria were used: a) cells with oval appearance and intact cytoplasm, b) oval nuclei with intact nuclear membrane, c) micronuclei less than or equal to one third the size of the main nuclei, d) micronuclei clearly separated from the main nuclei (Huber et al. 1983, Titenko-Holland et al. 1998). Results were recorded as percentage of cells containing micronuclei compared to the total number of cells counted. Statistical significances were assessed by using the Chi-square-test with Yates' correction (ISO/DIS-21427-2, Lovell et al. 1989) using SigmaStat 3.5 (SPSS-Jandel Scientific; Erkrath, FRG).

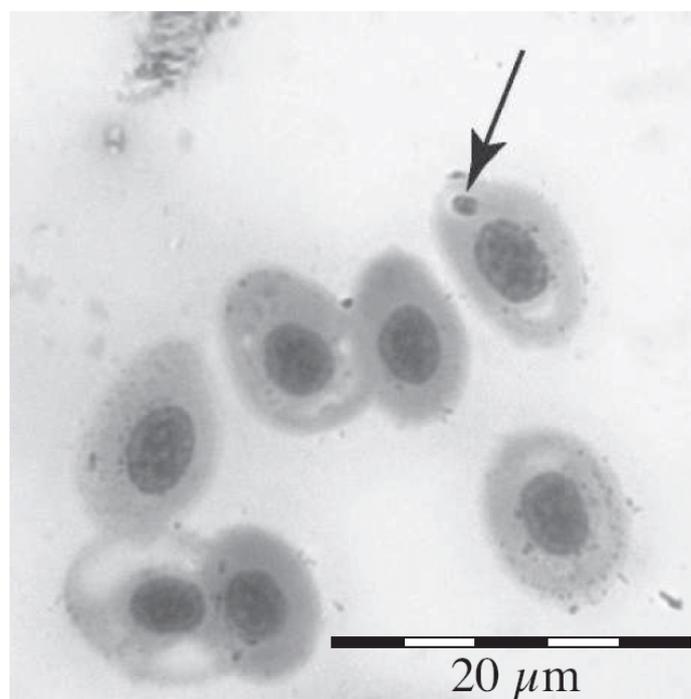


Fig. 4.2: Erythrocytes from *Oreochromis niloticus* collected in Billings reservoir, stained with Giemsa. Note micronucleus at arrow.

4.4 Results

4.4.1 Comet assay

For most sampling locations, the comet assay with RTL-W1 cells documented genotoxic effects with a positive dose-response relationship (Fig. 4.3, showing only data from two sites, near spring and Billings reservoir). Strong genotoxicity was detected in sediments from Billings reservoir near to São Paulo city from concentrations of 1.5 mg SEQ/ml, whereas the reference site near the spring had significant effects only at concentrations ≥ 100 mg SEQ/ml.

Ponte Nova samples showed significant effects at concentrations of ≥ 11 mg SEQ/ml. In sediments from Barra Bonita, Promissão and Três Irmãos

reservoirs, significant effects were detected in one replicate only, at concentrations ≥ 38.2 , 2.46 and 24mg SEQ/ml, respectively. Samples from the Bariri reservoir showed significant effects at concentrations of 7.8mg SEQ/ml; however, no effects were seen at any higher concentration.

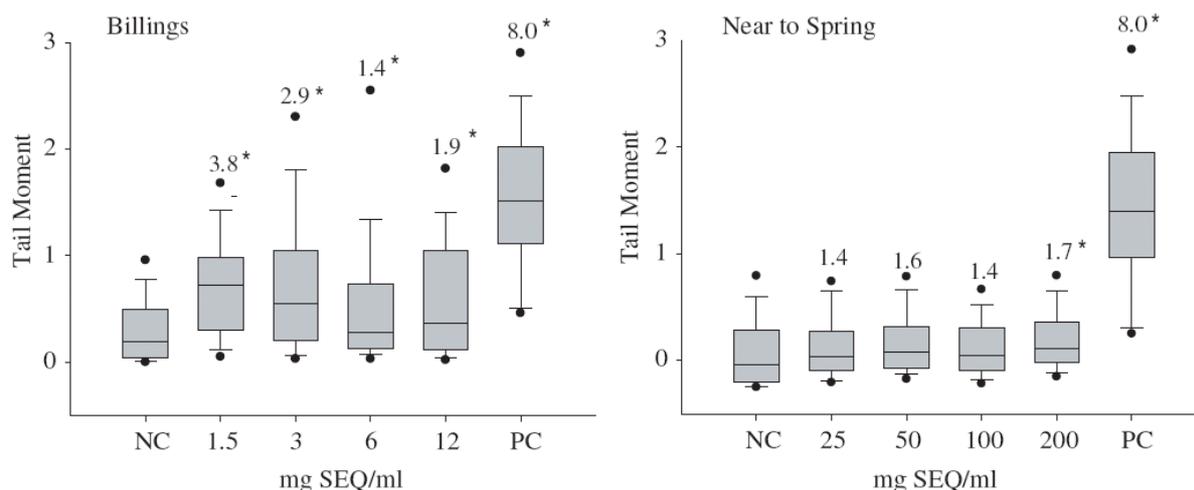


Fig. 4.3: Genotoxic effects of acetic sediment extracts from Billings reservoir (left) and near to the Tietê River spring (right), respectively, in the comet assay using RTL-W1 cells. Each box plot presents the tail moments of four different concentrations of extracts given in sediment equivalent (mg SEQ)/ml medium, as well as negative (NC) and positive controls (PC). Additionally, induction factors are indicated above each box plot. Significant genotoxic effects (post-hoc test according to Dunn; $p < 0.05$) are indicated by asterisks.

Maximum induction factors per mg SEQ/ml

For most of the samples, the highest genotoxicity was recorded for the 1:4 dilution of the highest test concentration. Therefore, maximum induction factors per mg SEQ/ml were calculated according to these concentrations. The analysis of the induction factors revealed an increase in the genotoxic potential from the spring to Billings reservoir followed by an abrupt decrease in Barra Bonita reservoir. Extracts from Barra Bonita showed the lowest genotoxic effects, comparable to those of the reference site (near spring). The locations downstream Barra Bonita showed an increase in their genotoxic potential again, indicating additional sources of genotoxic acting substances. From Promissão to Três Irmãos reservoir, a minor decrease in the genotoxic potential was recorded (Fig. 4.4).

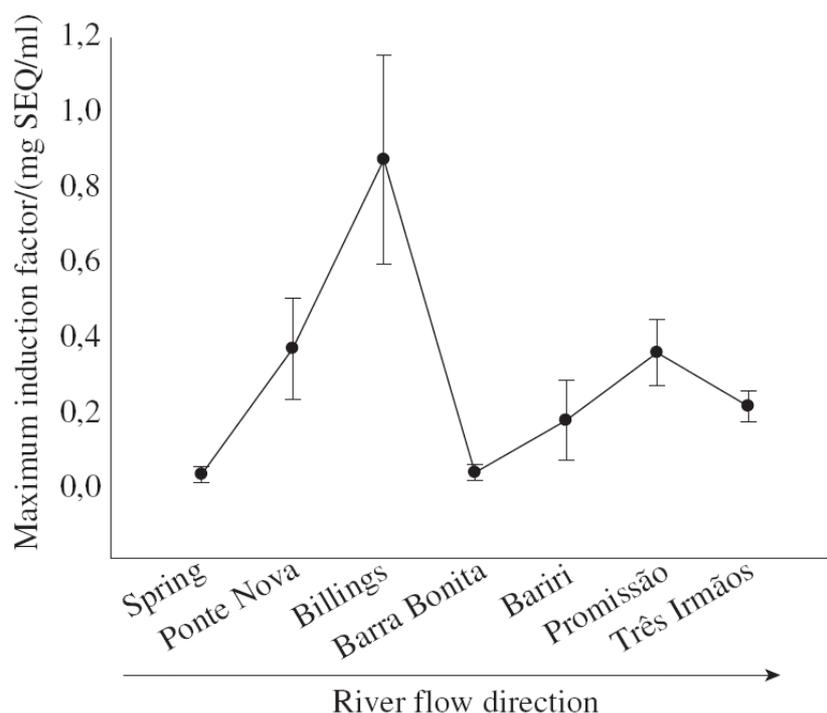


Fig. 4.4: Genotoxicity of sediment samples collected at different locations along the Tietê River in the comet assay, given as maximum induction factors in mg SEQ/ml (amounts of extract given in sediment equivalents, SEQs) for the 1:4 dilutions of the highest test concentrations. Highest IF/(mg SEQ/ml) could be observed in cells exposed to Billings reservoir samples, and a decrease of effects was evident in downstream direction. Data are given as means \pm SD from three independent experiments.

Concentration-dependant induction factor (CDI)

Fig. 4.5 gives a survey on the genotoxicity of the results presented as CDI factors of all locations based on three independent replicates. The CDI confirms that samples from near Tietê River's spring and Barra Bonita reservoir showed very low genotoxic effects (CDI values 0.14 and 0.17 respectively), followed by Três Irmãos and Bariri reservoirs (CDI values 0.64 and 0.72 respectively) and Ponte Nova e Promissão reservoirs (CDI values 1.10 and 1.33 respectively). The genotoxic potential of sediments from Billings reservoir samples becomes even more prominent than if expressed as maximum induction factors (CDI values 4.32, Fig. 4.4). Overall, however, values for CDI and IF closely paralleled each other.

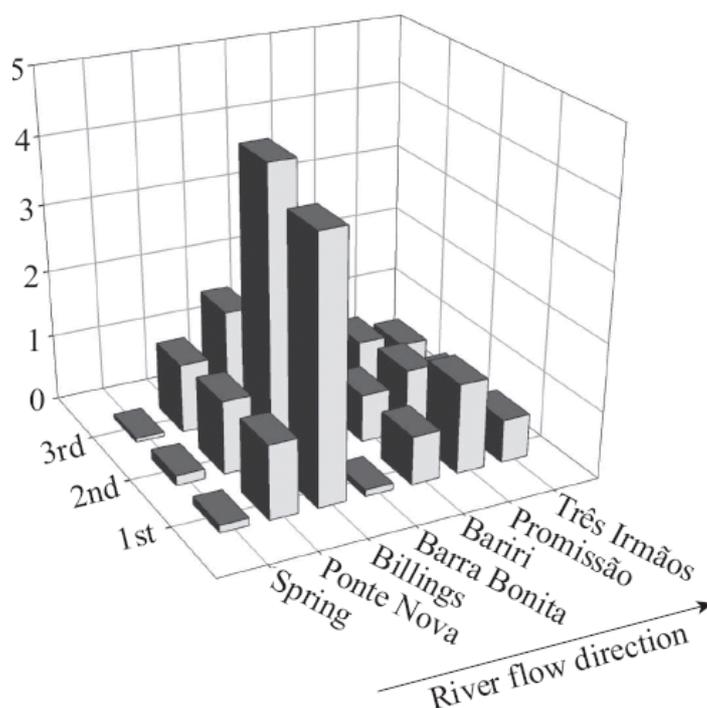


Fig. 4.5: Concentration-dependant induction factors (CDI; Seitz et al. 2008) of sediment samples collected from the Tietê River basin. Data are given as means \pm SD from three independent determinations. If compared to the maximum induction factors (Fig. 4), the differences in genotoxicity between Billings reservoir and the other sampling sites becomes even more prominent.

4.4.2 Micronucleus assay

In order to test the mutagenic potentials *in situ* (*in vivo*), the micronucleus test was applied to erythrocytes from Nile tilapia caught in the Tietê River (Fig. 6). For statistical analysis (Lemos et al. 2007), 2000 erythrocytes for each fish per location ($n = 4 - 9$) were analyzed. Due to the influence of the megacity of São Paulo, fish collected from Billings reservoir revealed by far the highest micronucleus frequencies with a median of 6.0 ‰. Clear-cut decreases in micronucleus frequencies were observed downstream from São Paulo. Bariri and Promissão reservoirs had the lowest micronucleus frequencies, with medians of 1.5 and 0.5 respectively.

The micronucleus frequencies significantly differed between individuals caught in the same area: although Ponte Nova and Barra Bonita reservoirs both showed median micronucleus frequencies of 3.5, blood samples from individuals collected at Ponte Nova varied in micronucleus frequency between 0 and 13, whereas fish from Barra Bonita only showed a range between 5 and 9. According to the Chi-square test, significant differences to Promissão, as a reference location with low contamination, could be observed for Ponte Nova, Billings and Barra Bonita ($p < 0.001$, Fig. 4.6). No significant difference in micronucleus formation was found between Bariri and Promissão.

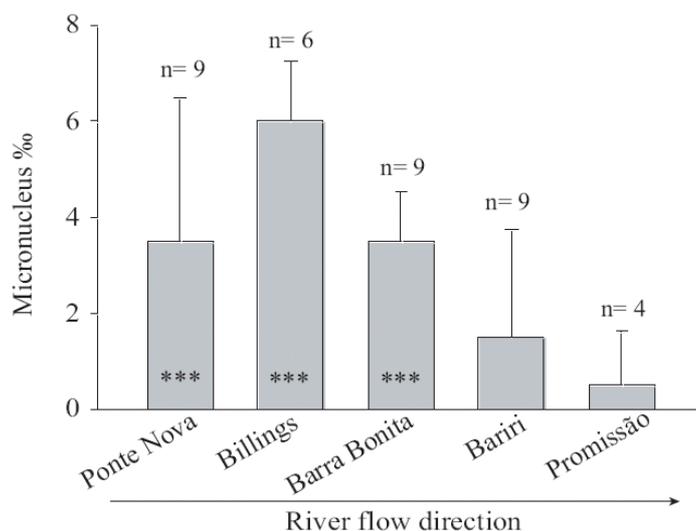


Fig. 4.6: Frequency of micronucleated cells (MN) scored from erythrocytes from fish at different sites along the Tietê River. Data are given as median \pm SD from 2000 erythrocytes per individual/location. n=number of individuals investigated at each location. *** = significantly different from the control group (Promissão; Chi-square test with Yates' modification, $p < 0.001$).

4.5 Discussion

The present study is the first comprehensive investigation into the genotoxic potential of sediments in the catchment area of the Tietê River basin.

In this study, the comet assay results (given as tail moment) documented a strong increase in genotoxicity from Billings extracts to the São Paulo city region, and a decrease further downstream. These results were confirmed by the calculation of the IF/(mg SEQ/ml); where only the concentration inducing highest effect was considered) and the CDI (taking into account all sediment dilutions) and indicated a strong influence of São Paulo city on the overall genotoxicity of the studied areas.

To appreciate the relative genotoxic potential of Billings and the others reservoirs, however, a comparison to well-know genotoxic water bodies may help: Seitz et al. (2008) studied the genotoxic potentials of various locations along the upper Danube River (Germany) by means of the comet assay, also exposing RTL-W1 cell to sediment extracts. They recorded the highest CDI value of 11.4 from Rottenacker sediment extracts, which is extremely high when compared to the highest value of CDI in the present study (sediment extract from Billings reservoir, with CDI values up to 4.32). However, extract of sediments from the localities Schwarzach and Ehingen, showed values comparable to those from Billings ($CDI \geq 3$). The genotoxic potentials of sediments from Ponte Nova and Promissão are comparable to those from Bad Abbach and Sigmaringen ($CDI \leq 1.73$), and from Bariri and Três Irmãos are

comparable to the Lauchert (CDI 0.66). CDI values from near Tietê River spring and Barra Bonita extracts (CDI 0.17) are considerably lower than all values recorded for upper Danube River sediments. In a fuzzy logic-based classification of sediments from the same locations in upper Danube River, based on data from several *in vitro* biotests, Keiter et al. (in press) classified sediments from Schwarzach and Ehingen as strongly toxic, those from Bad Abbach and Sigmaringen as moderately toxic and the samples from the Lauchert as non toxic. A direct comparison of the results obtained in the present study to results obtained by Keiter et al. (in press) is not possible, since their conclusions were based on effects in several *in vitro* biotests. However, taking into account both studies (Seitz et al. 2008 Keiter et al. in press), it is possible to rate sediments from Billings reservoir as strongly genotoxic, those from Ponte Nova and Promissão as moderately genotoxic and those from Bariri and Três Irmãos as less genotoxic.

The low genotoxicity by Barra Bonita extracts can be related to significant water input from more than 100 tributaries. However, it may also be related to the potential inefficiency of the sample preparation process for eutrophic sediments such as those from this area (for details, see, e.g., Matsumura-Tundisi and Tundisi 2005, Rodgher et al. 2005, Sotero-Santos et al. 2006). Interactions between eutrophication and contaminants may occur through many mechanisms: Eutrophication may cause dilution of contaminants by increasing the biomass, increased contaminant scavenging by dissolved organic carbon (DOC), increased sedimentation of contaminants and increased uptake in the food chain (Gunnarsson et al. 1995, Taylor et al. 1991, Koelmans et al. 2001). Deposition and recycling of contaminants from bottom sediments may be affected by the eutrophication status of the area (Skei et al. 1996), e.g., eutrophic water bodies contain a great phytoplankton biomass due to the excess of nutrients, causing persistent organic pollutants (POP) retention and greater sedimentation of these pollutants (Larsson et al. 2000). Contaminants and eutrophication factors may also interact to affect bioaccumulation as well as the growth, health and reproduction of benthic organisms (Skei et al. 1996).

Regarding the micronucleus assay data, all Billings fish showed an increase in micronucleus formation over specimens from all other locations. Under aquarium conditions, the micronucleus frequencies for negative controls of *Oreochromis niloticus* in previous studies ranged from 0.4 ‰ (Palhares and Grisolia 2002, Ventura et al. 2008) to 1.72‰ (Cavas and Ergene-Gözükara 2005). In specimens collected in the field, micronucleus frequencies in specimens from Promissão and Bariri reservoirs were in the same range. In *in vitro* experiments, Cavas and Ergene-Gözükara (2005) exposed *Oreochromis niloticus* to

cyclophosphamide (as a positive control) at a concentration of 4 mg/L for 3 and 9 days and recorded erythrocyte micronucleus frequencies of 4.12 and 5.9 ‰, respectively. Comparing these numbers to those recorded in the present study, the frequency of micronucleated cells in Billings fish (6.0 ‰) was comparable to the cyclophosphamide positive control, thus confirming the strong genotoxic potential in Billings reservoir.

During the last decades, many cases of contamination by heavy metals and organic compounds such as polychlorinated biphenyls (PCBs), organochlorine pesticides and polycyclic aromatic hydrocarbons (PAHs) have been observed in waters in and around São Paulo city, especially in the Billings reservoir (Bainy et al. 1999). In an earlier *in situ* (*in vivo*) study with *Oreochromis niloticus* from Billings reservoir, Bainy et al. (1996) recorded significantly higher levels of total microsomal cytochromes P450 and b5 in livers and kidneys of specimens from this reservoir, if compared to specimens from a reference site. These results were confirmed by Bainy et al. (1999) and Leitão et al. (2000), who, studying the same fish species, observed more than a 20-fold increase in 7-ethoxyresorufine-*O*-deethylase (EROD) in liver microsomes over fish from a non-polluted reservoir. In laboratory studies into the toxicity of Tietê River reservoirs, Almeida and Rocha (2006) applied biotests with *Chironomus xanthus* and *Hyaella azteca* as test organisms and recorded higher mortalities in organisms exposed to Billings reservoir sediments than in organisms exposed to Promissão samples. Assessing the toxicity of sediment samples from Tietê River reservoirs downstream São Paulo city, through chronic-partial toxicity bioassays with *Danio rerio* larvae as test-organisms, Fracácio et al. (2003) recorded inadequate conditions for the growth of the test organisms when exposed to the sediments of upstream reservoirs and also found an improvement of environmental conditions further down the river system. In an evaluation of the quality of water and sediment samples from the same reservoirs, Rodgher et al. (2005) recorded chronic toxicity for *Ceriodaphnia dubia* and acute toxicity for *Danio rerio* decreasing by one order of magnitude from Barra Bonita to Três Irmãos, demonstrating an environmental degradation gradient along the reservoirs. According to their findings and in agreement with the findings of the present study, sediment quality of the reservoirs improves with increasing distance from the metropolitan area of São Paulo, indicating the megacity to be the origin of most of the pollutants. However, based on the present findings on genotoxicity, the other locations can also not be considered as being free from anthropogenic impact, since although being less genotoxic than Billings reservoir, significant genotoxic effects were recorded in the other areas.

According to Kosmehl et al. (2004), testing of sediments and suspended particulate matters for genotoxicity should become a standard requirement for industrial wastewater discharge permits, as are routine aquatic toxicity and wastewater tests, since aquatic organisms exposed to wastewater discharges and particle-bound substances suffer an increased risk of genetic damage. Aquatic organisms such as fish accumulate pollutants directly from contaminated water or indirectly through the ingestion of contaminated aquatic organisms. Genotoxic pollutants may lead to the contamination not only of the aquatic organisms themselves, but also of the entire ecosystem and, finally, of humans *via* the food chain (Matsumoto et al. 2006). Thus, humans using water for drinking water purposes may suffer similar genetic or even carcinogenic risks as do fish (Kosmehl et al. 2004, 2008). Moreover, in recent years, there has been increasing concern about the risk for human health following consumption of contaminated fish, since fish are top predators in the food web and can metabolize, concentrate, store and also biomagnify contaminants in a way similar to humans. The assessment of sediment quality is, thus, essential for the understanding of processes governing the fate and availability of pollutants in water bodies, which are the final compartment for storage and transformation of most pollutants discharged by anthropogenic activities (Almeida and Rocha, 2006). For this reason, more specific environmental studies are required to elucidate the potential risk for environmental and common welfare, including that of humans.

4.6 Conclusions

Differences in genotoxic potentials between different locations could be identified by both the comet assay and the micronucleus test, with a very high correlation between *in vitro* and *in situ* results. The good correlation of these two tests is – in terms of weight-of-evidence approaches – an indication of the high ecological relevance of sediment genotoxicity for the situation in the field (cf. Böttcher et al. in press, Chapman and Hollert 2006).

In the present study, strong hazard potentials could especially be detected in samples from Billings reservoir, indicating a major impact of discharges of the megacity São Paulo. Dilution by tributaries further downstream resulted in a significant decrease in genotoxicity of sediment-bound contaminants. Genotoxicity studies thus corroborate conclusions drawn from several studies based on other toxicity parameters (Almeida and Rocha 2006, Bainy et al. 1996, 1999, Fracácio et al. 2003, Leitão et al. 2000, Rodgher et al. 2005).

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EDCs in river systems

The Danube, the second longest river in Europe, flows through several countries from where it receives discharges of agricultural, industrial, and urban effluents ¹. Sewage treatment plants, for example, receiving industrial and domestic waste waters constantly release a complex mixture of hormonally active chemicals into the aquatic environment. By investigating effluents from the municipal sewage treatment plant of Ulm/New Ulm a significant input of natural and synthetic steroidal estrogens into the river Danube could be determined ². The two most frequent estrogens excreted by humans, estrone and 17 β -estradiol, were most frequently detected in sewage treatment plants discharges pouring into the river Danube. The exogenous contraceptive hormones 17 α -ethynylestradiol and mestranol were also determined in several samples at a time showing about half the concentrations of the natural estrogens with mestranol occurring only in one sample. However, the concentrations commonly found for all estrogens were in the lower ng/L-range, indicating only a small burden in the pg/L-range resulting for a big river like the Danube. Additionally Grund and colleagues ³ detected estrogenic compounds in sediments of the Upper Danube River. Up to date, no estrogenic effects in aquatic species in the river Danube have been investigated or published. However, Grund *et al.* ³ revealed significant increases in the 17 β -estradiol production in H295R cells after exposure to sediments from the Upper Danube. For one sampling site, they detected also a significant decline in the testosterone level.

The fact, that no impacts on the endocrine system of aquatic species in the river Danube have been recorded so far, might be related to the occurrence of these endocrine disruptors only at local hotspots. Another reason might be the simple fact of missing investigations.

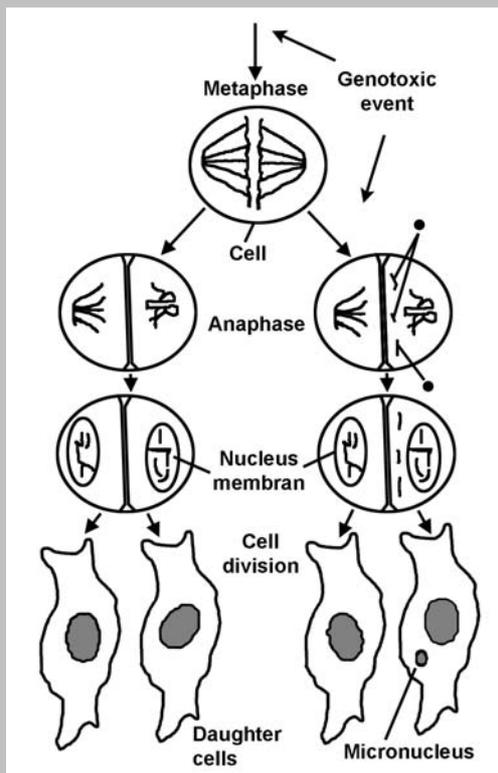
¹ Pringle, C., G. Vellidis, F. Heliotis, D. Bandacu and S. Cristofor (1993). "Environmental problems of the Danube Delta." *American Scientist* 81: 350-361.

² Kuch, H. M. and K. Ballschmiter (2000). "Determination of endogenous and exogenous estrogens in effluents from sewage treatment plants at the ng/L-level." *Fresenius. J. Anal. Chem.* 366: 392-395.

³ Grund S., Higley E, Schöneberger R, Suter M.J.-F., Giesy J.P., Braunbeck T., Hecker M., Hollert H. 2010. The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by in vitro bioassays and chemical analysis. *Environ. Sci. Pollut. Res.*

The micronucleus assay

The micronucleus assay is based on the loss of chromosomes or chromosome fragments during meiosis, which are not reincorporated into the nucleus after cell division and, therefore, are transformed into a smaller nucleus or micronucleus (see figure below ^{1,2}). The *in vitro* micronucleus tests are usually performed with continuously dividing cells, mostly established cell lines or human peripheral lymphocytes ^{3,4,5}.



Scheme of the micronucleus formation during cell division ⁶. * = Chromosome/-fragment without contact to spindle apparatus.

¹ Grisolia, C. K. (2002). "A comparison between mouse and fish micronucleus test using cyclophosphamide, mitomycin C and various pesticides." *Mutation Res.* 518 (2): 145-50.

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⁴ Müller-Tegethoff, Kerstin, Birgit Kersten, Peter Kasper and Lutz Müller (1997). "Application of the *in vitro* rat hepatocyte micronucleus assay in genetic toxicology testing." *Mutat. Res.* 392 (1-2): 125-138.

⁵ Reifferscheid, G., C. Ziemann, D. Fieblinger, F. Dill, R. Gminski, H.J. Grummt, C. Hafner, H. Hollert, S. Kunz, G. Rodrigo, H. Stopper and D. Selke (2007). "Measurement of genotoxicity in wastewater samples with the *in vitro* micronucleus test-Results of a round-robin study in the context of standardisation according to ISO." *Mutat. Res.*

⁶ Al-Sabti, K. and C. D. Metcalfe (1995). "Fish micronuclei for assessing genotoxicity in water." *Mutation Res.* 343 (2-3): 121-35.

Chapter 5

New and edited versions of the micronucleus assay with fish

Authors: Melanie Boettcher, Thomas Braunbeck

in preparation

5.1 Abstract

In fish, the micronucleus test proved to be a useful assay for genotoxicity testing. However, cell divisions are a prerequisite for the formation of micronuclei, most micronucleus tests in fish have been restricted to blood cells or have been based on systems with exogenous stimulation of cell division. Since there is an urgent need for genotoxicity tests in other organ systems of non-manipulated fish, the micronucleus assay was adopted to fixed liver tissues, an organ system central to numerous metabolic pathways in fish. The evaluation of micronuclei in fixed organ tissue enables an exact allocation of micronuclei in certain tissues. 7 d old zebrafish, all important organs have developed and show relatively high mitotic rates, thus it could be assumed that harmful substances should lead to the formation of micronuclei. However, newly hatched zebrafish do not have enough liver tissue to gain a sufficient amount of data and thus the present study was designed to develop a new/edited test protocols for the micronucleus assay with cell suspensions of *Danio rerio* embryos and fixed organs.

Keywords: Micronuclei, zebrafish, cell line

5.2 Introduction

Among others, genotoxicity of released substances is of special interest for regulatory issues, since it can be directly correlated with adverse reproductive effects (Anderson and Wild 1994) or even lead to an elevated extinction risk (Diekmann et al. 2004a). Previous studies on fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and fern (*Onoclea sensibilis*) have shown that exposure to mutagens in water or soil can enhance the frequency of heritable recessive lethal mutations (Diekmann et al. 2004b; Schoen et al. 2002; White et al. 1999). The accumulation of such deleterious mutations can contribute to the decline of small populations *via* a phenomenon known as mutational meltdown (Lynch et al. 1995).

In recent years, the *in vitro* micronucleus test has become an attractive tool for genotoxicity testing because of its simplicity of scoring and wide applicability in different cell types (Decordier and Kirsch-Volders 2006). Since the two basic mechanisms leading to the formation of micronuclei are chromosome breakage and disturbance of the chromosome segregation machinery, a mitotic or meiotic division and assessment of cell division in the presence of the test substance is obligatory for the development of micronuclei (Heddle 1988).

The detection of micronuclei has been widely used for years to evaluate *in vivo* mutagenic, clastogenic and aneugenic effects (Udroiu 2006). In the past, the high number of analysable cells, the simplicity of the technique, the possibility of automation, as well as the ability to more accurately detect aneugens, led to the dominant role of the *in vivo* micronucleus test compared to the *in vivo* chromosome aberration test (Adler et al. 1991; Mavournin et al. 1990; Miltenburger 1992; van Hummelen and Kirsch-Volders 1992; Vanderkerken et al. 1989). Although most of the studies published until now performed this assay on mammalian species (especially rodents), the micronucleus test showed to be a useful tool with samples taken from aquatic organisms (Boettcher *et al.* 2010; Rocha *et al.* 2010; Udroiu 2006). Both laboratory research (to evaluate the genotoxicity of xenobiotics) and *in situ* studies (to assess the water quality) have involved several invertebrate species (Barsiene and Barsyte Lovejoy 2000; Dolcetti and Venier 2002), amphibians (Zoll-Moreux and Ferrier 1999) and teleosts such as Cypriniformes (Al-Sabti 1986; Al-Sabti et al. 1994), Perciformes (Ieradi et al. 1996; Pelhares and Grisolia 2002; Rodriguez-Cea et al. 2003), Characiformes (Pantaleão et al. 2006), Anguilliformes (Rodriguez-Cea et al. 2003), Gadiformes (Barsiene et al. 2006), Pleuronectiformes (Barsiene et al. 2006), Salmoniformes (Rao et al. 1997; Rodriguez-Cea et al. 2003) and Siluriformes (Bahari et al. 1994).

Fish as test species are highly recommended, since central metabolic processes such as peroxisome proliferation (Yang et al. 1991) or oxidative damage induced by chemicals (Washburn and Di Giulio 1989) seem to be similar in fish and mammals. Thus, fish can not only be used as indicators for genotoxicity in aquatic systems, but also as model species for genotoxic properties of chemical and physical agents in vertebrates in general (Udroiu 2006).

Therefore, two new/edited versions were applied to extend the field of application and to elucidate the impact of differential biotransformation capacities: (1) The micronucleus test with liver tissue from adult fish and (2) with fish embryos. Although the micronucleus test with liver-derived cell suspensions of exposed animals has already been used for many years (Williams and Metcalfe 1992), the present study proposes a new approach to evaluate micronuclei in intact liver tissue. One advantage of using the intact liver-complex compared to its suspension is that the hepatocytes can be distinguished easily from endothelial and other surrounding cells. Furthermore, the production of artifacts due to the treatment and cell preparation is unlikely. Since the perfusion of the fish can be done easily on site, an *in situ* investigation of the micronucleus frequency of any target organ is possible.

In the second approach, zebrafish larvae were exposed to the model substance nitroquinoline-*N*-oxide, sacrificed, homogenized and evaluated in the micronucleus assay. After 7 days, zebrafish larvae have developed all important organs for metabolising substances, and due to the high mitotic rates in tissues of growing organisms, it can be assumed that harmful substances will lead to the formation of micronuclei. The *in vivo* micronucleus test with homogenised zebrafish larvae can be combined with the extended version of the fish embryo assay designed for examination of teratogenic effects (Braunbeck *et al.* 2005; Nagel 2002), or with the short-term toxicity test on embryo and sac fry stages according to OECD TG 212 (OECD 1998). Thus, in addition to the higher ecological relevance of whole organism tests compared to cell line approaches, these new approaches offer the multiple usage of test animals and can, therefore, be considered as a contribution to the reduction of animal tests.

To conclude, the objective of this study were (1) to develop new versions of the micronucleus assay to broaden its field of application, (2) to compile data from different applications of the micronucleus assay to compare the results obtained by the ‘new’ approaches with the results from the established test systems, and (3) to elucidate the impact of variable biotransformation capacities in different organs on the formation of micronuclei.

5.3 Materials and methods

5.3.1 Nitroquinoline-*N*-oxide

The chemical 4-nitroquinoline-*N*-Oxide (NQO; CAS number 56-57-5) is a water soluble, *N*-heterocyclic organic compound (nitroaromatic compound, *N*-PAH). The \log_{KOW} is 1.09 (Hansch *et al.* 1995). NQO has a well-known mode of action (Galiegue-Zouitina *et al.* 1985; Menichini *et al.* 1989; Nunoshiha and Demple 1993; Yang *et al.* 1991; Yano *et al.* 1995) and is frequently utilised as genotoxic model substance (Erbes *et al.* 1997; Walton *et al.* 1983; Walton *et al.* 1984; Walton *et al.* 1985), and methods for analytical detection have been described (Lund *et al.* 1981). Technical NQO was purchased from Steraloids (Newport, USA).

5.3.2 *In situ* analysis of micronuclei

Liver tissue sampling

Liver tissue samplings were performed on wild barbel (*Barbus barbus*) immediately after catch from the Upper Danube River at Ehingen (Germany). Ehingen was selected, since previous bioassays had indicated elevated genotoxic potentials of this site (Seitz 2007, Keiter 2006). As a reference, fish from a less contaminated site, Riedlingen, were caught, transferred to and maintained in the fish keeping facilities at Heidelberg University for at least 60 days under constant flow-through conditions (4 L/h) in 400 L tanks with continuous air supplies. The maintenance water had the following characteristics: 14.0 ± 1.0 °C temperature, 744 μ S conductivity (370 mg/L CaCO₃ hardness), pH 7.5 ± 0.2 , 10.5 ± 0.5 mg/L O₂ (95 % saturation), and a 12 h light/12 h dark photoperiod. Ammonia, nitrite, and nitrate were below detection limits (≤ 5 , 1 and 150 mg/L, respectively). Once a week, barbels were fed with red chironomids, otherwise with commercially available trout flake food (Trouvit pro aqua, Milkivit, Burgheim, Germany).

Length ranged between 11 and 15.5 cm, weight was between 13 and 33.3 g. At each sampling site, five mature barbel were collected by electric fishing. For fixation of the liver tissue (Braunbeck *et al.* 1987), fish were anaesthetized by suspension in an aqueous solution of ethyl-4-aminobenzoate (benzocaine) and perfused *in situ* through the ventricle, with 1.5 % glutardialdehyde and 1.5 % formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.6) containing 2.5 % PVP. Afterwards, the liver was incubated in perfusion fixative for at least 30 min at 4 °C. The fixation was continued in 2.5 % glutardialdehyde in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4 % PVP and 0.05 % calcium chloride for 20 min at 4 °C. After rinsing in cacodylate buffer, tissue blocks were dehydrated in a graded series of ethanol and embedded in Spurr's medium (Spurr 1969). Semithin sections of 100 to 120 nm thickness were mounted on glass slides and were allowed to dry. For optimal staining quality, Spurr's medium was removed by incubating the slides over night in a NaOH-ethanol mixture (Weissenfels 1982). Staining was performed with iron hematoxylin according to Weigert (1904). After dehydration in a graded series of ethanol, the slides were covered with DePeX (Serva, Heidelberg, Germany).

Micronucleus test with liver tissue from field fish

From each barbel specimen, 3 slides were prepared. On each slide, 2000 hepatocytes were scored for micronuclei under a light microscope (Typ, Hersteller) equipped with an oil-

immersion lens at 1200 x magnification. Criteria for identification of micronuclei in were set as follows: a) Maximum size of micronuclei must not exceed 30 % of the main nucleus. b) Micronuclei and main nuclei should stain similarly. c) Micronuclei should be clearly separated from the main nucleus. d) Only cells with good cytoplasmatic outlines are used for reading (ISO21427 2003).

Results were recorded as percentage of cells containing micronuclei compared to the total number of cells scored. The induction factor for each site was calculated by computing the percentage of cells showing micronuclei relative to the negative controls. Statistical significances were assessed by using the Chi-square test with Yates' correction (ISO21427 2003; Lovell et al. 1989). For each concentration, the number of cells with micronuclei was compared with those without micronuclei and with the corresponding negative control by means of SigmaStat 3.5 (SPSS-Jandel Scientific). Values differing significantly from negative controls were indicated in the respective graph with * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ with three asterisks (***).

5.3.3 *In vivo* laboratory tests: Micronucleus assay with zebrafish embryos

Maintenance and egg production of zebrafish

Sexually mature zebrafish (*Danio rerio*) were kept in communities of up to 20 individuals in 25 L tanks under flow-through conditions in water with a temperature of 27 ± 0.5 °C; other water characteristics were identical to those described above for barbel laboratory controls. Fish were fed a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany) or nauplii from *Artemia* sp. (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Ogden, USA) twice daily. The day before a test, 10 males and 10 females were placed in 18 L breeding chambers (water conditions as above) immediately before dawn. Artificial plants served as breeding stimulant and substrate. Spawning and fertilization took place within the first 30 min after the onset of light in the following morning. A fertilization rate of ≥ 80 % served as a quality control for adequate test materials.

NQO exposure

About 1 h post fertilization, the fertilized eggs can easily be separated from non-fertilized opaque eggs. By using a 2 ml plastic pipette with a widened opening, 20 fertilized eggs were selected and transferred to a 100 ml Petri dish containing 50 ml artificial water and the model

substance 4-nitroquinolin-N-oxide (NQO) at concentrations of 47.5, 95, 190, 380 and 760 µg/L. A survival rate of 80 % was a prerequisite for further use in the micronucleus assay. Eggs were incubated at 27.0 ± 0.1 °C in the dark to prevent photooxidation of NQO. After 7 days, 7 to 8 embryos were processed for cell isolation and subsequently for the micronucleus assay.

Cell isolation

Subsequent to NQO exposure, the micronucleus assay was carried out in exposure groups with ≤ 20 % mortality. In any case, overlapping of acute toxicity and mutagenicity had to be excluded.

Cell isolation was carried out mechanically according to the protocol by Kosmehl et al. (2006). For each exposure group, 7 to 8 zebrafish embryos served as donors for one slide. A glass/glass tissue grinder (Potter-Elvehjem-type, Braun Biotech, Sartorius, Goettingen, Germany) with a defined pestle/wall distance of 50 - 70 µm was used to isolate the cells in 2 ml PBS (Sigma) and 5 % FCS. Grinding was carried out with four gentle manual strokes with a maximum rotation of 90° per stroke. The resulting cell suspension was resuspended in xxx medium and filtered through 70 µm gauze (Verseidag, Krefeld, Germany) into one reaction tubes in order to separate the individual cells from remaining pieces of connective tissue. This was followed by centrifugation at 150 g in a centrifuge at 4 °C for 10 min.

Micronucleus assay

The cell suspensions were fixed within the reaction tube with 200 µl of a 4:1 mixture of methanol and acetic acid spread on a slide. After drying, the slides were either analyzed directly or stored in a glass box for at maximum 8 days. Immediately before scoring, the DNA was stained with 20 µl of 20 µM acridine orange in aqua bidest. and covered with a coverslip. Statistical significances were assessed by using the Chi-square-test with Yates correction (ISO21427 2003; Lovell et al. 1989).

5.4 Results

5.4.1 Micronucleus test in liver tissues from barbel after *in situ* exposure to Danube River water

Laboratory-maintained control barbel collected at the reference site Riedlingen had a hepatocellular micronucleus frequency of 0.18 ± 0.019 %, whereas the micronucleus frequency in liver tissues from barbel collected at Ehingen (Fig. 5.1 and 5.2) had a significantly elevated micronucleus rate of 0.45 ± 0.053 % ($p \leq 0.05$).

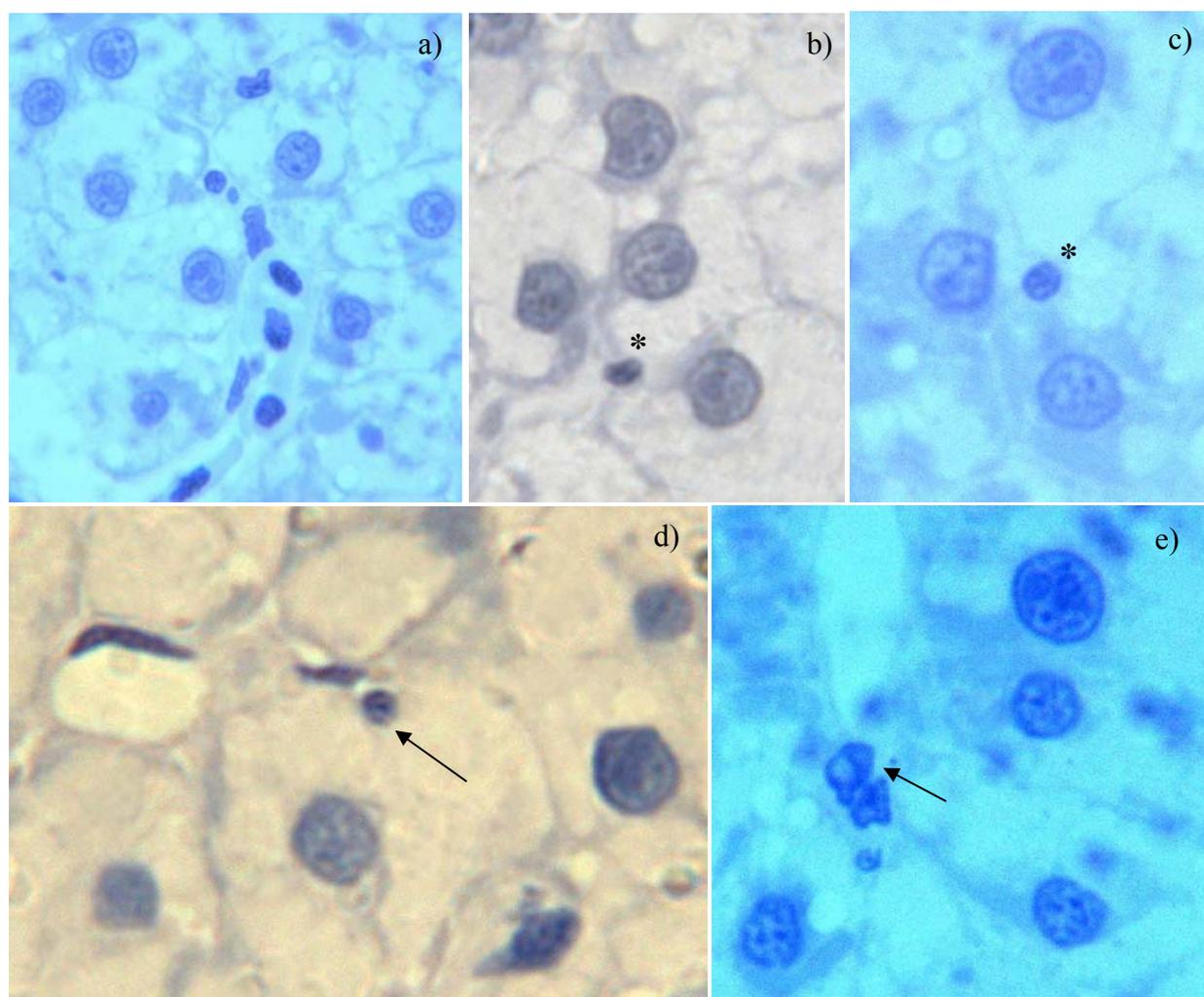


Fig. 5.1: Hepatocytes from the liver of *Barbus barbuis* from Ehingen. **5.1 a:** Hepatocytes with cells from the hemal system. **5.1 b:** Three normally developed nuclei and one nucleus with micronucleus. **5.1 c:** Three normally developed nuclei and one nucleus with micronucleus. **5.1 d and e:** Hepatocytes and endothelial cells (fixation acc. to Spurr 1969; iron hematoxylin; x 1220). * = micronucleus; \rightarrow no micronucleus.

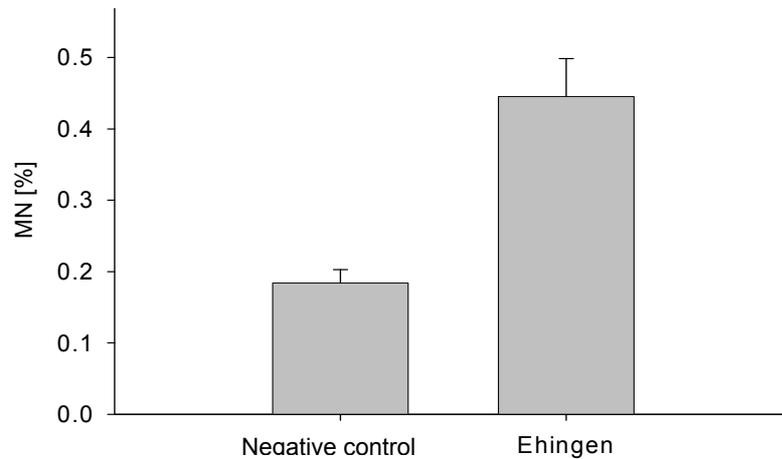


Fig. 5.2: Genotoxic activity of in the micronucleus assay with fixed liver tissue from barbel (*Barbus barbus*) caught at Ehingen, expressed as percentage of micronucleus formation (MN [%]). For each sampling site 3 barbels were evaluated. NC = negative control.

5.4.2 Micronucleus test in whole body cell suspensions from zebrafish exposed *in vivo*

The micronucleus frequency in the whole body cell suspensions (Fig. 5.3) increased in all replicates in a dose-dependent fashion, finally reaching induction factors around 4. However, only the highest NQO concentration of 760 $\mu\text{g/L}$ differed significantly from the negative control (Fig. 5.4).

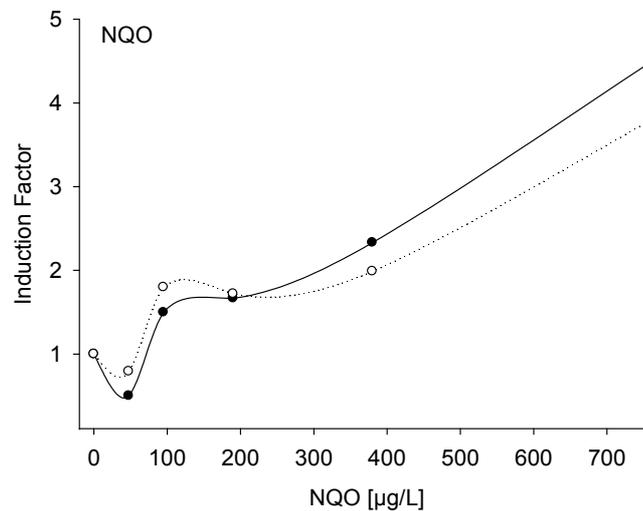
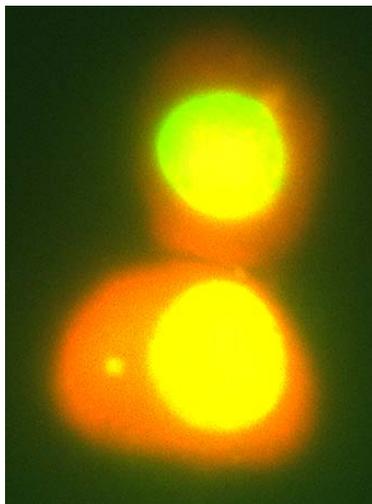


Fig. 5.3 (left): Two cells from the whole body cell suspensions from a 7-day old zebrafish. The lower cell has a small micronucleus, whereas the upper one has none (fixation: methanol / acetic acid; dye: acridine orange).

Fig. 5.4 (right): Dose-response curves for the mutagenic activity of NQO in the micronucleus assay with whole body cell suspensions of zebrafish. Data are given as induction factors over controls. Asterisks: Values differ significantly from the negative control (Chi-square test with Yates correction; ** $p \leq 0.01$; *** $p \leq 0.001$).

5.5 Discussion

The micronucleus assay *in vivo* was performed with liver tissue from barbel and zebrafish embryos. Aim of the present study was not only to investigate their suitability for mutagenic testing, but also to elucidate the impact of different biotransformation capacities.

5.5.1 The embryonic micronucleus test

The protocol for the fish embryo micronucleus assay uses zebrafish embryo-derived cell suspensions. Suspended tissues have frequently been used for detecting micronuclei in mussels (Siu et al 2004, Dolcetti and Venier 2002, Burgeot et al. 1995), sea urchins (Hose et al 1983), fish (Williams and Metcalfe 1992, Takai et al 2004, Hayashi et al 1998, Ueda et al 1992, Barsiene et al 2006, Rao et al 1997, Pelahres and Grisolia 2002), rats (Ribero et al 2004) and humans (Daley et al 2003). Fish embryos (*Carassius* sp., *Zacco platypus*, *Leiognathus nuchalis* and *Ditrema temmincki*, *Rhodeus ocellatus ocellatus*) have previously been applied in the micronucleus test, which proves their overall suitability for detecting the mutagenic potential of chemical substances (Hayashi et al 1998, Ueda et al 1991). Since the embryos used in these two studies were only allowed to develop for 24 h, the chorion could restrict the bioavailability especially of highly lipophilic substances (Braunbeck et al. 2005). Furthermore, the biotransformation of the embryos in the gastrula stage is strongly limited, due to an incomplete developed detoxification system: The zebrafish arylhydrocarbon-receptor-2 mRNA (zfAhR2 mRNA), for example, cannot be detected before 24 h post fertilization and expression increases throughout early development (Tanguay et al. 1999). CYP1A1 mRNA was observed already after 15 h of development, whereas the CYP1A1 protein and monooxygenase activity, however, were not detected until 3 days post fertilization and after successful hatching (Mattingly and Toscano 2001). Since both components provide essential functions for the biotransformation of endogenous and exogenous elements (Goksoyr and Husoy 1998), it can be assumed that bioassays with zebrafish embryo, performed before hatching might not cover toxic effects of such promutagens, which need to be bioactivated to exert their toxicity. With respect to a good comparability to adult fish studies and as the chorion can function as a barrier for certain substances (Henn and Braunbeck 2011), we propose to use at least 3 days old, hatched larvae for the micronucleus assay. Nevertheless, the exposure of very early embryos might be necessary, if early developmental stages such as organogenesis should be covered. Considering the relatively low standard deviation of the replicate experiments, a good reproducibility of the results can be expected.

5.5.2 Micronucleus assays in fixed liver tissues from barbel after *in situ* exposure

To the best of our knowledge, the present study is the first one to perform the micronucleus assay with fixed liver tissue from fish after *in situ* exposure. Several other studies already conducted the piscine micronucleus assay with liver-derived cell suspensions after exposure under laboratory conditions (Rao et al. 1997; Williams and Metcalfe 1992). One advantage by using the micronucleus test with fixed tissues is that different organs even of very small fish can be scored separately after perfusion of the fish and isolated embedding of the tissues. Perfusion with fixatives of electron microscopy quality was found indispensable in order to facilitate perfect fixation of all tissues for optimal identification of micronuclei and nuclear abnormalities.

Once the pre-fixed organs are dissected and immersed in the cooled fixative, they can be stored for several days. This opportunity allows *in situ* sampling with subsequent processing of the samples in the laboratory without any loss of quality. The cells remain in their original constitution and position within the tissue. This allows the restriction to score for micronuclei in otherwise undamaged cells exclusively. Furthermore, fixed organs can also be used for normal histopathological surveys. Consequently, as more information can be drawn from a single animal, this version of the micronucleus can also be seen as a reduction in animal experiments according to the three Rs (Reduction, Refinement and Replacement; Russel and Burch, 1959).

5.5.3 Sensitivity of the applied micronucleus assays

Recently, NQO was also tested in the permanent fish liver-derived cell line RTL-W1 (Böttcher *et al.* 2010). The maximum induction factor in zebrafish embryos of 4.15 was recorded after exposure to 760 µg/L NQO, which was also the lowest observed effect concentration (LOEC; whereas the LOEC in RTL-W1 cells exposed to NQO was < 12 µg/L (own data)). The maximum induction factor in RTL-W1 cells, however, was only slightly higher in RTL-W1 cells (4.95), but was observed at a lower concentration of 190 µg/L NQO already.

These findings can be confirmed for NQO in the comet assay: Schnurstein and colleagues (2001) tested NQO in the comet assay with the rainbow trout cell lines RTL-W1 and RTG-2. Compared to the results obtained for genotoxicity in cell suspensions from zebrafish embryos by Kosmehl and colleagues (2006), the cell lines turned out to be more sensitive: The LOEC for RTG-2 cells exposed to these substance was 119-fold lower than for zebrafish embryos.

For RTL-W1 cells, the difference was less conspicuous than for RTG-2 cells; the LOECs were approximately 7 lower than for zebrafish-derived cell suspensions.

Sensitivity of the micronucleus test with fixed liver tissues relative to micronucleus assays with erythrocytes

Since metabolic activation usually occurs in the liver, the hepatocellular micronucleus assay was expected to be a more sensitive system for detecting genotoxic responses in fish than micronucleus assays with fish erythrocytes (Rao et al. 1997, Williams and Metcalfe 1992). Since only one site along the River Danube (Ehingen) was sampled, it is difficult to assess the relative sensitivity of the micronucleus test with fixed liver tissues in general. Significant inductions in the number of micronucleated cells could be detected for both liver cells and erythrocytes (Böttcher et al. 2010). Unexpectedly, the relative micronucleus induction in liver cells was lower than in erythrocytes (2.4 versus 4.1, respectively; Böttcher et al. 2010), whereas the relative percentage of cells containing micronuclei was higher in liver (= 0.45 %) than in erythrocytes (= 0.28 %). This may lead to the assumption, that in this case detoxification processes in liver tissues dominated over bioactivation of xenobiotics. The lower proliferation rate of mature liver tissue compared to the lifespan of erythrocytes could be a further explanation. From the literature, it appears that a peak in micronucleated erythrocytes occurs 1 – 5 days after exposure, but in most species it takes place after 2 or 3 days. Therefore, they are more suitable for the detection of actual hazardous events, whereas the micronucleus rate in liver tissue rather reflects effects of long-term exposure.

Hayashi and colleagues (1998) reported a higher micronucleus frequency in gill cells than in erythrocytes. However, since their study did not include a negative control, no statement in regard to the sensitivity can be made.

Similar findings were reported for peripheral blood smear samples in tilapia (*Oreochromis mossambicus*; (Manna and Sadhukhan 1986) and for sister chromatid exchanges in the mud minnow (*Umbra krameri*; Sayato et al. 1992).

The micronucleus test can be carried out in any active tissue. The proliferation rate of erythrocytes is very high compared to other tissues. Even though cell division can be stimulated by damaging the target tissue (e.g. epithelial cell division; (Arkhipchuk and Garanko 2005) or by proliferating substances (e.g. allyl formate in liver; Rao *et al.* 1997), every additional process entails a risk of enhancing the formation of artefacts.

As a conclusion, based on our results, we suggest using piscine erythrocytes in the micronucleus test for short-term exposure of xenobiotics or *in situ* monitoring of water quality rather than any other tissue. The relatively high proliferation rate of the erythrocytes allows to detect the mutagenic hazard potential even of indirectly acting mutagens like NQO or benzo[*a*]pyrene (Diekmann et al. 2004b) and of environmental samples (Al-Sabti and Metcalfe 1995; Minissi et al. 1996).

However, since the standard deviation for the hepatic micronucleus test (10 - 12 %) was lower than for the micronucleus test with erythrocytes (16 - 80 %; Böttcher et al. 2010), the micronucleus assay with liver tissue might be more suitable for assessing effects of mutagens after long-term exposure.

5.6 Conclusions

The micronucleus assay with fixed (liver) tissues and the modified micronucleus test with zebrafish embryos represent valuable tools for the *in vivo* identification of the mutagenic potentials of pure substances, environmental samples and field locations. In particular, given the longevity of liver cells in adult fish, the identification of micronuclei in fixed tissues provides a tool to record long-term genotoxic effects in field fish. Even though zebrafish embryos have proven to be less sensitive to pure substances (own data) and to environmental samples (Seitz et al. 2007) than RTL-W1 cells, their ecological relevance is much higher. *In vivo* studies of mutations at either the gene or chromosomal level are of particular relevance for estimating the carcinogenic potential of a genotoxic agent. Such events demonstrate not only that the substance has penetrated to critical targets *in vivo*, but that it, or its biologically active metabolites, have escaped host protective mechanisms to produce the kinds of irreversible genetic effects that underlie most forms of cancer or heritable disorders.

However, further studies are required to confirm the validity of these micronucleus assay versions. Although the protocols for both the micronucleus assay with fixed liver tissues as well as that with fish embryos make a contribution to the reduction of animal experiments, future studies should put focus on the comparison of *in vivo* micronucleus assays with *in vitro* versions of the micronucleus assay with fish cell lines in order to further minimize animal testing.

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Genotoxicity of endocrine disruptors

Even though modern sewage plants manage to decrease the concentration of endocrines in the aquatic system, contamination with endocrine disruptors such as tributyltin and nonylphenol is still alarmingly high at several sites ¹. The impact may be greatest on species of aquatic fauna other than fish which we have yet to study. Tributyltin, for example, acts as a biocide and is mainly used as a slow-release antifouling agent incorporated in paints for ships. It had little effect on fish, but had a disastrous impact on the female mollusc population due to a masculinisation effect ². Furthermore, the polybrominated flame retardants may become an increasingly undesirable constituent of some sediments and animal tissue, but its significance is still difficult to evaluate ¹. Bioassays performed with H295R cells exposed to sediments from the river Danube reveal also an endocrine potential ³. However, the estrogens (nonylphenol, bisphenol, estrone) detected in these sediments mostly occur at low concentration ranges ³ and cannot be responsible for the measured effects. In contradiction to the common opinion, an increase in the estradiol concentration and/or a decrease in the testosterone level are not necessarily associated to an estrogenic or anti-androgenic compound. Gracia *et al.* ⁴, for example, showed a dose-dependent increase in estradiol (up to 10-fold), in correlation with a significant decrease in testosterone after exposing H295R cells to the strong androgen trenbolone.

¹ Johnson, A. and M. Jürgens (2003). "Endocrine active industrial chemicals: Release and occurrence in the environment." *Pure Appl. Chem.* **75** (11-12): 1895-1904.

² Schulte-Oehlmann, U., J. Oehlmann, B. Bauer, P. Fiorini, M. Oetken, M. Heim and B. Markert (1999). *Ökosystemare Ansätze und Methoden. Ökotoxikologie.* B. Markert and J. Oehlmann. München-Landsberg, Ecomed Verlagsgesellschaft mbh: 350–363.

³ Grund S., Higley E., Schönenberger R., Suter M., John P. Giesy J., Braunbeck T, Hecker M., Hollert H. (2010) The endocrine disrupting potential of sediments from the Upper Danube River (Germany) as revealed by *in vitro* bioassays and chemical analysis

⁴ Gracia T., Hilscherova K., Jones P., Newsted J., Higley E., Zhang X., Hecker, M., Murphy M., Yu R., Lam P., Wu R. and J. Giesy (2007). "Modulation of steroidogenic gene expression and hormone production of H295R cells by pharmaceuticals and other environmentally active compounds." *Toxicol. Appl. Pharmacol.* **225**: 142-153.

Temporal increases in the incidence of certain hormone-dependent cancers and the relationship between reproductive health and cancer may be linked to rising levels of endocrine-disrupting contamination in the environment ⁵. These suggestions indicate that common environmental pollutants are capable of disrupting reproductive and developmental processes by interfering with the actions of endogenous hormones ⁶. Many reports of endocrine disruption describe changes in the normal development of organs and tissues that are consistent with genetic damage, and recent studies confirm that many chemicals classified to have hormone-modulating effects also possess carcinogenic and mutagenic potential ⁶.

⁵ Weir, H.K., L.D. Marrett, N. Kreiger, G.A. Darlington and L. Sugar (2000). "Pre-natal and pen-natal exposures and risk of testicular germ cell cancers." *Int. J. Cancer* **87**: 438-443.

⁶ Hagger, J.A., Depledge M., Oehlmann J, Jobling S. and T.S. Galloway (2006). "Is there a causal association between genotoxicity and the imposex effect?" *Environ. Health Perspect.* 114 (1): 20-26.

Chapter 6

Low-dose Effects and Biphasic Effect Profiles: Is Trenbolone a Genotoxicant?

Authors: Melanie Boettcher, Thomas Kosmehl, Thomas Braunbeck

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6.1 Abstract

Over the last years, extensive research has documented endocrine-disrupting activities for a significant number of substances including, among others, hormones, pharmaceuticals, pesticides and surfactants. Nonetheless, for most endocrine disruptors, toxicological profiles are still incomplete or even lacking. A systematic review has shown that a number of endocrine disruptors with steroid-modulating effects may also exert mutagenic and carcinogenic activities. For trenbolone, an androgenic compound, there is controversy about its genotoxic properties in literature, apparently with a strong dependency on the choice of the test system. Since fish and other aquatic animals run a risk of exposure to runoffs from cattle feedlots or sewage discharge containing trenbolone, potential consequences to aquatic ecosystems need to be assessed. For this end, the genotoxic hazard potential of trenbolone was tested *in vitro* in the permanent fish cell line RTL-W1 as well as in primary cell cultures derived from zebrafish (*Danio rerio*) embryos after *in vivo* exposure. In either test system, a genotoxic hazard potential characterized by biphasic dose-response curves could be documented even from concentrations of 30 µg/L. Results thus confirm the conclusion that the steroid trenbolone may act as a genotoxic substance.

Keywords: Genotoxicity, micronucleus assay, comet assay, *in vivo*, *in vitro*, trenbolone

6.2 Introduction

Over the last two decades, research has documented endocrine-disrupting activities for various environmental pollutants including hormones, pharmaceuticals, pesticides and surfactants [1-3]. Many of these chemicals are for direct human use; others serve as veterinary drugs such as growth promoters and antibiotics and are extensively used in agriculture and released to the environment. Therefore, ecotoxicological studies frequently focused on investigating the effects of estrogenic compounds on (non-target) higher eukaryotes. E.g., 17 α -ethinylestradiol, the active component of contraceptive pills, and nonylphenol, a derivative of industrial chemicals, are among the most commonly studied endocrine-disrupting chemicals (EDCs; [4]).

In contrast, although very high androgenic activities have been detected, e.g., in sediment samples [5], the effects of androgens have been investigated less intensively. In fact, the potential of androgenic substances to influence sexual development of animals have already been shown at environmentally relevant concentrations by Jegou *et al.* [6], who reported

masculinization of fathead minnow (*Pimephales promelas*) in rivers downstream cattle feedlots. The androgenic activity observed in cattle feedlots could be attributed to exposure to major metabolites (17 α -trenbolone, 17 β -trenbolone, and triendione) of the highly potent androgen trenbolone acetate, which is used US-wide as a growth promoter in cattle [7-8]. The synthetic androgen 17 β -trenbolone is applied to cattle in the form of its ester, 17 β -trenbolone acetate. Since there is immediate hydrolysis in the blood, subcutaneous implants of 17 β -trenbolone acetate pellets provide a continuous supply of 17 β -trenbolone to cattle [9]. In cattle bile, the predominating metabolite of 17 β -trenbolone acetate is 17 α -trenbolone. In contrast, rat bile preferentially contains 16 α -hydroxylation products of 17 β -trenbolone and of the 17-keto compound triendione. Thus, species differences in the oxidative metabolism of 17 β -trenbolone exist at least between rat and cattle. Human urine contained mostly 17 α -trenbolone together with some 17 β -trenbolone and triendione, resembling the bovine metabolites more closely than the rat metabolites of 17 β -trenbolone acetate [10]. Furthermore, at least five more polar human metabolites were observed, which have not been identified to date [10]. However, if α -trenbolone is administered to human liver microsomes, it is converted at significant amounts into the strong androgen β -trenbolone [11]. Apart from this, however, little is known about the metabolism and possible pathways of trenbolone in mammals and, even more so, in other vertebrates or even invertebrates.

In fact, to the best of our knowledge, nothing is known about the metabolism of trenbolone in fish or other aquatic animals, which can be directly exposed to runoff from farms using large concentrations of pharmaceutical agents, such as cattle feedlots. Previous studies detected 12 % of previously applied 17 β -trenbolone acetate in liquid manure and 20 % in solid dung after application of implants to heifers [11].

The active compound 17 β -trenbolone is a potent anabolic steroid and is known to masculinize fish after exposure *via* food and water [12]. Concerning its androgenic mode of action, it can be assumed that trenbolone acts like other androgens; however, in contrast to most endogenous androgens, 17 β -trenbolone cannot be metabolized to estrogens. If compared to the most active endogenous hormone dihydrotestosterone (receptor binding activity = 100), the affinity of 17 β -trenbolone to the androgen receptor is even higher (receptor binding activity = 109). The gestagenic activity may not contribute to the anabolic effect, but must be considered for residue evaluation. The strong growth-promoting activity of trenbolone is based on the anabolic activity as an androgen and the anticatabolic activity as an antigluccorticoid [13].

Given the hormonal nature of 17 β -trenbolone and given the fact that such substances have frequently been shown to produce tumors *via* epigenetic mechanisms, different assays have been conducted to elucidate whether 17 β -trenbolone causes genotoxic effects. The genotoxic potential of trenbolone has been discussed controversially in literature, however, with a trend to assume that trenbolone does not exert a genotoxic potential (for review see [14]). However, particularly in fish, only little information has been made available on its genotoxic potentials. Therefore, the present study has been initiated (1) to elucidate if very low doses of trenbolone cause genotoxicity in fish or a fish cell line and (2) how different biotransformation capacities influence the genotoxic potential of trenbolone.

6.3 Materials and methods

6.3.1 Trenbolone

Trenbolone (CAS No. 10161-33-8) is an anabolic steroid hormone with a water solubility between 340 and 380 mg/L without solvent (25 °C; [15]) and a half-life in liquid manure of > 250 days [3,16]. Technical grade trenbolone was purchased from Steraloids Inc. (Newport, USA).

6.3.2 *In vitro* tests

Cell culture conditions

RTL-W1 cells originally derived from rainbow trout liver (*Oncorhynchus mykiss*; [17]) were cultured according to Klee et al. [18] in Leibovitz L15 medium (Sigma-Aldrich, Deisenhofen, Germany). Prior to use in the *in vitro* assays, RTL-W1 cells were washed twice with phosphate-buffered saline (PBS; Sigma-Aldrich) and trypsinized according to Kosmehl et al. [19], using 0.05 % trypsin (Sigma-Aldrich) and 0.02 % ethylenediamine tetraacetic acid (EDTA; Sigma-Aldrich).

Micronucleus test with RTL-W1

For exposure, cells were transferred to 6-well plates containing ethanol-cleaned 12 mm diameter glass cover slips (Assistant, Sondheim, Germany) and incubated for 12 h in pure medium to allow for complete cell attachment. Subsequently, the medium was changed to trenbolone concentrations of 0.013 to 32 mg/L, which, in previous acute cytotoxicity tests,

had been shown to not induce more than 10 % lethality in the neutral red assay [20]. Exposure was carried out for 24 h at 20 °C. Post-incubation and fixation were conducted as described by Schnurstein and Braunbeck [21]. Subsequent to fixation, the slides were stained for 1 min with undiluted Giemsa stain (Gurr, BDH Laboratory Supplies, Poole, UK) and covered with DePeX (Serva, Heidelberg, Germany). After 24 hours of solidification, the slides could be scored.

Per slide, 2000 cells were analyzed. Micronuclei were counted under an Aristoplan light microscope (Leica, Germany) equipped with an oil-immersion lens at 1200x magnification. Criteria for micronuclei in RTL-W1 cells were set as follows: a) Maximum size of micronuclei must not exceed 30 % of the main nucleus. b) Micronuclei and nuclei should stain similarly. c) Micronuclei should be clearly separated from the nucleus. d) Only cells with good cytoplasmatic outlines were used for reading [22]. Results were recorded as percentage of cells containing micronuclei relative to the total number of counted cells. The induction factor for each site was calculated by computing the percentage of micronuclei from exposed cells to the percentage of micronuclei in the negative controls. Statistical significances were assessed by using the Chi-square test with Yates correction [22-23]. For each concentration, the number of cells with micronuclei was compared to that of cells without micronuclei and to the corresponding negative controls by means of SigmaStat 3.5 (SPSS-Jandel Scientific, Erkrath, Germany). Values differing significantly from the negative control were marked in graphs with degrees of freedom from $p \leq 0.05$ with one asterisk (*), for $p \leq 0.01$ (**) and for $p \leq 0.001$ (***)

Comet Assay

The comet assay was performed under alkaline conditions following the original procedure of Singh et al. (1988) with modifications according to Schnurstein and Braunbeck [21] using trenbolone concentrations at a range between 0.125 and 32 mg/L.

UV light (240 - 280 nm for 5 min) was used as a positive control. For exposure to trenbolone, cells were transferred to 6-well plates (TTP Renner, Dannstadt, Germany) and incubated for 12 h in uncontaminated medium to allow for complete cell attachment. Afterwards, the medium was changed to different trenbolone concentrations, which, in previous acute toxicity tests, had been shown to not induce more than 20 % lethality in the neutral red assay [20]. Exposure was carried out for 24 h at 20 °C. After incubation, cells were washed with PBS, trypsinized and processed for the comet assay.

In order to guarantee for optimal adhesion, fully frosted slides (Langenbrink, Emmendingen, Germany) were used; they were cleaned in 99 % ethanol and coated with 1 % normal melting agarose (NMA; SeaKem, FMC Bioproducts, Rockland, USA), which was hardened for 5 min at 37 °C and scraped off afterwards. This procedure increases the adhesion of the following 0.5 % NMA layer. Cells were embedded in 0.7 % low melting agarose (LMA; SeaKem, FMC Bioproducts) layers on the pre-coated slides and again coated with an additional layer of 0.7 % NMA. Slides were cooled on ice for 3 min and dried at 37 °C for 5 min, followed by lysis in 100 mM EDTA, 2.5 M NaCl, 1% Triton X-100 and 10 % DMSO (pH 13.0) for 1.5 h in the dark at 4 °C. After electrophoresis in the same buffer at 25 V and 310 mA for 20 min, samples were neutralized by incubation in 400 mM Tris at pH 7,4 for 2 min. Slides were either analyzed directly or stored in a humid box (PBS) for at maximum 8 days at 4 °C. Immediately before scoring, the DNA was stained with 75 µl of 20 µM ethidium bromide (Sigma-Aldrich) and covered with a cover slip.

All slides were examined at 320 x magnification using a Leica Aristoplan fluorescent microscope equipped with an excitation filter of 518 nm and an image analysis system (Bfi Optilas, Munich, Germany) with a grey-scale CCD camera (Optilas) and the Comet 5.5 software (Kinetic Images, Liverpool, UK). For each concentration, the tail moments of 100 randomly selected cells were analyzed on each of 3 slides.

For statistical analysis, data were analyzed with the H-test according to Kruskal and Wallis (SigmaStat 3.5). In cases of significant differences, a post-hoc test according to Dunn was used to identify groups differing significantly. The induction factor (IF) was calculated by dividing the median of each concentration by the median of the corresponding control group.

6.3.3 Genotoxicity tests with zebrafish embryos

Maintenance and egg production of parent zebrafish

Sexually mature zebrafish (*Danio rerio*) were kept in communities of up to 18 individuals in 25 L tanks under flow-through conditions in water with the following characteristics: 27.0 ± 0.5 °C temperature, 744 µS conductivity (370 mg/L CaCO₃ hardness), pH 7.5 ± 0.2 , 10.5 ± 0.5 mg/L O₂ (95 % saturation), and a 12 h light/12 h dark photoperiod [24]. Ammonia, nitrite, and nitrate were kept below the detection limits (0 - 5, 0.025 - 1 and 0 - 150 mg/L, respectively). Fish were fed twice daily a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany) or nauplii from *Artemia* sp. (Great Salt Lake Artemia Cysts, Sanders, Ogden, USA). The day before a test, 9 males and 9 females were placed in 22 L

breeding chambers (water conditions as above) immediately before dusk. Artificial plants served as breeding stimulant and substrate. Spawning and fertilization took place within the first 30 min after the onset of light in the morning. A fertilization rate of $\geq 80\%$ served as a quality control for adequate test materials.

Trenbolone exposure

About 1 h post fertilization, the fertilized eggs can easily be separated from non-fertilized opaque eggs [24]. By using a 2 ml plastic pipette with a widened opening, 12 fertilized eggs were selected and transferred to a 100 ml Petri dish containing 50 ml artificial water according to ISO 7346/3 and trenbolone at concentrations of 0.03, 0.06, 0.13, 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/L. A survival rate of 80 % at any concentration was a prerequisite for use of the egg batch in the micronucleus assay. Eggs were incubated at 27.0 ± 0.1 °C in the dark to prevent photooxidation of trenbolone. After 7 days, 7 - 8 embryos were processed for cell isolation and the micronucleus assay.

Cell isolation

Cell isolation was carried out mechanically according to the protocol by Kosmehl *et al.* [25]. For each slide in the comet assay, 7 - 8 zebrafish embryos were used as donors. A glass/glass tissue grinder (Potter-Elvehjem-type, Braun Biotech, Sartorius, Goettingen, Germany) with a defined pestle/wall distance of 50 - 70 μm was used to separate the cells in 2 ml PBS (Sigma-Aldrich) and 5 % fetal calf serum (Sigma-Aldrich) from each other. Grinding was carried out with four gentle strokes, each with a maximum rotation angle of 90°. The resulting cell suspensions were resuspended and filtered through 70 μm gauze (Verseidag, Krefeld, Germany) into reaction tubes in order to separate the individual cells from remaining pieces of connective tissue. This was followed by centrifugation at 150 g at 4 °C for 10 min (Multifuge 1 S-R, Heraeus-Fisher Scientific, Hanau, Germany).

Micronucleus assay with cell suspensions obtained from zebrafish embryos

Following exposure of embryos to trenbolone, the micronucleus assay was carried out with trenbolone concentrations inducing less than 20 % mortality (i.e., ≤ 8 mg/L). In any case, overlapping of acute toxicity and mutagenicity had to be excluded.

The cell suspensions were fixed within the reaction tubes with a 4:1 methanol/acetic acid-mixture. In order to ensure optimal adhesion, slides (Langenbrink, Emmendingen, Germany) were pre-cleaned in 99 % (v/v) ethanol. After drying, the slides were either analyzed directly or stored in a glass box for at maximum 8 days. Immediately before scoring, the DNA was stained with 20 µl of 20 µM acridine orange (Sigma-Aldrich) in aqua bidest. and covered with a cover slip. Statistical significances were assessed by using the Chi-square test with Yates correction [22-23].

Comet assay with cell suspensions obtained from zebrafish embryos

The comet assay was conducted in parallel to the micronucleus assay. The cell suspension was treated as described for the permanent cell line RTL-W1.

6.4 Results

6.4.1 Micronucleus assay

Genotoxicity of trenbolone in the micronucleus test with RTL-W1 cells

In a range-finding test, RTL-W1 cells were exposed to concentrations between 0.01 and 32 mg/L trenbolone. At 4 mg/L, the micronucleus induction rate was not significantly elevated over controls, whereas between 0.03 and 1 mg/L each concentration showed significant increases. Therefore, in subsequent experiments, RTL-W1 cells were exposed to concentrations of 0.01 to 0.2 mg/L trenbolone in three independent replicates, whereas concentrations between 0.25 and 32 mg/L were tested in two replicates. Previous studies had documented an onset of cytotoxicity from 33 mg/L for trenbolone (details not shown).

Exposure to 0.025 - 1 mg/L trenbolone resulted in a significant increase in micronucleated cells (Fig. 6.1). At very low concentrations of 0.1 and 0.125 mg/L, each replicate showed a distinct peak in micronucleus induction with induction factors of approx. 2.2. At 0.2 mg/L, the micronucleus rate decreased to an induction factor of 1.5. The second induction peak (induction factor = 2.3) was observed at 1 mg/L trenbolone. At 2 mg/L, the micronucleus frequency of only one replicate was elevated significantly; at higher concentrations, micronucleus rates decreased to control levels.

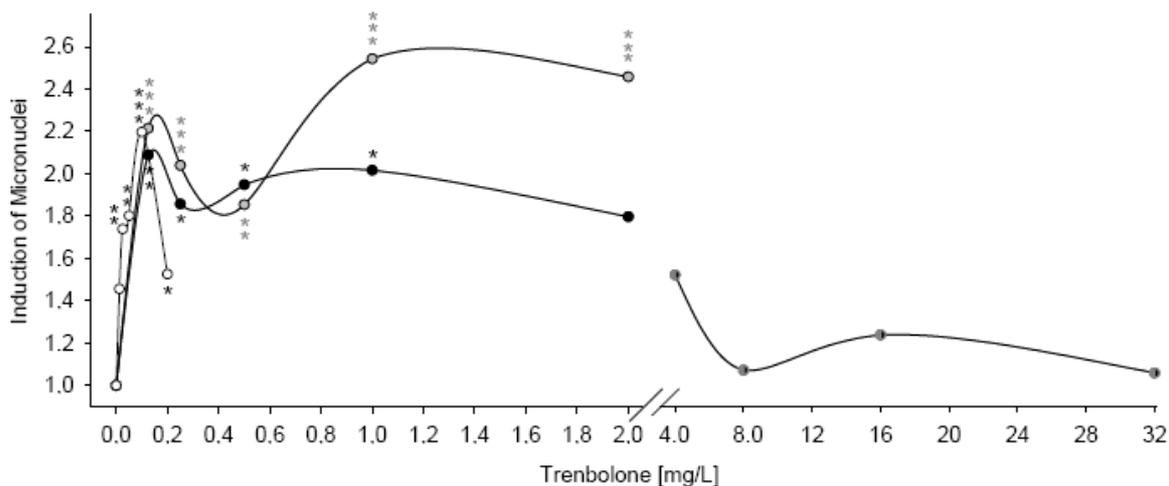


Fig. 6.1: Induction of micronucleus formation in RTL-W1 cells by trenbolone. Each line represents the induction of micronuclei after exposure to trenbolone at a range of concentrations. Asterisks: Values differ significantly from the negative control (Chi-square test with Yates correction; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

*Genotoxicity of trenbolone in the micronucleus test with primary cell cultures from *Danio rerio* embryos*

The micronucleus assay with zebrafish embryo-derived cell suspensions was carried out in duplicate at concentrations between 0.03 and 8 mg/L (Fig. 6.2). Since previous range-finding tests had revealed no significant elevation in the frequencies of micronuclei at concentrations between 0.5 and 8 mg/L trenbolone, the two highest concentrations (4 and 8 mg/L) were tested only once. A significant, but consistent increase in micronuclei over negative controls was observed at 0.25 mg/L trenbolone only.

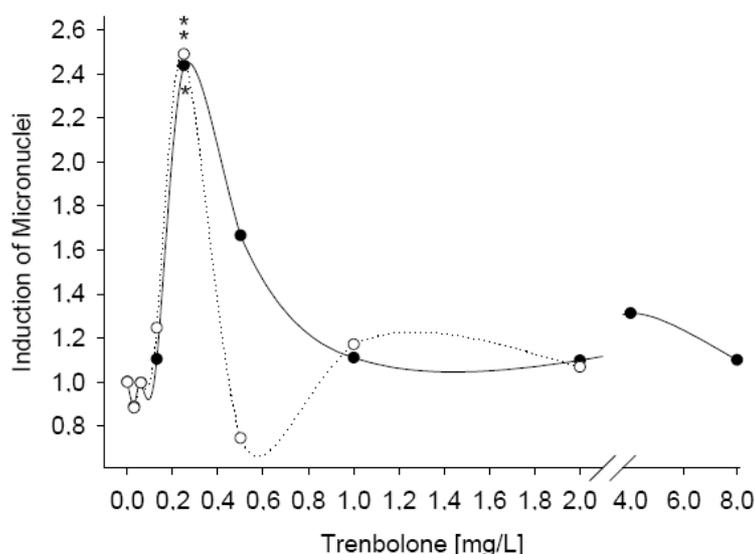


Fig. 6.2: Induction of micronucleus formation in primary cells derived from 168 h old zebrafish (*Danio rerio*) embryos exposed to trenbolone. Each line represents the induction of micronuclei after exposure to trenbolone at a range of concentrations. Asterisks: Values differ significantly from the negative control (Chi-square test with Yates correction; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

6.4.2 Comet assay

Genotoxicity of trenbolone in the comet assay with RTL-W1 cells

Two independent replicates for concentrations between 0.125 and 2 mg/L revealed a significant increase in comet induction rates at 0.5 mg/L trenbolone (Fig. 6.3). RTL-W1 cells were also exposed to concentrations from 4 to 32 mg/L, however, without significant increases in tail moments

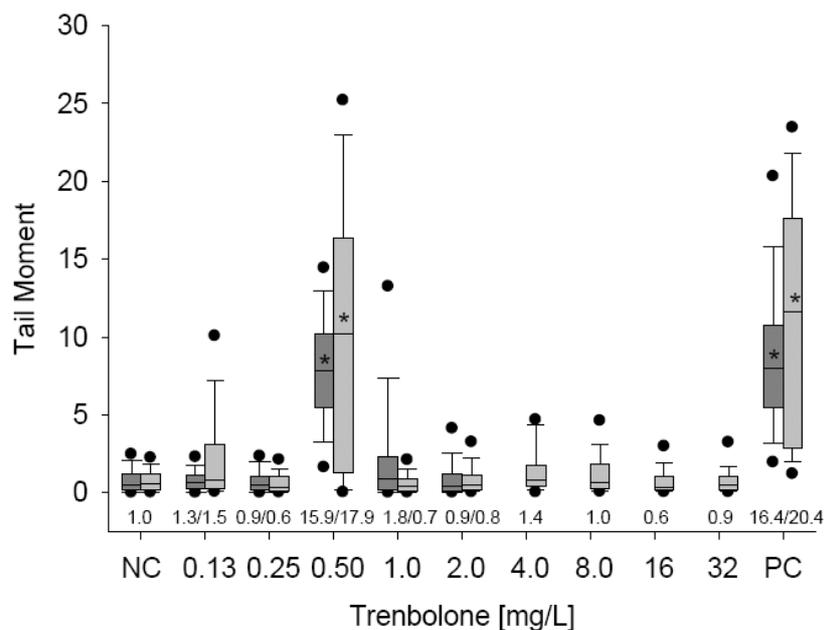


Fig. 6.3: DNA damage expressed as tail moments (bars) and induction factors (digits) in RTL-W1 cells exposed to a concentration range of trenbolone. Genotoxicity is given as box plots of 100 cells per concentration from three replicate slides (100 cells each). As a positive control (PC), cells were exposed to UV light (240 - 280 nm for 5 min). The box plots show the medians, standard deviations, 25 % and 75 % percentiles (upper and lower limit of the bar), 5 % and 95 % percentiles (dots). * Significantly different from negative control (NC; Dunnett's test, $p < 0.05$).

Genotoxicity of trenbolone in comet assay with suspensions from zebrafish embryos

Zebrafish embryos were exposed to concentrations of 0.03 – 2.0 mg/L trenbolone in two independent replicates (Fig. 6.4); only one test run was performed for 4 and 8 mg/L trenbolone. The tail moment was significantly elevated at the two lowest concentrations of 0.03 and 0.06 mg/L, but no significant increase was observed for 0.12 mg/L. A second positive result was obtained at 0.25 mg/L trenbolone. Even though the induction was lower than the induction levels at 0.03 and 0.06 mg/L, there was a significant difference in comparison to the negative control in both test runs. At a concentration of 0.5 mg/L, one replicate was significantly increased over controls. No significant increase in comet formation could be revealed at any higher concentration.

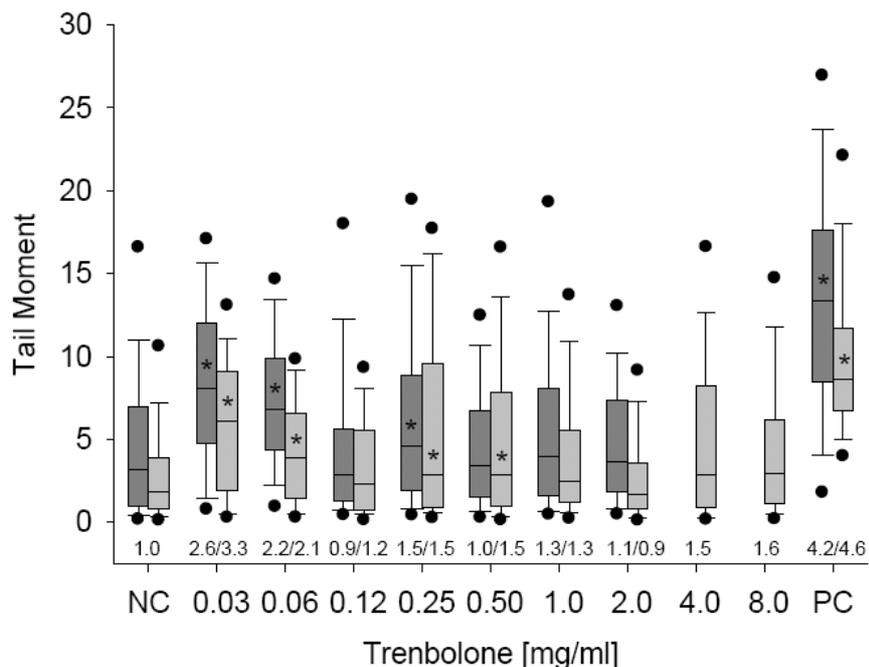


Fig. 6.4: DNA damage expressed as tail moments (bars) and induction factors (digits) in primary cells derived from 168 h old zebrafish (*Danio rerio*) embryos exposed to a concentration range of trenbolone. Genotoxicity is given as box plots of 100 cells per concentration from three replicate slides (100 cells each). As a positive control (PC), embryos were exposed to 0.1 % H₂O₂ for 1 h. The box plots show the medians, standard deviations, 25 % and 75 % percentiles (upper and lower limit of the bar), 5 % and 95 % percentiles (dots). * Significantly different from negative control (NC; Dunnett's test, $p < 0.05$).

6.5 Discussion

6.5.1 Mutagenicity of trenbolone in the micronucleus assay

Trenbolone induced micronuclei both in RTL-W1 cells and in primary cell suspension derived from zebrafish embryos. Nevertheless, the micronucleus assay with freshly isolated zebrafish cells proved less sensitive with an LOEC (Lowest Observed Effect Concentration) 10-fold higher than the LOEC for RTL-W1 cells (0.25 and 0.025 mg/L, respectively). Most importantly, however, the LOEC of 0.25 mg/L for zebrafish embryo-derived cells was also the only concentration with a significant increase in micronuclei. In the micronucleus assay with RTL-W1 cells, two peaks with significant micronucleus inductions were observed at 0.1 and at 1 mg/L. In contrast, at a concentration range between 4 and 32 mg/L, no significant induction of micronuclei could be observed. This biphasic induction of micronuclei might be explained by assuming different modes of genotoxicity at different trenbolone concentration ranges. These findings may be in line with reports by Schiffmann et al. [26], who revealed a strong cell line-dependent mutagenicity for trenbolone: There was a dose-related increase of micronuclei in Syrian hamster embryo (SHE) cells, but no effects in mouse embryo

fibroblasts (C3H10T1/2) in the same concentration range. For SHE cells, they determined an LOEC of 1.3 mg/L trenbolone, which is about 52-fold higher than the LOEC determined for RTL-W1 cells. However, the lowest test concentration of Schiffmann *et al.* ([26]; 0.27 mg/L) was within the range between 0.2 and 0.5 mg/L, i.e. exactly where our experiments also showed a decline in micronucleated cells. Since Schiffmann *et al.* [26] did not test lower concentrations, the existence of a second peak of micronucleus formation in SHE cells at lower concentrations cannot be excluded.

In fact, trenbolone has been tested repeatedly for its mutagenicity in the micronucleus test [10,27], but except for Schiffmann *et al.* [26], lower concentrations have never been tested. Therefore, the general conclusion by Schiffmann *et al.* [26] has been that trenbolone, if at all, induces micronuclei only at very high concentrations. Our results confirm that trenbolone does not induce micronuclei in RTL-W1 cells at high concentrations between 4 and 32 mg/L. Further testing of the mutagenic potential of trenbolone, however, should clearly extend to concentration ranges below 1 mg/L in order to also cover low-dose effects.

6.5.2 Genotoxicity of trenbolone in the comet assay

Trenbolone revealed a significant genotoxic potential in comet assays with both RTL-W1 cells and zebrafish embryo-derived cell suspensions. The tail moment for RTL-W1 cells exposed to 0.5 mg/L trenbolone consistently exceeded control values by factors of 16 to 18. Surprisingly, this very strong induction was the only significant increase in tail moments; neither exposure to lower (0.13 - 0.25 mg/L) nor to higher concentrations (1 - 32 mg/L) produced an induction of DNA fragmentation. The result thus corroborates trends observed in the micronucleus assays with primary zebrafish cells. The similarity of all bioassays applied in the present study is, that, in contrast to low-dose effects, they showed no or hardly any effect of trenbolone exposed to the test systems in concentrations exceeding 1 mg/L. The inverse U-shaped dose-response relationship in the comet assay with *Danio rerio* was, therefore, comparable to that of micronucleus induction rates in RTL-W1 cells. Thus, both test systems followed a biphasic induction pattern with a first peak at very low concentrations followed by a second peak at much higher concentrations. These findings are in accordance with Schiffmann and colleagues [26] who observed a maximum transformation frequency in SHE cells at concentration of 2.5 - 5 mg/L trenbolone, whereas at higher concentrations (7.5 - 15 mg/L), a marked decline in cloning efficiency was noted. Although the effect

concentrations in the present study are located in a lower concentration range, the shape of the dose-response curve appears similar.

However one point which should be taken into consideration is that neither in the micronucleus nor in the comet assays were steps taken to observe the influence of trenbolone on the duration or success of cell proliferation in RTL-W1 cells. If the cell cycle was not completed, the cells did possibly not pass through the sensitive phase (e.g. the synthesis phase), and, thus, trenbolone did not cause any detectable genotoxic or mutagenic effect at higher test concentrations. Therefore, it is possible that the lack of genotoxicity in RTL-W1 cells at the higher test concentrations is related to altered division kinetics caused by the cytotoxic or cytostatic effect of the test agent. However, this explanation is not applicable for the comet and the micronucleus assays with zebrafish embryos, since the embryos showed normal development at all concentrations with any sign of a delay in cell proliferation. However, no genotoxic or mutagenic effect at higher concentrations was observed. A next step would be to check if concentrations above 1 mg/L alter cell division, e.g. by applying the cytokinesis-block micronucleus technique [28].

6.5.3 Do different biotransformation capacities affect genotoxicity of trenbolone?

Permanent cell lines and primary zebrafish cell suspensions may be assumed to have different biotransformation capacities: The exposure of intact fish for example always represents a more realistic exposure scenario with higher ecological relevance than *in vitro* exposures. Nevertheless, in several studies, the cell line RTL-W1 showed good transformation potentials to bioactivate indirect mutagens *via* CYP 1A monooxygenases [17,29-31]. In the present study, the different sensitivities of a permanent cell line compared to a primary cell suspension were investigated.

Differences in exposure time and test system had remarkable consequences on the rate of genotoxicity of trenbolone. However, relative sensitivities of the test systems strongly depended on the endpoints assessed: RTL-W1 cells were significantly more sensitive for the induction of micronuclei than for the induction of a tail moment in the comet assay. The relatively higher sensitivity of the cell line to micronucleus formation might be due to mutational events resulting from misrepaired DNA lesions, which may not be detected by the comet assay, as the DNA chain is not disrupted [32]. The micronucleus assay on the other hand detects misrepaired DNA double-strand breaks, which can lead to symmetrical and

asymmetrical chromatid and chromosome exchanges as well as chromatid and chromosome fragments [33].

This mechanistic explanation does not comprise the micronucleus assay and the comet assay with primary zebrafish embryo cells, since the micronucleus assay showed a lower sensitivity than the comet assay with zebrafish exposed *in vivo*. However, the differences between the test systems might be correlated not only to higher detoxification capacities of the embryos, but also to differences between DNA repair mechanisms in seven day old zebrafish and the permanent cell line RTL-W1. With respect to bioavailability of trenbolone, first the chorion and second the epidermis of the zebrafish could serve as barriers for xenobiotics, whereas the liver-derived fibroblasts from the rainbow trout are directly exposed to trenbolone. Additionally, the zebrafish embryo-derived cell suspension mostly consists of muscle cells and fibroblasts, since they form the major part of the fish body. However, since metabolization of trenbolone mainly takes place in endothelial and liver cells, these organs might provide a more sensitive response than muscle cells. Additional reasons might be found at the cellular level: DNA damage measured in the comet assay does not necessarily lead to a mutation of the genome. Some of the lesions detected *via* the comet assay are reparable, or may even be caused by actual repair mechanisms [34]. In contrast, the micronucleus test detects only irreplaceable losses of DNA. Therefore, the tail moments measured in the comet assay after exposure to trenbolone might be based on repair mechanisms. A disorder of the spindle apparatus is unlikely, since this would not explain the genotoxicity measured in the comet assay. If trenbolone affects the spindle apparatus, zebrafish embryos are obviously able to induce repair mechanisms before karyogenesis is affected. The high activity in the comet assay with primary cells, but the little reaction in the micronucleus assay could also be explained by a defect in the genome leading to an induction of repair mechanisms.

Yet, the mechanism behind the very concentration specific induction of micronuclei in *Danio rerio* and of tail moments in RTL-W1 remains unclear. A similar inverted U-shaped dose-response curve has been observed for the expression of *CYP19a* mRNA in several warm water species after exposure to 17 α -ethinylestradiol (EE2). Relatively low concentrations of 10 to 100 ng/L caused an up-regulation of *CYP19a* expression in fathead minnow and Japanese medaka (*Oryzias latipes*) [35-37]. Other studies have found that exposure to relatively high concentrations of estrogens resulted in down-regulation of *CYP19a* expression in adult zebrafish (5 μ g/L of EE2 [38]), juvenile zebrafish (app. 30 μ g/L of EE2 [39]), and embryonic zebrafish (app. 270 μ g/L of EE2 [40]). The mechanism of EE2 interaction with *CYP19a* expression is unknown, as well. However, one suggestion is that the down-regulation

is a direct effect of EE2 on gametogenesis in teleost zebrafish [39, 41]. This example confirms the existence of inverted U-shaped dose-response curves in fish after exposure to endocrine active substances. However, further work is required to understand the mechanism of action.

6.6 Conclusions

Results provide evidence of non-linear genotoxic and mutagenic potentials of trenbolone in a very low concentration range. Since fish and other aquatic organisms are potentially exposed to trenbolone in runoff from cattle feedlots, manure or sewage discharge [16], genotoxic/mutagenic effects by trenbolone in aquatic ecosystems cannot be ruled out. Fish take a top position in the aquatic food web and serve as a food source for man; thus, there might be a risk to both environment and man. In the US, the total number of cattle and calves was 100.8 million head in July 2010 [42]. The estimated recovered fraction of 17 β -trenbolone ranges between 0.3 and 2.7 % in solid dung, 0.4 % in liquid manure and 0.001 % in effluent (calculated according to Schiffer [16]). Given that > 90 % of the beef cattle raised in the US receive one or more implants during their growth period [43], at least 4 to 20 tons of trenbolone acetate is applied every year. In addition, recent trends in the US indicate a shift from small diversified farms to large-scale intensive and confined breeding and feeding operations [44], which in many cases will include a concomitant increase in the rate of manure applied due to the lack of available nearby land [45]. Thus, it is not unlikely that concentrations of trenbolone causing genotoxic effects can occur in the environment. Since trenbolone metabolism in fish and the mechanisms leading to genotoxicity in fish are only poorly understood, further experiments are required to fully assess the genotoxic hazard potential of trenbolone to humans and the aquatic environment.

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Chapter 7

Uptake and Distribution Pattern of Trenbolone in 72 h – 2 Weeks Old Zebrafish

Authors: Melanie Boettcher, Thomas Braunbeck

in preparation

7.1 Abstract

In the United States, 11.8 million cattle and calves are maintained in large feedlots in November 2007. 90% of these animals are treated with natural and/or synthetic hormones. Trenbolone acetate, used in combination with estradiol is the most effective growth promoter available to cattle feeders. Nevertheless, almost nothing is known about the metabolism and distribution pattern of trenbolone in fish or other aquatic animals which can be directly exposed to runoffs from farms using large concentrations of pharmaceutical agents, such as cattle feedlots.

Since the zebrafish has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology it was chosen as object to study. The embryonic development of zebrafish was described in detail, so that possible target organs and tissues of trenbolone are easily to trace.

The present study aims to give an insight of the distribution pattern and target organs of trenbolone in different developmental stages of zebrafish. Trenbolone accumulated in tissues at different tissues of the zebrafish, without any linkage to the developmental stage. At this, the heterogeneously stained muscle fibres of the hatched larvae hints to a attachment of trenbolone mainly to the actin fibres in the cells. Furthermore, the cytoplasm of various tissues responded to the antibody staining, while the nucleus remained unstained. Actin is ubiquitous in the cytoplasm as it participates in many important cellular functions. The fact that steroids seems to accumulate in the cytoplasm is in accordance with the investigations made for a human cell line: Only approximately 12% of a previously radioactive labelled steroid internalized by the cells appeared bound to androgen receptor in the nucleus. The unbound steroid remained largely in the cytoplasmic fraction. This study demonstrates that trenbolone is easily accessible for all tissues of the embryonic and larval stages of zebrafish.

7.2. Introduction

Trenbolone is an alternative model androgen that recently has been used in fish studies (Ankley *et al.*, 2003; Peterson *et al.*, 2001). Trenbolone is a high affinity ligand for the fathead minnow androgen receptor (AR) and is quite potent with regard to effects on reproductive endocrinology in this species (Ankley *et al.*, 2003). Additionally, trenbolone shows strong binding affinity to the human androgen, the bovine progestin and the bovine glucocorticoid receptor (Bauer *et al.*, 2000). As opposed to methyltestosterone, the former

model androgen, trenbolone is not a suitable substrate for conversion to an estrogen analogue by CYP19 aromatase (Ankley and Johnson, 2004). Extensive studies evaluating trenbolone as a model endocrine disrupting chemical (EDC) recently have been conducted with the fathead minnow, Japanese medaka, and zebrafish (OECD, 2004).

Previous studies detected 12 % of previously applied 17 β -trenbolone acetate in liquid manure and 20 % in solid dung after application of implants to heifers (European Commission, 2002). The active compound 17 β -trenbolone belongs to the group of most efficient anabolic steroids and is known to masculinise fish after exposure *via* food and water (Holbech *et al.*, 2006). Nevertheless, almost nothing is known about the metabolism and distribution pattern of trenbolone in fish or other aquatic animals which can be directly exposed to runoffs from farms using large concentrations of pharmaceutical agents, such as cattle feedlots.

The zebrafish was chosen as object to study, it has become a major model in neurobiology and toxicology as well as in general molecular and developmental biology (Ekker and Akimenko, 1991; Goolish *et al.*, 1999; Hisaoka and Battle, 1958; Kimmel *et al.*, 1995; Laale, 1977; Lele and Krone, 1996; Nüsslein-Vollhard, 1994; Roosen-Runge, 1938; Sander and Baumann, 1983; Westerfield, 2000; Wixon, 2000). The species is easily obtainable, inexpensive, readily maintainable and, under appropriate conditions, yields a large number of non-adherent, fully transparent eggs (Laale, 1977). The embryonic development of zebrafish was described in detail (Hisaoka and Battle, 1958; Kimmel *et al.*, 1995; Roosen-Runge, 1938; Thomas and Waterman, 1978), so that possible target organs and tissues of trenbolone are easily to trace.

The present study aims to give an insight of the distribution pattern and target organs of trenbolone in zebrafish. As the eggshell is suggested to serve as possible barrier for lipophilic substances, not hatched stages as well as hatched stages of the zebrafish were investigated.

7.3 Material and methods

7.3.1 Maintenance and egg production of zebrafish

Sexually mature zebrafish (*Danio rerio*) were kept in communities of up to 18 individuals in 25 L tanks under flow-through conditions in water with the following characteristics: 27.0 \pm 0.5 °C temperature, 744 μ S conductivity (370 mg/L CaCO₃ hardness), pH 7.5 \pm 0.2, 10.5 \pm 0.5 mg/L O₂ (95 % saturation), and a 12 h light/12 h dark photoperiod. Ammonia, nitrite, and nitrate were kept below the detection limits (0 – 5, 0.025 – 1 and 0 – 150 mg/L, respectively).

Fish were fed a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany) or nauplii from *Artemia* sp. (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Ogden, USA) twice daily. The day before a test, 9 males and 9 females were placed in 22 L breeding chambers (water conditions as above) immediately before dusk. Artificial plants served as breeding stimulant and substrate. Spawning and fertilization took place within the first 30 min after the onset of light in the morning. A fertilization rate of $\geq 80\%$ served as a quality control for adequate test materials.

7.3.2 Trenbolone exposure

About 1 h post fertilisation, the fertilised eggs can easily be separated from non-fertilised opaque eggs. By using a 2 ml plastic pipette with a widened opening, 50 fertilized eggs were selected and transferred to a 100 ml Petri dish containing 4.5 ng trenbolone diluted in 50 ml artificial water (90 ng/L). Eggs were incubated at 27.0 ± 0.1 °C in the dark to prevent photooxidation of trenbolone. After 70 hours (immediately before hatch), 72 hours (immediately after hatch), one week and 2 weeks 10 eggs were 7 days, 10 embryos each were processed for kryostat sectioning and subsequently for the immunohistological staining.

Fixation and sectioning and antibody staining of the samples

The exposed fish were anesthetized with 1,1,1-trichlor-2-methyl-2-propanol-hemihydrate (10 g/L). Afterwards the animals were aligned, snap-frozen in nitrogen (at -120 °C) and immediately stored at -20 °C until sectioning. The sectioning was performed with knife (knife profile C; Leica Microsystems, Nussloch, Germany) in a cryostat at -20 °C. Fish were sectioned into 10 μm thick slices, mounted on gelatine coated microscope slides (Firma Ort). Cryostat sections (10 μm) were fixed in acetone for 10 minutes at room temperature, blocked with Chemiblocker (5 % FCS, 0.5 % triton x-100 diluted in PBS) for 1 h. Additionally the detergents triton x-100 permeabilised the cells. Afterwards, sections were incubated with 1:200 diluted sheep polyclonal antibodies (abcam, Cambridge, UK) over night. Normal fetal calf serum was used as negative control.

The slides were then stained for 90 min at room temperature using a 1:400 dilution of Alexa Fluor 488 donkey antisheep IgG (invitrogen GmbH Karlsruhe, Germany). After washing the slides with PBS and drying the sections were covered with aqueous mounting medium (Firma, Ort) and a cover slip and dried over night.

7.4 Results

7.4.1 Not hatched embryo

An overview of the immunohistological staining revealed an accumulation of trenbolone at the chorion and at the yolk. By taking a closer look at the eggshell, it can be seen that trenbolone accumulates especially at the boundaries of the chorion pore channels (fig. x). Head and tail of the embryos are stained less, but still show an immunohistological response to the antibodies. In the head region, the cytoplasm of the brain cells is coloured, whereas the cell nucleus remains unstained. Compared to that the muscle fibres are coloured quite homogeneously. Nevertheless the outside of the fibres are coloured more intensive than the inside.

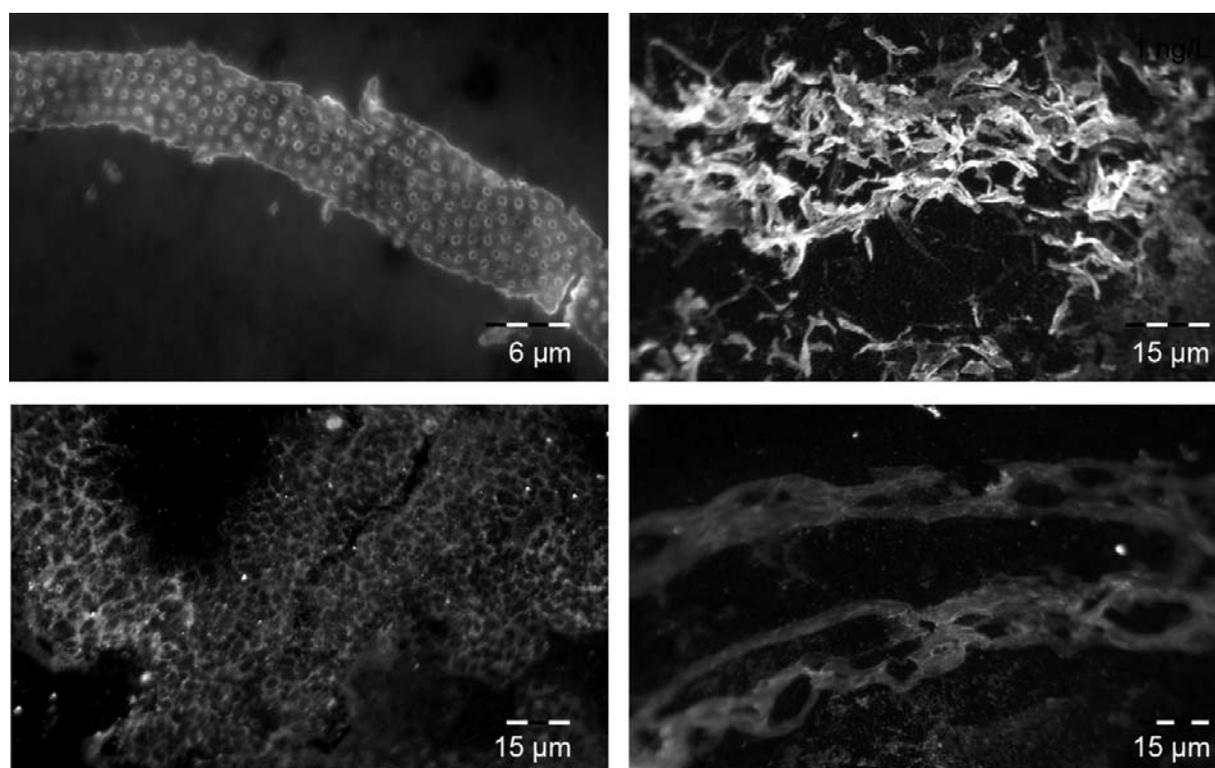


Fig. 7.1: Longitudinal sections (head to tail) of 72 h old embryos immediately before hatch. A. Inner side of the eggshell with chorion pore canals. B. Yolk sac of the not hatched embryo. C. Detail picture of the head region. D. Arterial canal surrounded by tail musculature. Trenbolone antibody staining with Alexa 488.

7.4.2 Hatched embryos

No differences in immunohistological staining sensitivity or distribution for any of the observed tissues were observed at the different times of investigations after hatch.

The brain cells were dyed as described for the not hatched embryos. The cartilage in the branchial arches was stained equally in the gills and the jaws. Depending on the age and the branchial arch investigated, different parts were ossified. The not ossified parts were stained at the boundaries and/or all over (fig x.). The heart is located in the ventral pharyngeal region. The immunohistological staining showed an illumination of the atrium wall and the epithelial cells. Looking at the ventricle wall the staining is much less intense. By studying the antibody stained liver sections it could be observed that only the cytoplasmatic part of the hepatocytes was stained. The nucleus as well as the cell boundaries remained unstained. Taking a closer look to the antibody distribution in the muscle fibres it can be seen that in the longitudinal section only the outer boundaries, consisting of actin filaments are stained (fig. x). These observations can be confirmed by observing the cross section (fig. x). As shown for the longitudinal section, the myosin filaments are not stained, while the actin filaments, as well as the connective and nervous tissue are coloured. The gut also displays stronger and less coloured parts. Regarding the position and the histological toluidin stained sections, the inner strongly illuminated parts of the gut are the supranuclear vacuoles in the enterocytes, whereas the nucleus remains unstained as well.

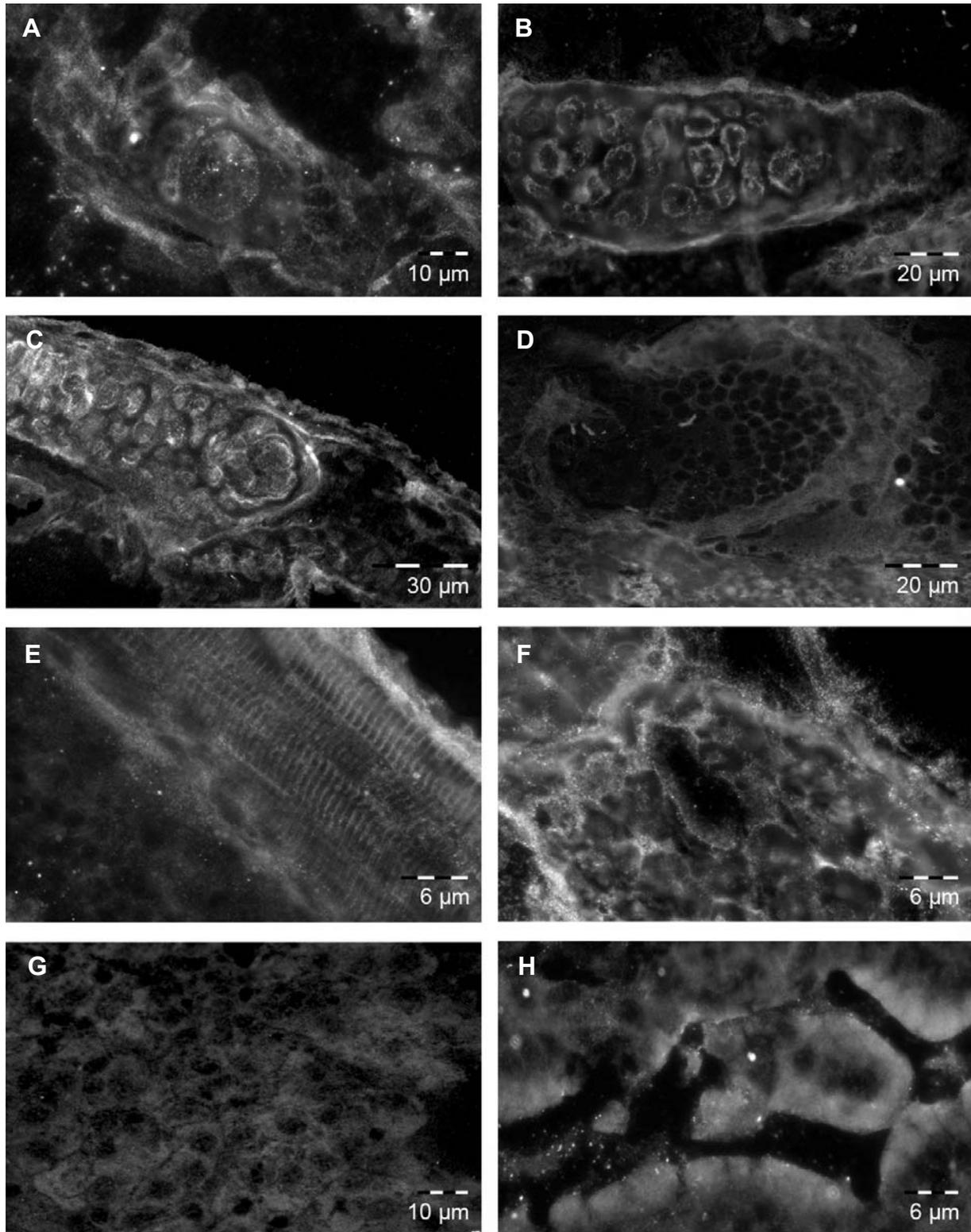


Fig. 7.2: Longitudinal sections (dorsal-ventral) of 8 (A, B, D-H) and 14 day (C) old embryos. A and B. Light optical microscopic pictures of a branchial arches in different developmental stages. C. Cartilage of a lower jaw. D. Atrium of the heart. E and F. Skeletal and smooth musculature in the zebrafish tail. G. Liver tissue. H. Longitudinal section of the mid gut. Trenbolone antibody staining with Alexa 488.

7.5 Discussion

Based on the partition coefficients of 1040 ($\log K_{ow} = 3$), 17 β -trenbolone is expected to have a medium lipophilicity and therefore a low potential for bioaccumulation in organisms in the environment (EPA 1984). Nevertheless, compared to the remaining tissues, the most lipophil tissue, the yolk, showed the strongest respond to the antibody staining. Besides that, trenbolone accumulated as well in the boundaries of the chorion pore canals. As the actual pore remained unstained the molecule must have attached to the walls of the canal. Braunbeck *et al.* (2005) assumed that the barrier function of the chorion may increase with lipophilicity. As the present method is not suitable for quantification of trenbolone residues within the eggshell, it cannot be clarified if the chorion partly functioned as barrier. The brain cells showed no differences in trenbolone distribution between the hatched and not hatched embryos. On the other hand, trenbolone showed no specific distribution pattern in the muscles of the not hatched embryo trenbolone, whereas the musculature of the hatched larvae was heterogeneously stained: The actin fibres responded to the antibody staining whereas the myosin parts remained unstained. The assumption that trenbolone binds to actin, could also account for the fact that in major part of the studied tissues (brain, liver, cartilage, heart/veins, gut, musculature), the cytoplasm responded to the antibody staining, while the nucleus remained unstained. Actin is ubiquitous in the cytoplasm as it participates in many important cellular functions (including cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signalling, and the establishment and maintenance of cell junctions and cell shape). Recent studies indicated association of actin with multiple nuclear complexes; however, the existence of actin in the cell nucleus is still controversial (Chen and Shen 2007). Furthermore, the fact that steroids seems to accumulate in the cytoplasm is in accordance with the investigations made by Fedoruk *et al.* (2004) for a human HeLa cell line: Only approximately 12% of the radioactive labelled steroid Dihydrotestosterone ($[^3\text{H}]\text{DHT}$) internalized by the cells appeared bound to androgen receptor (AR) in the nucleus. The unbound $[^3\text{H}]\text{DHT}$ remained largely in the cytoplasmic fraction (Fedoruk *et al.* 2004). These observations suggest that DHT may be held within the cell by macromolecules other than AR, but that AR may be required in order for $[^3\text{H}]\text{DHT}$ to be efficiently internalized. Furthermore, Fedoruk *et al.* (2004) suggested a complex relationship between the androgen transport through cell membrane, androgen accumulation in the cell, and the AR.

The variable strong illuminated parts of the sections might depend on a different concentration of trenbolone in the tissues and therefore with a varying cross reaction with the antibody staining. The heterogeneous distribution of trenbolone in the fish body can depend

on a number of parameters including the presence of multiple receptor subtypes with different binding affinities and differential tissue distributions of the receptor subtypes (Thomas 2000). The ER in croaker testis, for example has a higher affinity than the croaker liver ER for estrogens and xenoestrogens and may be more susceptible to chemical interference.

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Chapter 8

Environmental Effect Assessment for Sexual Endocrine-Disrupting Chemicals: Fish Testing Strategy

Authors: Thomas Knacker, Melanie Boettcher, Tobias Frische, Hans Rufli, Hans-Christian Stolzenberg, Matthias Teigeler, Sabine Zok, Thomas Braunbeck, Christoph Schäfers

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8.1 Abstract

Current standard testing and assessment tools are not designed to identify specific and biologically highly sensitive modes of action of chemicals, such as endocrine disruption. This information, however, can be important to define the relevant endpoints for an assessment and to characterize thresholds of their sublethal, population-relevant effects. Starting a decade ago, compound-specific risk assessment procedures were amended by specifically addressing endocrine-disrupting properties of substances. In 2002, the Conceptual Framework, agreed upon by OECD's Task Force on Endocrine Disrupters Testing and Assessment, did not propose specific testing strategies, and appropriate testing methods had not yet been developed and approved. In the meantime, the OECD Test Guidelines Programme has undertaken important steps to revise established and to develop new test methods, which can be used to identify and quantify effects of endocrine-disrupting chemicals on mammals, birds, amphibians, fish, and invertebrates. For fish testing of endocrine-disrupting chemicals, the first Test Guidelines have recently been adopted by the OECD and validation of further test systems is under progress. Based on these test systems and the experience gained during their validation procedures, we propose a 3-step fish testing strategy: 1) Weight-of-evidence approach for identifying potential sexual endocrine-disrupting chemicals; even after advanced specification of systematic criteria, this step of establishing initial suspicion will still require expert judgment; 2) *in vivo* evaluation of sexual endocrine disrupting activity in fish by applying *in vivo* fish screening assays; sufficient data are available to diagnose the aromatase inhibition and estrogen-receptor agonist mechanisms of action by indicative endpoints (biomarkers), whereas the ability of the respective biomarkers in the screening assay to identify the estrogen-receptor antagonists and androgen-receptor agonists and antagonists requires further validation; 3) characterization of sexual endocrine-mediated adverse effects including threshold concentrations; in cases when the most sensitive population-relevant endpoints and the most sensitive time window for exposure are known for the mechanisms of action, the fish full life-cycle or 2-generation test, which are the normal definitive tests, might be abbreviated to, e.g., the fish sexual development test. In the European Union, the measurement of indicative endpoints in the definitive test might be crucial for the authorization procedure under REACH and plant-protection products. The results of the definitive tests can be used in existing schemes of compound-specific environmental risk assessments

8.2 Introduction

Before entering into the subject of this article, and by analogy to the definitions provided by Escher and Hermens(2002), we would like to specify the use of the terms “mode of action” and “mechanism of action.” Mode of action in this paper is a set of physiological and behavioral signs characterizing the biological response caused by sexual endocrine disrupting chemicals (SEDCs); mechanism of action refers to the biochemical process or xenobiotic–biological interaction (e.g., estrogen-receptor agonism or aromatase inhibition) underlying a given mode of action. SEDCs are substances that interfere with the sexual development and reproduction of fish as outlined in more detail in the following sections of this paper.

The issue of endocrine disruption (ED) was brought onto the agenda of regulatory authorities by environmentalists and researchers dedicated to studying the impact of chemicals on aquatic organisms under field conditions (Smith 1981; Woodward et al. 1993; Purdom et al. 1994; Sumpter and Johnson 2005). Data from these and other authors raised concern, because it became obvious that current testing and assessment tools are not appropriate to identify specifically designed and biologically highly active chemicals, which may result in sublethal population-relevant effects insufficiently addressed by available standard test methods. To improve the basis for regulatory and responsible care-driven decisions in chemicals safety management, activities were initiated for compound-specific environmental risk assessments (ERAs) to amend testing tools and assessment concepts specifically addressing endocrine-disrupting properties. The need for such amendments is common to all major types of substances, i.e., industrial chemicals, active ingredients of plant-protection and biocidal products, and environmentally relevant human and veterinary pharmaceuticals. The starting point for an ED-specific testing strategy is the identification of a substance as potentially endocrine disruptive, which depends on varying information levels according to the legal requirements of compound-specific ERAs.

The main objective of this paper is to propose a strategy for how to use the new and revised OECD testing methods with fish, for a comprehensive and efficient ERA for SEDCs. Before doing that, we outline the current state of risk assessment schemes with regard to EDCs, and we summarize the latest status of the OECD Conceptual Framework for testing of EDCs with emphasis on fish tests, which are designed to identify subtle, population-relevant effects caused by SEDCs.

8.3 Status of risk assessment schemes with regard to ED issues

ERA schemes address endocrine-disrupting properties of compounds as an indication for possibly increased risks to the environment (see, e.g., EC 2002a, 2002b, 2003). In most cases, however, the tiered standard risk assessment starts with data from short-term ecotoxicological studies, which cannot provide safe effect thresholds for endocrine disruptors.

This conceptual shortcoming can be illustrated by, e.g., the human drug 17 α -ethinylestradiol. Regulatory acceptable environmental risks would be indicated if the risk characterization were based exclusively on short-term aquatic toxicity data; unacceptable risks become obvious only when using data from long-term effect studies with fish (Lange et al. 2001; Wenzel et al. 2001). In the guideline on ERA of medicinal products for human use, the European Medicines Agency (EMA) recommends long-term instead of short-term ecotoxicity tests as the starting point for effects assessment (EMA/CHMP 2006). This modification provides an improved level of information (e.g., information on reproduction) for revealing ED-related effects for human drugs. The rationale provided by EMA for proposing long-term tests, however, is based not on the interest in revealing ED activities of a compound but on the fact that human pharmaceuticals are continuously released to the aquatic environment via effluents from sewage treatment plants, so long-term exposure of organisms is a given, which should be compared with results from long-term effect studies. Moreover, pharmaceuticals are designed to exert biological highly specific sublethal activities, which are not adequately covered by standard testing methods for acute toxicity.

In cases when there is evidence that a compound shows endocrine-disrupting properties, adaptations of the ERA schemes are proposed in several other regulatory frameworks and corresponding guidance documents. For example, for plant-protection products, a nonstandard avian reproduction test and a fish life-cycle test should be considered, if there is evidence for endocrine-disrupting effects (EC 2002a, 2002b). Recently, the upcoming EU regulation on the placing of plant-protection products on the market passed the European Parliament, which calls for a nonauthorization of plant-protection products that have endocrine-disrupting properties (EU 2009).

Currently, the new European Chemicals Regulation REACH (EC 2006) is being implemented by comprehensively amending the existing Technical Guidance Documents for risk assessment (EC 2003). Highly concerning substance properties, for example, persistence, bioaccumulation, and endocrine disruption, are of particular significance as a trigger for

entering the authorization procedure in REACH (Article 57 of EC 2006). Thus, it will become a key aspect for ERAs of industrial chemicals to identify, test, and assess substances that show endocrine activities. So far, more detailed technical guidance in regulatory frameworks is limited (see, e.g., ECHA 2008).

On the other hand, authorities of major economic regions have set up strategies to promote screening, assessment, and management of EDCs across regulatory schemes, such as the US Endocrine Disruptor Screening Program (EDSP; see <http://epa.gov/endo/index.htm>) or the EU strategy for EDs (see http://ec.europa.eu/environment/endocrine/strategy/index_en.htm, DG Environment; and http://ec.europa.eu/research/endocrine/activities_strategy_en.html, DG Research). Implementing these overarching strategies in substance-specific regulations, e.g., REACH or pesticide-approval regulations, requires operationalizing testing and assessment for individual substances in specific regulatory procedures.

Despite the progress made by implementing new regulation and the fact that a plethora of research papers have recently been published on endocrine effects (Sumpter and Johnson 2008), none of the guidance documents cited above provides technical details or consistent concepts on EDspecific testing and assessment.

8.4 The OECD conceptual framework for the testing of EDCs

The OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) has agreed on a Conceptual Framework that “attempts to identify tests of different levels suitable for assessing potential endocrine disrupters in situations of increasing biological complexity in both toxicological and ecotoxicological areas” (Huet 2000; <http://www.oecd.org/dataoecd/17/33/23652447.doc>). Also casually called toolbox, the Framework is based on input from OECD member states, in particular the United States, Japan, and the European Union. With regard to the OECD Test Guidelines Programme, the principle of mutual acceptance of data requires that the selection of instruments for the toolbox is based on international consensus. The Framework consists of 5 levels:

1. Sorting and prioritization based on existing information
2. *In vitro* assays providing mechanistic data
3. *In vivo* assays providing data on single endocrine mechanisms and effects
4. *In vivo* assays providing data on multiple endocrine mechanisms and effects
5. *In vivo* assays providing data on effects from endocrine and other mechanisms

The hierarchical structure of the proposed levels implies a stepwise approach from simple to more complex and toxicologically or ecologically more relevant testing (OECD 2002), although this is not compulsory. Entering into and exiting from the Framework at any level are explicitly foreseen, depending on case-specific considerations of existing as well as legally required information and testing data. This flexible approach is justified when considering the diverse complexities of specific regulatory cases and the need for efficient use of resources within ERA strategies (Fava et al. 1987; OECD 1995). The selection of tools for identifying and assessing EDCs might vary depending on compound-specific legislations in the OECD member countries.

Initiated by the Conceptual Framework, the OECD Test Guidelines Programme has undertaken important steps to amend established methods and develop new test methods that can be used to identify, characterize, and quantify effects of EDCs on mammals, birds, amphibians, fish, and invertebrates (OECD 1999, 2002; Gourmelon and Ahtiainen 2007). To integrate these ED-related testing methods into ERA schemes, 2 basic considerations are important.

First, endocrine-disrupting properties of a compound have to be evaluated in a transparent weight-of-evidence approach (see, e.g., ECHA 2008). This will be a key issue for regulatory testing and assessment procedures and requires particular consideration, which is, however, not included in this paper. The crucial point is to agree upon what existing or newly generated screening information could provide the basis for initial concern of a given substance to act as an endocrine disruptor. For fish, such concern would be confirmed or falsified by testing and assessment procedures proposed below.

Second, in regulatory risk assessment, the risk characterization is a result of comparing exposure (e.g., predicted environmental concentrations; PEC) with effect concentrations (e.g., predicted no effect concentrations; PNEC), and risk is defined as $PEC - PNEC$ (Vermeire and Van Der Zandt 1996). This regulatory principle also applies to EDCs, given that all endocrine-mediated, population-relevant adverse effects are appropriately covered in PNEC derivation.

8.5 ED-related fish test methods

Two OECD expert consultation meetings (OECD 1999, 2000) developed a concept for *in vivo* screening and definitive testing of EDCs, which would affect predominantly the

endocrine regulation of sexual development and reproduction. Specifically, the concept aims at investigating the impact of substances acting as estrogens, androgens, or aromatase inhibitors. EDTA recommended that the test methods should be applicable to the fish species fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), and zebrafish (*Danio rerio*); in addition, the three-spined stickleback (*Gasterosteus aculeatus*) has been recommended as a test fish (Hahlbeck, Katsiadaki, et al. 2004; Hahlbeck, Griffiths, et al. 2004; Katsiadaki et al. 2006). The OECD Validation Management Group for the Screening and Testing of Endocrine Disrupters for Ecotoxicological Effects (VMG-Eco) coordinates revision, development, and validation of several methods focusing on the sexual hormone system of fish.

The progress made so far by VMG-Eco is based mainly on research with compounds used as active ingredients in medicinal products and pesticides. Hence, the mechanisms of action described for these compounds, i.e., estrogenic or antiestrogenic, androgenic or antiandrogenic, and aromatase inhibiting, are known from drug development or toxicological studies with mammals and are related mainly to interactions with the reproductive system of vertebrates. Consequently, when proposing to use the OECD toolbox for fish testing with EDCs for regulatory purposes, it is important to keep in mind that impacts of chemicals on other mechanisms of action under endocrine control, which are related to physiological processes such as stress, maintenance, and regulation of energy, have been almost neglected. The only exception is the amphibian metamorphosis assay, which serves as a model for studying thyroid axis functions in vertebrates (OECD 2004a, 2004b). More recently, Gourmelon and Ahtianinen (2007) in the journal *Ecotoxicology*, as part of a special issue on endocrine disruption in invertebrates, described the status of and need for aquatic invertebrate test methods for the assessment of chemicals including potential endocrine-active substances.

Table 8.1 shows important features of the *in vivo* fish screening assays and the definitive fish tests, and Figure 8.1 presents a scheme of the OECD concept for *in vivo* fish screening assays and definitive tests of EDCs. For reasons of acceptability in all OECD member countries, rather than fulfilling a scientific need, 2 *in vivo* fish screening assays have been adopted by the OECD (OECD 2009a, 2009b), which are both conducted with reproductively active fish and correspond to level 4 of the Conceptual Framework.

Tab. 8.1: Comparison of important features determining the design of *in vivo* fish screening assays and definitive fish tests

	In vivo fish screening assays		Definitive fish tests		
	STRA (fish short-term reproduction assay)	21d-FA (21-d fish assay)	FSDT (fish sexual development test)	FLCT (fish full-life cycle test)	2-GenT (fish 2-generation test)
Duration of exposure	21 d	21 d	60 d	4–5 Months	5–6 Months
Nr of treatments	3	3	5	5	5
Nr of replicates	2–4	2–4	4	4	4
Nr of fish per replicate	10	10	40 (introduced as fertilized eggs)	P generation:	P generation:
				Start with 50 fertilized eggs, reduction to 20 (adults)	20 adults
				F1 generation:	F1 generation:
				Start with 50 fertilized eggs	Start with 50 fertilized eggs, reduction to 20 (adults)
					F2 generation:
					Start with 50 fertilized eggs
Important endpoints	Vitellogenin, secondary sex characteristics, fecundity, fertility, histology	Vitellogenin, secondary sex characteristics	Vitellogenin, hatch, survival of early life stages, sex ratio, histology	Hatch, survival of early life stages, juvenile growth, fecundity, fertility, sex ratio, histology	Hatch, survival of early life stages, juvenile growth, fecundity, fertility, sex ratio, histology
OECD Guideline	229	230	Validation ongoing	Draft guideline (not yet validated)	Draft guideline (not yet validated)

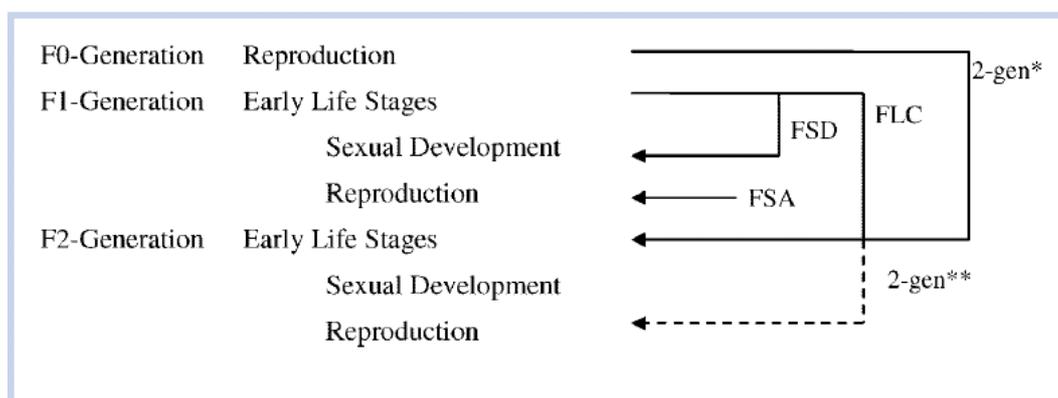


Fig 8.1: Schematic presentation of OECD’s Test Guidelines Programme to amend established and to develop new test methods, which can be used to identify and quantify effects of endocrine-disrupting chemicals on fish (solid lines). F0 = parental; F = filial; 2-gen* = 2-generation test according to US-EPA (from F0, reproduction, to F2, early life stages); 2-gen** = 2-generation test proposed in this paper (from F1, early life stages, to F2, reproduction; dotted lines indicate the extension of the FLC to 2-gen test); FLC = fish full life cycle test; FSD = fish sexual development test; FSA = fish screening assays (OECD Guidelines 229 and 230).

According to the guideline OECD 229 Fish Short Term Reproduction Assay (STRA; OECD 2009a), the biomarker endpoints vitellogenin and secondary sexual characteristics are determined. Additionally, fecundity is quantitatively monitored, and gonads are preserved for histopathological evaluation to assess the reproductive fitness of the test animals. Neither fecundity nor histopathology is intended to identify unequivocally specific cellular mechanisms of endocrine action. The STRA provides data on multiple endocrine mechanisms. Fathead minnow is the preferred test species, because only for this species have all endpoints been validated.

The guideline 230 21-day Fish Assay: A Short Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition (21d-FA; OECD 2009b) serves as an indicator for the 3 specific endocrine mechanisms of action by determining the same biomarker endpoints as in OECD 229. For fathead minnow, medaka, and zebrafish, the validation study confirmed the mechanisms of action for potent (17 β -estradiol) and weak (4-tert-pentylphenol) estrogenic substances, aromatase inhibitors (fadrozole, prochloraz), and potent androgenic substances (17 β -trenbolone, androstendione). Estrogenic substances induced dose-dependent vitellogenin (VTG) synthesis in males. Androgenic activity could be determined by measuring secondary sex characteristics in females of fathead minnow and medaka, which are sexually dimorphic species, in contrast to zebrafish. Aromatase inhibitors caused a dose-dependent decrease of VTG levels in females of all 3 fish species, together with cessation of spawning. For the antiandrogen flutamide, especially in zebrafish, histological investigation of the gonads or measurements of 11-ketotestosterone were needed for detecting this mechanism of action, as secondary sex characteristics and VTG measurements did not show treatment-related responses (OECD 2006a, 2006b, 2007, 2008; Owens 2007; USEPA 2007).

Proposed definitive fish tests corresponding to level 5 of the Conceptual Framework are the Fish Full Life Cycle/ 2-Generation Test (FLCT/2-genT) and the Fish Sexual Development Test (FSDT). The FSDT can be seen as an extension of the existing OECD Test Guideline 210 (OECD 1992) by continued exposure until the fish reach sexual maturity. The enhancement allows—in addition to the endpoints mortality; malformations; and growth of embryos, larvae, and juveniles—recording of the sexual development, i.e., sex ratio as determined via histological examination of the gonads, and VTG production. The test aims at studying the impact of sexual endocrine disruptors in fish at a very sensitive stage of their life (Holbech et al. 2006).

Both the FLCT and the 2-genT allow assessing the effects on developmental and reproductive endpoints. Additionally, the 2-genT allows determining transgenerational transfer of effects. Depending on the species used, the total test duration may vary considerably. Measurement endpoints in both definitive tests include developmental and reproductive endpoints (hatching, sex ratio, survival, growth, time to first spawn, fecundity, fertility, and behavior), as well as biochemical, histological, and morphological markers that are indicative of specific mechanism of endocrine disruption.

Recently, 2-generation studies with zebrafish exposed to flutamide, trenbolone, and tamoxifen have been completed (Braunbeck et al. 2009; see also Supplemental Data S1–S3). Apart from sexual reproduction, the stages of sexual differentiation are considered to be most sensitive for SEDCs.

In Table 8.2, effects of ED substances representing different mechanisms of action induced in the 21d-FA on indicative endpoints (biomarkers) are compared with effects measured for population-relevant endpoints in definitive tests (FLCT/2-genT) with the same zebrafish strain, mostly conducted in the same laboratory; this means that data with the same test substances, but different fish species or zebrafish strains, are not taken into consideration for this comparison. Also, not all studies shown in Table 2 might be in compliance with standards (e.g., number of analytical measurements during the exposure period of fish; statistical evaluation: EC₅₀ vs. NOEC) applied to studies used for regulatory purposes. Nevertheless, the data show that, for all mechanisms of action except androgen-receptor agonists, the level of sensitivity of measured biomarkers and population-relevant endpoints is rather similar, the EC₅₀ diverging by a factor of less than 3. In consequence, for zebrafish, the combination of VTG and 11-ketotestosterone measurements seems to be appropriate to indicate all mechanisms of sexual endocrine action except for one, namely, androgen-receptor agonists. For the latter, as long as a suitable biomarker remains to be found, the use of fish species with secondary sex characteristics should be recommended, or gonad histology should be included.

Tab. 8.2: Effect concentrations (EC₅₀) determined for zebrafish when exposed to different sexual endocrine-disrupting substances

SED-MoA	Substance	21-FA (biomarker)		FLCT/2-genT (population-relevant endpoint)	
		EC50	Endpoint	EC50	Endpoint
ER agonist					
Strong	Ethinylestradiol	3.1 ng/L	VTG+	1.1 ng/L ^d	Fertility
Weak	Bisphenol A	640 µg/L	VTG+	1410 µg/L ^e	Fertility
ER antagonist	Tamoxifen-citrate ^a	3.0 µg/L	VTG-	1.5 µg/L	Sex ratio
AR agonist	Trenbolone ^b	220 ng/L	VTG-	2.2 ng/L ^f	Sex ratio
AR antagonist	Flutamide ^c	560 µg/L	11kT+	760 µg/L	Fecundity
Aromatase inhibitor	Azole fungicide	62 µg/L	VTG-	90 µg/L ^g	Sex ratio
Inhibitor of basic steroid synthesis	3,4-DCA	150 µg/L	VTG-	140 µg/L ^h	Fecundity
Inhibitor of basic steroid synthesis	Atrazin	730 µg/L	VTG-	30–4300 µg/L ⁱ	Fecundity

Data (based on measured concentrations) obtained from Teigeler *et al.* (2007) and Braunbeck *et al.* (2009). Further data sources are explained in footnotes below.

SED-MoA = sexual endocrine-disrupting mechanisms of action; ER = estrogen receptor; AR = androgen receptor; DCA = dichloroaniline. Biomarker: VTG+ = increase of vitellogenin in both sexes; VTG- = decrease of vitellogenin in females; 11kT+ = increase of 11-ketotestosterone in males. Population-relevant endpoint: fertility (decreased percentage of fertilized eggs), fecundity (decreased number of eggs due to decreased mating), sex ratio (increased number of males).

^a Supplemental Data S1: 2-generation test with zebrafish (*Danio rerio*); Teigeler and Schäfers. EC₅₀ values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

^b Supplemental Data S2: 2-generation test with zebrafish (*D. rerio*); Böttcher *et al.* EC₅₀ values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

^c Supplemental Data S3: 2-generation test with zebrafish (*D. rerio*); Teigeler. EC₅₀ values were determined by applying probit analysis (maximum likelihood regression) according to Finney (1971).

^d Wenzel *et al.* (2001)

^e Raw data from IDEA (Segner *et al.* 2003). Semistatic exposure; 3 renewals per week; data based on mean measured initial concentrations.

^f Values are nominal concentrations. During the exposure period of 6 months, four analytical verifications of all treatment levels showed mean recoveries ± SD between 35 ± 12% and 50 ± 14%.

^g Raw data from a confidential GLP study; for the SED-MoA confirmed by Schäfers (2007).

^h Raw data from Ensenbach (1991), only fecundity (ELS more sensitive, different MoA).

ⁱ Nagel *et al.* (2004).

8.6 Proposal for a testing strategy: Regulatory use of OECD fish-testing tools for SEDCs in ERA schemes

The proposed testing strategy for SEDCs in fish is based on data compiled in Table 2 and the outcome of a workshop held at Berlin, Germany, on 10 and 11 December, 2007 (Schäfers et al. 2008). The conceptual background as provided by Huet (2000) and more general considerations on screening assays and definitive tests as published by Hutchinson et al. (2006) are essentials of the proposed testing strategy. It consists of 3 distinctive steps (Schäfers and Teigeler 2005): 1) weight-of-evidence approach for identifying potential SEDCs, 2) in vivo confirmation of sexual endocrine-disrupting activity associated with specific mechanisms of action, and 3) characterization of sexual endocrine-mediated adverse effects, including their thresholds. Each of the 3 steps will be outlined briefly in the following sections and is schematically presented in Figure 8.2.

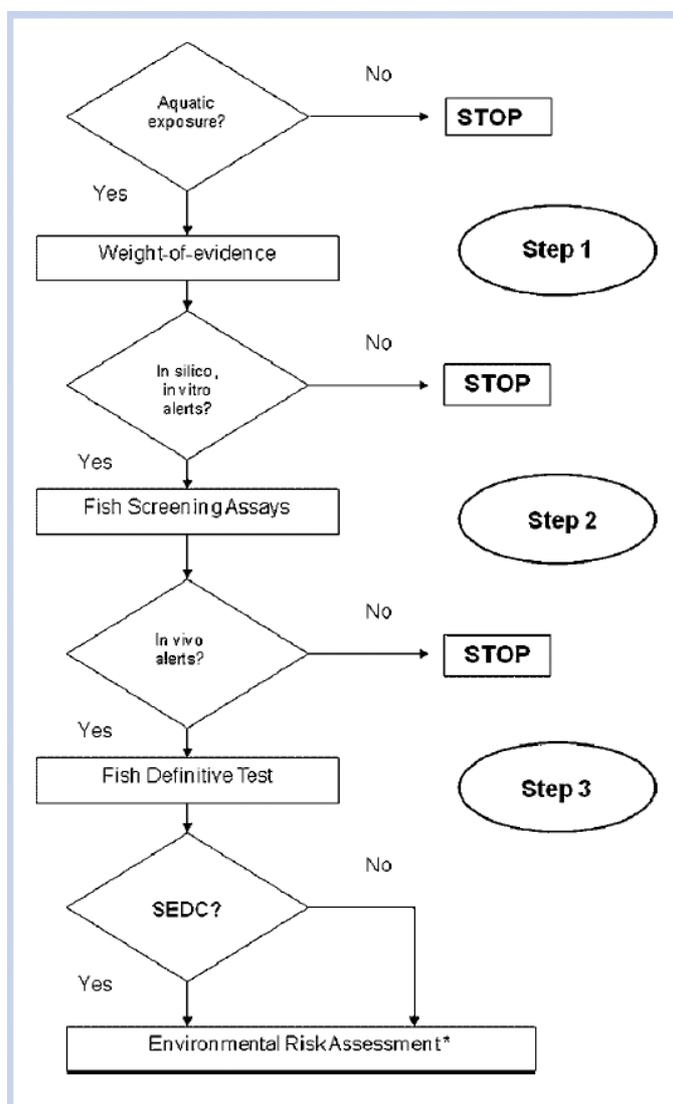


Fig 8.2: Schematic presentation of the proposed testing strategy with fish for sexual endocrine-disrupting chemicals. In some legislation, the identification of a substance as an SEDC may initiate an authorization procedure or may lead to a nonregistration of the substance.

8.6.1 First step: Weight-of-evidence approach for identifying potential SEDCs

There is, so far, no standardized and cost-efficient screening method to identify all potential ED properties of chemical substances comprehensively without risking false-negative responses. Hence, for a substance released into the aquatic environment, all available information has to be assessed to evaluate the possibility of whether the substance might

cause endocrine effects in fish (weight-of-evidence approach). When exposure of the aquatic compartment to the substance in question is demonstrated not to be relevant, the evaluation of data and specific testing to assess endocrine effects can be waived. Based on legal requirements, the competent authorities request a specific set of data according to the use of a chemical. This results in varying information levels for substances with different use patterns when evaluating the possibility of a substance of being a potential SEDC. Important information to justify the assumption of endocrine-disrupting properties of a chemical is in principle available from the following.

- Large acute to chronic ratio (ACR) with regard to the toxicity in fish (threshold value for $ACR > 20$ recommended by participants of the workshop Characterisation of Endocrine Mediated Effects on Fish held in Berlin, Germany, on 10 and 11 December, 2007)
- Evaluation of studies with mammals and birds with regard to endocrine-specific effects
- Reliable and relevant (preferably fully validated) in vitro assays
- (Q)SARs, read-across, chemical categories
- Knowledge of the mechanism of action (normally applicable only to active ingredients)

The ECHA (2008) gives advice on what data can be useful to evaluate the endocrine-disrupting potential of a substance. The guidance emphasizes that (Q)SARs, read-across, and chemical categories provide nontesting data, whereas estrogen- and androgen-receptor binding assays, reporter gene assays, and VTG and steroidogenesis assays provide in vitro screening data. However, both the nontesting and in vitro screening methods are still under development. For compounds other than industrial chemicals, in particular, for pesticides and pharmaceuticals, extensive ecotoxicological data are usually available and can be reviewed. The complete set of compiled information requires a comprehensive, transparent evaluation to decide whether there is sufficient evidence to move on to the second step of the testing strategy.

8.6.2 Second step: *In vivo* confirmation of sexual endocrinedisrupting activity associated with specific mechanisms of action

When the weight-of-evidence approach reveals potential endocrine effects of the substance on fish, this finding has to be assessed by testing the substance in in vivo screening assays. The biomarkers measured according to the OECD guidelines for the screening assays (OECD 2009a, 2009b) allow the identification of 4 mechanisms of action related to sexual endocrine

disruption. The measurement of 11-ketotestosterone, the androgen-receptor antagonist, which indicates the fifth mechanism of action, is not yet included as a validated endpoint in the screening assays. The following list denominates the mechanisms of action and their biomarkers, which, when affected by the exogenous substance, would indicate that the compound is an SEDC.

1. Estrogen-receptor agonist: Substance increases the VTG level in both sexes of fish, most clearly indicated in male fish.
2. Estrogen-receptor antagonist: Substance reduces the VTG level in fish; however, particularly in male fish, the reduction is difficult to measure, because VTG levels are already rather low under normal physiological conditions.
3. Androgen-receptor agonist: Substance is indicated by secondary sex characteristics for fathead minnow and medaka, whereas for zebrafish histopathological analyses are required.
4. Androgen-receptor antagonist: Substance causes compensatory increase of 11-ketotestosterone levels in fish.

Hutchinson et al. (2006) proposed a stepwise procedure for measuring the various biomarkers in the screening assays, starting with the biomarker that requires the least resources. Whenever a specific impact on a biomarker is indicated, the analysis of the remaining biomarkers can be terminated, and the third step of the testing strategy, the definitive testing, can be initiated.

The 21d-FA, if subjected to further amendment by integrating the measurement of 11-ketotestosterone, would identify all SEDCs with regard to their intrinsic properties and thus contribute to the definition of the European Commission that “an endocrine disrupter is an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations” (EC 1999, 2007). However, the identification of a compound as an SEDC in either the extended 21d-FA or the STRA does not allow conducting a quantitative ERA, because population- relevant endpoints are not adequately addressed by the in vivo screening assays (Hutchinson et al. 2006). For substances without indication for an endocrine activity via the mechanisms covered by the extended 21d-FA, no further endocrine-specific fish testing is considered necessary. Eventually, this approach requires that the extended 21d-FA can confirm all of the 5 sexual endocrine mechanisms of action, which, for the time being, has been sufficiently demonstrated only for aromatase-inhibiting substances and estrogen-receptor agonists. Hence, when step 1 would not provide sufficient evidence to specify a

probable mechanism of endocrine action, or as long as the assumed mechanism is not covered by available step 2 test systems, step 2 might be skipped, with direct progression to a life-cycle study according to step 3.

8.6.3 Third step: Characterization of sexual endocrine-mediated adverse effects, including their thresholds for regulatory use

A positive response of one of the in vivo screening assays triggers the performance of a definitive test to determine regulatory useful threshold concentrations, e.g., no observed effect concentration (NOEC), for population-relevant endpoints, i.e., survival, growth, and reproduction, including sexual development, which are required for ERAs. Publicly available experimental data from fish life-cycle studies (Teigeler et al. 2007; Braunbeck et al. 2009) showed that, for the already-mentioned sexual endocrine mechanisms of action, the following population-relevant endpoints were most sensitive.

- Aromatase inhibition: Sex ratio shift toward males, delays in growth and sexual development, impaired fecundity (number of eggs); the variability between the NOECs of these endpoints was in the range of a factor of 3.
- Estrogen-receptor agonist: Fertilization rate, growth, time to first spawn, and sex ratio shift toward females (medaka, fathead minnow) or delay of male sex development (zebrafish); the sensitivity of these endpoints was rather similar; however, depending on the fish species tested, the sequence from the most to the least sensitive endpoint can vary.
- Estrogen-receptor antagonist: Sex ratio shift toward males.
- Androgen-receptor agonist: Sex ratio shift toward males.
- Androgen-receptor antagonist: Spawning and fecundity (number of eggs) are affected.

The number of tested aromatase-inhibiting substances and estrogen-receptor agonists is large enough to justify acceptable confidence in the identified most sensitive population-relevant endpoints (Teigeler et al., 2007). For both mechanisms of action, tests exposing different developmental time windows demonstrate the sexual development phase to be the most critical life stage with respect to sensitivity and maintenance of effects. The OECD Test Guidelines Programme has not yet finalized the protocols for definitive fish tests. However, definitive studies to be used in ERA should cover the following population-relevant endpoints.

- Mortality (e.g., survival of embryos, larvae, juveniles and adults)
- Growth and development (e.g., time to hatch, success rate of hatch, body weight and length, malformations, time to first spawn, secondary sex characteristics, sex ratio)
- Reproduction (e.g., fecundity; i.e., number of eggs laid; fertility, i.e., rate of fertilized eggs; sexual behavior, i.e., courtship)

Apart from sex ratio, time to first spawn, secondary sex characteristics, and behavior, all endpoints are also covered by USEPA's fish life-cycle test method (USEPA 1996). From a general point of view, the measurement of indicative endpoints (biomarkers) such as VTG, secondary sexual characteristics, histological findings, and 11-ketotestosterone is not required in definitive tests. If, however, observed population-relevant effects in a definitive test can be associated with other than sexual endocrine-disrupting mechanisms of action, this might be important, e.g., for the authorization procedure according to article 57(f) under REACH (EC 2006) and according to the upcoming EU regulation on the placing of plant-protection products on the market (EU 2009). Indicative endpoint measurements can exclude or substantiate that adverse effects observed in a definitive test are caused by endocrine mechanisms (cf. Weybridge definition of EDs). Additionally, the integration of indicative endpoints into definitive tests can improve the differentiation between effects caused by an endocrine mode of action and effects caused by systemic toxicity. Crane et al. (2009) describe an approach using multicriteria decision analyses (MCDA) for selecting test endpoints in fish life-cycle studies.

The limited data available do not reveal conclusively whether the 2-genT is more sensitive than the FLCT (Schäfers et al. 2007). However, the 2-genT is required to identify potential transgenerational effects, i.e., effects that become obvious in the second generation after lifetime exposure of the parental generation. Substances with high bioaccumulation potential, which might result in in ovo exposure (i.e., intrinsic exposure to a contaminant deposited in the egg following maternal exposure), and SEDCs, which affect the quality of eggs or sperm during early developmental stages, might be candidates for causing transgenerational effects. The proposed 2-genT design starts with the exposure of reproducing adults (P) and includes exposure of the first filial generation (F1) and the early life stages of the second filial generation (F2).

Alternatively, as with the FLCT, the 2-genT should start with the exposure of fertilized eggs and end with the sex determination of the following generation. This design has several advantages: 1) the number of test fish would be reduced by one-third, 2) the number of

reproductive phases, which are very labor-intensive as well as causing high variability in the test results, would be reduced from 2 to 1 and, 3) the number of sexual maturation phases as the most important time windows of exposure would be enhanced from 1 to 2. In comparison with starting the 2-genT with reproductive adults, this would improve the statistical homogeneity of the first life-time-exposed generation. Starting with fertilized pooled eggs from several clutches improves the genetic homogeneity of eggs exposed to the different treatment levels. Properly planned and prepared, each FLCT can be transformed to a 2-genT when extended to the reproductive phase of the filial generation (Figure 10.1).

Instead of the FLCT or 2-genT, shorter tests might be acceptable as definitive test as long as the most sensitive time window of exposure and the most sensitive endpoints related to the specific sexual endocrine-disrupting mechanism of action of the tested substance are covered. For example, for aromatase-inhibiting substances, it has been shown that the NOEC values derived from FLCT and the NOEC values derived from FSDT are almost identical, because the most sensitive endpoints linked to sexual development are measured in both tests (Teigeler et al. 2007). Hence, if the introductory information and the in vivo screening assays reveal the mechanism of action as being aromatase inhibition for a chemical, the definitive test can be the FSDT. Possibly, for estrogen-receptor agonists, the FSDT can also replace the FLCT when the slightly lower sensitivity observed in the FSDT (fertilization rate not covered; Teigeler et al. 2007) is compensated for by a higher assessment factor. For the remaining sexual endocrine-disrupting mechanisms of action, the available data to compare results obtained from the FLCT with results from partial life cycle tests are still scarce.

8.7 Conclusions

The proposed testing strategy is a comprehensive concept that uses components of OECD's Conceptual Framework in such a way that sexual endocrine-disrupting chemicals can be identified and their ecotoxicological effects on fish measured and integrated into established compound-specific ERA schemes. With regard to fish testing, a similar, less specific approach has been proposed by ECETOC, which focuses on hazard rather than risk assessment and which, in addition to fish, includes other groups of organisms (ECETOC 2009).

In particular, the first step (weight-of-evidence approach) of the testing strategy, which describes the rules, the nontesting and in vitro screening methods for identifying potential sexual endocrine-disrupting chemicals, still requires more specific guidance, criteria, and

supportive data. Another example for future validation work is the measurement of 11-ketotestosterone as an additional endpoint, which would allow the indication of androgen-receptor antagonistic action and could be used in all 3 recommended fish test species, i.e., fathead minnow, medaka, and zebrafish, in the in vivo fish screening assays or definitive fish tests. Moreover, for some mechanisms of endocrine action, i.e., the estrogen-receptor antagonist and androgen-receptor agonist and antagonist, more data generated with the endocrine-specific fish tests will be needed to confirm the assumed capacity of biomarkers to predict specific mechanisms. Several elements of the available guidance for FLCT and 2-genT should be harmonized, validated, and eventually transferred to an internationally accepted OECD Test Guideline or Guidance Document. The proposed testing strategy has to prove its practicability in real cases from different regulatory frameworks. Based on accumulating experience, the strategy should be subjected to further amendments and specification when necessary.

Testing strategies and ERA procedures related to substances that are not SEDCs should be based on endpoints such as mortality, growth, reproduction, and behavior, which integrate across all potential impacts of endocrine-disrupting chemicals at the organizational level of organisms (see, e.g., Ankley and Johnson 2004). In other words, because currently available screening assays cover only mechanisms of endocrine action related to SEDCs, for substances other than SEDCs there is no possibility to apply a tiered testing strategy. Such cases directly require the performance of life-cycle and/or multigenerational tests to eliminate reasonable suspicion of an endocrine mode of action.

8.8 Supplemental data

Supplemental Data A. Tamoxifen Citrate SEDC fish testing strategy.

Supplemental Data B. Trenbolone SEDC fish testing strategy.

Supplemental Data C. Flutamide SEDC fish testing strategy.

8.9 References

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Chapter 8: Supplement A

Two Generation Test with zebrafish (*Danio rerio*)

Test substance: Tamoxifen citrate

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Authors:

Matthias Teigeler

Christoph Schäfers

Fraunhofer Institut für Molekularbiologie

und Angewandte Oekologie (IME)

Auf dem Aberg 1

57392 Schmallenberg

Germany

Content

A-1. Introduction.....	A-5
A-2. Study conditions.....	A-5
A-2.1 Test organism.....	A-5
A-2.2 Test substance.....	A-6
A-2.3 Test system.....	A-6
A-2.4 Chemical analysis.....	A-7
A-3. Performance of the study.....	A-8
A-3.1 Description of the different life phases.....	A-8
A-3.2 Observations.....	A-10
A-3.3 Specific methods.....	A-10
A-3.3.1 Egg collection.....	A-10
A-3.3.2 Photography and image analysis.....	A-11
A-3.3.3 Blood collection and preparation.....	A-11
A-3.3.4 Measurement of Biomarkers using ELISA.....	A-11
A-3.3.5 Histopathology.....	A-13
A-3.3.6 Statistical Data Analysis.....	A-14
A-4. Results.....	A-15
A-4.1 Chemical analysis.....	A-15
A-4.2 P-generation.....	A-19
A-4.2.1 P-generation, Observations.....	A-19
A-4.2.2 P-generation, Reproduction.....	A-19
A-4.2.3 P-generation, Biomarker.....	A-21
A-4.3 F ₁ -generation.....	A-23
A-4.3.1 F ₁ -generation, Observations.....	A-24
A-4.3.2 F ₁ -generation, Hatch/Survival.....	A-24
A-4.3.3 F ₁ -generation, Juvenile Growth.....	A-25
A-4.3.4 F ₁ -generation, Reproduction.....	A-26
A-4.3.5 F ₁ -generation, Biomarker.....	A-28
A-4.3.6 F ₁ -generation, Sex ratio.....	A-30
A-4.3.7 F ₁ -generation, Histopathology.....	A-30
A-4.4 F ₂ -generation.....	A-32
A-4.4.1 F ₂ -generation, Hatch/Survival/Length.....	A-32
A-5. Summary and Conclusions.....	A-34
A-6. References.....	A-37

List of tables and figures

Table A-1: Fish two generation test with <i>Danio rerio</i> , important endpoints of the study	A-9
Table A-2: Categories of maturation of zebrafish (<i>Danio rerio</i>) gonads.....	A-14
Table A-3: Summary of results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate	A-15
Table A-4: Detailed results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate	A-16
Table A-5: P-generation, Summary of all endpoints.....	A-23
Table A-6: F ₁ -generation, Early Life Stage, Juvenile Growth and Reproduction, Summary of all endpoints	A-31
Table A-7: F ₁ -generation, Sex ratio and Biomarker, Summary of all endpoints	A-32
Table A-8: F ₂ -generation, Early Life Stage, Summary of all endpoints	A-34
Table A-9: Two generation test with tamoxifen citrate, Summary of effect data (mean measured values).....	A-36
Figure A-1: Tamoxifen citrate, Chemical structure	A-6
Figure A- 2: P-generation, Egg numbers per day and female.....	A-19
Figure A- 3: P-generation, Cumulative number of fertilised eggs.....	A-20
Figure A- 4: P-generation, Fertilisation rate [%]	A-20
Figure A- 5: P-generation, VTG concentration in female blood plasma [ng/μg]	A-21
Figure A- 6: P-generation, VTG concentration in male blood plasma [ng/μg]	A-21
Figure A- 7: P-generation, 11-keto testosterone concentration in male blood plasma [pg/mL].....	A-22
Figure A- 8: F ₁ -generation, Hatch of fish larvae [%].....	A-24
Figure A- 9: F ₁ -generation, Survival of fish larvae at day 28pf [%].....	A-25
Figure A- 10: F ₁ -generation, Pseudo specific growth rate based on length measurements on day 28 and day 63.....	A-27
Figure A- 11: F ₁ -generation, Egg numbers per day and female [n].....	A-27
Figure A- 12: F ₁ -generation, Cumulative number of fertilised eggs [n].....	A-27
Figure A- 13: F ₁ -generation, Fertilisation rate [%]	A-29
Figure A- 14: F ₁ -generation, VTG concentration in male blood plasma [ng/μg]	A-29
Figure A- 15: F ₁ -generation, Concentration of 11-keto testosterone in male blood plasma [pg/mL].....	A-29
Figure A- 16: F ₁ -generation, Sex ratio of adult fish [%].....	A-30
Figure A- 17: F ₂ -generation, Survival of fish larvae at day 35pf [%].....	A-33
Figure A- 18: F ₂ -generation, Length of fish larvae at day 35pf [cm]	A-33

List of abbreviations

11 kT	11-keto testosterone
AR	androgen receptor
ER	estrogen receptor
F ₁	Filial 1 generation
F ₂	Filial 2 generation
LOEC	Lowest observed effect concentration
MOA	Mode of Action
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
P	Parental generation
pf	post fertilisation
UBA	German Federal Environment Agency (Umweltbundesamt)
VTG	Vitellogenin

A-1. Introduction

From August 15, 2006 to July 25, 2007, a two generation study with tamoxifen citrate as test substance was performed at Fraunhofer IME, Schmallingenberg, Germany. Tamoxifen represents the endocrine mode of action estrogen receptor antagonist.

The aim of the study was the evaluation and the assessment of effects after continuous exposure with tamoxifen citrate on all life stages of zebrafish. This includes the parental generation (P-generation), early life stages (life phase directly post hatch), juvenile growth and reproduction of the Filial 1 (F₁) generation and finally, the early life stage phase of the Filial 2 (F₂) generation. Furthermore, molecular biomarkers of the P- and F₁-generation were measured, including the yolk protein precursor vitellogenin and the sex steroid 11-keto testosterone.

A-2. Study conditions

A-2.1 Test organism

The zebrafish (*Danio rerio*, Teleostei, Cyprinidae) was used as test organism. This fish is recommended by the OECD TG 210 (OECD, 1993) as test fish for Fish Early Life Stage Toxicity (FELS) studies. It is also one of the four test fish species recommended for fish two generation studies (Anonymous 2002).

The source of the fish was the laboratory culture of the Fraunhofer IME, Schmallingenberg, Germany. The origin of the used strain was a commercial fish breeder, namely the West Aquarium GmbH in Bad Lauterberg, Germany.

Up to the test start, the adult zebrafish were kept in communities of approximately 300 individuals in 150 L tanks under flow-through conditions with a 12 h light/12 h dark photoperiod. The temperature of the water was kept constant at $25.0 \pm 1^\circ\text{C}$.

Fish were fed a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany) *ad libitum* and nauplii from *Artemia ssp.* (Great Salt Lake Artemia Cysts, Sanders Brine Shrimp Company, Utah, USA) twice a day.

Purified drinking water was used in the rearing tanks. The purification includes filtration with activated charcoal, passage through a lime-stone column and aeration until oxygen saturation. Carbonate hardness of the water was nearly 90 mg/L CaCO₃, pH was in the range of 7.5-8.5.

The animals used in the two generation test did not exceed the age of 6 month.

A-2.2 Test substance

Tamoxifen citrate (purity $\geq 99\%$; Sigma Aldrich, St. Louis, MO, USA) was used as a test substance. It is a representative of the estrogen receptor antagonist. Tamoxifen has been used as a therapeutic drug for the treatment of breast cancer. It is listed under the term of selective estrogen receptor modulators (SERMs).

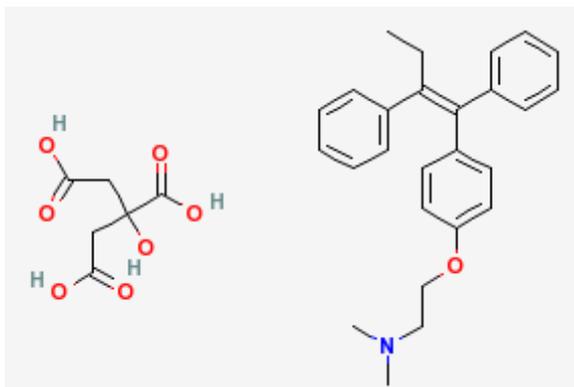


Fig. A-1. Tamoxifen citrate, Chemical structure (Source: PubChem Database)

A-2.3 Test system

For the test performance, a flow-through system was implemented. Three replicates per treatment were arranged.

Full glass aquaria of 42 x 28 x 28 cm (L x W x H) with approx. 25 litres of test medium were used. To adjust the water temperature in the test system, the vessels were placed in a water bath. To start the early life stage phases, the fertilised eggs were placed in fry chambers made of plastic with a nylon net as bottom of the cage.

Five concentrations of the test item and the untreated dilution water as control were tested under flow-through conditions at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ and a light/dark cycle of 12 h/12 h. No additional aeration was introduced. For the first two replicates per treatment, an individual dosage system was used. For the third replicate a second pump system was implemented. Dilution water was pumped by a water dosage pump (membrane pump, Prominent, Heidelberg, Germany) into a mixing chamber, placed on a magnetic stirrer. An adequate amount of the stock solution was added into the mixing chamber by a dosage pump (membrane pump with a stainless steel head, Prominent, Heidelberg, Germany). The prepared test solution entered the test vessels via flexible tubes, distributed to the two vessels by an electronically regulated distributor driven by compressed air. For the third replicate a tube no splitting was necessary.

The daily water exchange rate was adjusted to at least 5 test vessel volumes.

The nominal test item concentrations were 0.16, 0.50, 1.6, 5.0 and 16 µg tamoxifen citrate/L. The main stock solution was prepared by adding 2 L of dilution water to 20 mg of tamoxifen citrate salt. After ultrasonic treatment for 15 minutes, the solution was adjusted to pH 3 to keep the test item stable. To achieve adequate concentrations for the use in the dosing system, the stock solution was further diluted. Depending on the delivery rate of the dosing pumps, the stock solution was diluted by a factor of 82, 250 and 1250 for the high, medium and low test concentrations, respectively. After the pre-treatment phase, which included only water dosage pumps, exposure was started by starting the stock solution dosage pumps.

The temperature was measured daily, pH-values and oxygen concentrations were measured twice a week. The oxygen concentration did not fall below 60% through the whole exposure period.

A-2.4 Chemical analysis

An on-line analysis of water samples taken from the test system was not possible during the study. Thus, water samples had to be stored deep frozen until analysis. At each sample date, water samples of 200 mL were taken from each test vessel including controls acidified to pH 3 and stored at -20°C.

Samples were analysed using LC-MS with a Limit of Quantification (LOQ) of 0.15 µg tamoxifen citrate /L. There were 10, 3 and 1 sampling dates covering the P-generation, the F₁-generation and the F₂-generation, respectively. At each sampling date, samples of all vessels including the controls (n=18 vessels) were sampled and stored deep frozen until they were analysed.

A-3. Performance of the study

A-3.1 Description of the different life phases

A detailed study plan with the durations of the evaluated life phases is shown in Table A-1.

The parental generation (P-generation) was generated with 8 males and 8 females per tank aged at least 150 days. The fish were kept under test conditions for at least three weeks to adjust a consistent state of fecundity over all groups (n=18), and to re-sort the spawning groups in case of low egg numbers.

When all groups were spawning daily, exposure was started. Freshly spawned eggs were collected daily, counted and the number of fertilised eggs determined. The P-generation was sacrificed when the F₁-generation successfully reached an age of 28 days.

For the start of the F₁-generation, 100 fertilised and randomised eggs spawned by the parental fish of each replicate were placed on nylon nets forming the bottom of cages fixed at the water surface of each test vessel. Starting from day 6 of age, larvae were fed daily *ad libitum* with breeding food (Tetra, AZ 000). From day 9 of age, brine shrimp nauplii (*Artemia salina*) were added *ad libitum*. From day 16 of life, ground TetraMin flake food was added *ad libitum* to the daily food. After 35 days, the fish were randomly reduced to 50 individuals and transferred from the fry chambers to the main vessels. After 28 and 63 days of age, length was measured. After 63 days of age, the fish number was randomly reduced to 30 individuals in order to create identical conditions for reproduction.

Reproduction was measured in the same way as described for the P-generation. The F₁-generation was sacrificed when the F₂-generation successfully reached an age of 21 days.

For the start of the F₂-generation, 100 fertilised and randomised eggs spawned by the F₁-fish of each replicate were placed on nylon nets forming the bottom of cages fixed at the water surface of each test vessel. Larvae were fed daily *ad libitum* as described above for the F₁-generation.

After 35 days, the fish were measured for length and weight, and the test was terminated.

Chapter 8: Supplement A

Table A-1. Fish two generation test with *Danio rerio*, important endpoints of the study

Day after start of exposure	Phase	Course (related to each of three replicates per concentration)	Endpoints
Pre-treatment phase, at least 7d		Start with 8 males and 8 female fish, reorganisation of groups	Reproduction: - Egg numbers - Fertilisation rate
0 - 20	P-generation Reproduction	Transfer to exposure tanks (run for 48h) Sampling of eggs for F ₁ phase	
21 -49	F₁-generation Early Life Stage (28 days)	Introduction of 100 fertilised eggs in fry chambers	Daily observation of : - mortality - survival rate - hatching rate - time to 90 % hatch
		Begin of hatch (day 23)	
		End of hatch (day 25)	
		Transfer to the total vessel Randomly reduced to 50 (day 49) Blood/Liver/Gonad sampling of P fish (Sex-Determination of excess fish)	Weekly observation of - mortality - survival rate - length Vitellogenin 11-keto testosterone Gonad histology
50 - 84	F₁-generation Juvenile Growth	Randomly reduced to 30 (day 63) (Sex-Determination of rest fish)	Weekly observation of - mortality - survival rate - length
85 - 120	F₁-generation Reproduction	Sampling of eggs for F ₂ phase	Reproduction: - Start - Egg numbers - Fertilisation rate
120	F₂-generation Early Life Stage (35 days)	Introduction of 100 fertilised eggs in fry chambers	Daily observation of : - mortality - survival rate - hatching rate - time to 90 % hatch
122		Begin of hatch	
124		End of hatch	
126			Weekly observation of - mortality - survival rate - length
155		Test termination Blood/Liver/Gonad sampling of F ₁ fish (Sex-Determination of excess fish)	Recording of: - survival - weight - length Vitellogenin 11-keto testosterone Gonad histology Sex ratio

A-3.2 Observations

Observations on fish of all life stages were recorded daily. Dead eggs, larvae, juvenile and adult fish were recorded and removed as soon as observed.

Hatching rates were estimated by daily counting of non-hatched eggs in the period between two days of age and the time, when 90 % of the fish had hatched.

Appearance of any change in behaviour (e.g. loss of equilibrium, change in swimming behaviour, change in mating behaviour, etc) compared to the control was recorded.

Between hatch and 28 to 35 days of age of the F₁- and F₂-generation, respectively, larvae/juvenile fish were photographed weekly and the survival rates were estimated. The data were confirmed by counting the fish on day 28 (F₁) and day 35 (F₂) when transferred to the main aquaria (F₁) or sacrificed (F₂). Lengths of F₁-fish were measured by digital photography after 28 and 63 days of life. For this time interval, the pseudo-specific growth rate based on length was calculated according to the respective OECD technical guidance (OECD 2000). The time of first spawning, identified as first day at which eggs were found in the spawning trays, was recorded. The reproductive endpoints like egg number and fertilisation rate were observed for 20 daily counts. After successful start of the F₂-generation, the adult F₁-fish were sacrificed and sexed by inspection of the gonads. The sex ratio was calculated. The number of females per groups was determined, and used to calculate the number of total and fertilised eggs per female and day. Growth of F₂-fish until day 35 was measured as individual lengths and weights.

A-3.3 Specific methods

A-3.3.1 Egg collection

Spawned eggs were collected daily in all glass spawning-trays placed at the bottom of the test vessels. The tray was covered with a lattice (stainless steel), to prevent the eggs from being predated by the adult fish. Artificial plant substrate was placed on the lattice to stimulate spawning into the tray. The switch on of lighting (neon lamps with a light intensity of approximately 1000 lux, measured 5 cm above the water surface in the middle of the test vessel) induces mating of fish. Within two hours after spawning, eggs were transferred from the spawning-tray onto a sieve, rinsed with clean water in order to remove faeces and remainings of food, put into glass dishes, and counted as total number and number of

fertilised eggs. Fertility was determined by observing cleavage stages using a binocular according to Nagel et al. (1986).

A-3.3.2 Photography and image analysis

For fry counts and length measurements, photographs were made using a digital camera (Canon Snap Shot). Digital image processing was performed using UTHSCSA ImageTool Version 2.0, Alpha 2; University of Texas Health Science Center at San Antonio, 1997.

Fish larvae were photographed by putting a vessel beneath a fry chamber to maintain the water level, dislocate the fry chamber with the vessel to the photo device (light plate with additional illumination from above), and re-introducing the fry chamber in the aquarium after being photographed. Juvenile and adult fish were netted and placed in glass vessels with a low water level in the photo device.

A-3.3.3 Blood collection and preparation

At termination of P- and F₁-generation, a blood sample was taken from each fish. The samples were taken by cardiac puncture using syringes with a fixed thin needle. To avoid coagulation of blood and degradation of proteins, the syringes were prefilled with Phosphate-buffered saline (PBS) containing heparin (1000 units/mL) and the protease inhibitor aprotinin (2 TIU/mL). As ingredients for the buffer, heparin as ammonium-salt (Sigma-Aldrich, Munich, Germany) and lyophilised aprotinin (Roth, Karlsruhe, Germany) were used.

Before sampling fish were anaesthetized within a water bath containing chloro butanol (20 g/L). Blood samples with volume of 10 to 30 µL were taken. Plasma was separated from the blood via centrifugation (30 min; 5000 rpm; 4°C) and immediately stored at -80°C until further analysis.

A-3.3.4 Measurement of Biomarkers using ELISA

Vitellogenin measurement

The measurement of vitellogenin levels was performed using commercially available enzyme-linked immunoabsorbant assays (ELISA) raised to zebra fish (*Danio rerio*) VTG (homologous ELISA kit) purchased from Biosense, Bergen, Norway.

The VTG-analysis used is based on a sandwich assay utilizing specific binding between antibodies and VTG. The wells of micro-titer plates are coated with a specific capture antibody that binds to VTG in samples added to the wells. Unbound components are washed out, and a different VTG-specific antibody (detecting antibody) is added. Unbound detecting antibody is washed out, and an enzyme-labelled secondary antibody is added. After a last wash, the enzyme activity is determined by adding a substrate being metabolised to a coloured product. The enzyme activity (colour intensity) measured by a microplate reader is directly proportional to the concentration of VTG in the sample. The assay is calibrated using purified VTG from zebra fish as standard, which was provided by the producer.

For measurement of VTG levels in plasma it was necessary to dilute the samples to reach the linear part of the respective calibration curve. Each sample was diluted in two steps depending on the sex of the sampled fish. Each dilution step was measured in duplicate, resulting in 4 measurements per fish. Female plasma samples (control fish) were diluted by factors of 30000 and 60000, male samples (control fish) by factor of 75 and 150. The inter-assay variation of the VTG-ELISA was estimated to be between 14 and 18% (Nilsen, 2004). The intra-assay variation was determined to be between 3 and 10%. The intra-assay variation of the measurements performed in this study was based on a comparison of all standard curves and determined to be 6 and 11% and thus was in line with the reference values.

Whole protein measurement

In order to minimize variability generated by the blood sampling methods (e.g. by taking up tissue liquid), the measured vitellogenin concentrations were normalized against the blood plasma protein content, expressed as ng VTG/ μ g protein. Total protein was quantified by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). The method of the BCA Protein Assay combines the reduction of Cu^{2+} to Cu^{+} and allows a selective colorimetric detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid. The coloured reaction is formed by the chelation of two molecules of BCA with one cuprous ion. This complex shows a strong absorbance at 562 nm, that is nearly linear to increasing protein concentration.

11-keto testosterone measurement

The measurement of 11-keto testosterone (11-kT) levels was made using a commercially available enzyme-linked immunoabsorbant assay (ELISA) raised to fish 11-keto testosterone purchased from Cayman Chemicals (Ann Arbor, Michigan, USA).

The assay is based on the competition between 11-kT and an 11-kT-acetylcholinesterase (AChE) conjugate (11-KT tracer) for a limited number of 11-kT -specific rabbit antiserum binding sites. Because the concentration of the 11-kT tracer is held constant, while the concentration of 11-kT varies, the amount of 11-kT is inversely proportional to the concentration of 11-kT in the well. This rabbit antiserum-11-kT complex binds to a mouse monoclonal antirabbit IgG antibody that has been previously attached to the well. After a washing step, Ellmann's reagent which contains the substrate to AChE, is added to the well. The product of this enzymatic reaction shows a strong absorbance at 412 nm. The intensity of this colour, determined by spectrophotometry, is proportional to the amount of 11-kT tracer bound to the wells, and inversely proportional to the amount of free 11-kT present in the well during the incubation.

Only samples of male fish were measured. To meet the linear part of the respective calibration curve it was necessary to dilute the plasma samples. Each sample was diluted in two steps, 10 and 20fold. Each dilution step was measured in duplicate, thus, every sample was measured 4 times. The inter-assay variation was estimated to be 4-12 %, the intra-assay variation was determined to be 10-19 %. The intra-assay variation of the measurements performed in this study, based on a comparison of all standard curves, was determined to be between 5 and 7 % and thus was in line with the given reference values.

A-3.3.5 Histopathology

The analysis of histological alterations was carried out according to the protocols by Wolf (2003) and Knörr (2005). In case of abnormal observations in zebrafish gonads, the abbreviation system developed within the OECD standardisation procedure for histological analyses of gonadal aberrations was used. For details see Table A-2.

Table A-2. Categories of maturation of zebrafish (*Danio rerio*) gonads

	Females	Males
Stage (Stg) 0 entirely immature	Oogonia (OOA) up to perinucleolar oocytes (PND)	Spermatogonia (SPA), spermatocytes (SPC) and spermatids (SPT)
Stage (Stg) 1 early vitellogenic/ spermatogenic phase	Main part of the germ cells are pre-vitellogenic follicles; mostly perinucleolar oocytes (PND) and alveolar oocytes (CAO)	immature phase is dominating; spermatozoa are present
Stage (Stg) 2 middle vitellogenic/ spermatogenic phase	At least half of the follicles are early (EVO) to late vitellogenic (LVO)	Spermatocytes (SPC), spermatids (SPT) and spermatozoa (SPZ) are present in roughly equal portion
Stage (Stg) 3 late vitellogenic/ spermatogenic phase	The majority of follicles in the ovary are late vitellogenic (LVO) and mature/spawning follicles (MSO).	All maturation grades are present, though the spermatozoa are dominating
Stage (Stg) 4 late vitellogenic/ hydrated	At stage 4, the majority of follicles are late vitellogenic and mature/spawning follicles. Follicles are larger as compared to Stg 3.	-

A-3.3.6 Statistical Data Analysis

The evaluation of the effect concentrations was based on nominal test item concentrations.

All biological response data were statistically analysed and reported separately for both males and females, except for endpoints of juvenile fish. Each data set was evaluated for normal distribution and homogeneity of variances.

If the prerequisites for parametric methods were met – normal distribution and variance homogeneity - effects were compared between treatments versus control group using analysis of variance (ANOVA). A Dunnett's or Williams' test on multiple pair-wise comparisons was performed subsequently.

If the prerequisites for parametric methods were not met - non-normal distribution or heterogeneous variance - , consideration was given to transform the data to achieve homogeneous variances prior to performing the ANOVA. In a few cases, where variance homogeneity could not be achieved, a Welch t-test with Bonferroni adjustment was performed. The significance level was chosen to be alpha = 0.05.

All statistical analyses were conducted using a PC based computer program (ToxRat[®] Professional (Alsdorf, Germany)).

A-4. Results

A-4.1 Chemical analysis

After collection, all samples were stored and analysed together after completion of the exposure phase of the study. There were 10, 3 and 1 sampling dates covering the P-generation, the F₁- generation and the F₂-generation, respectively. The results of the chemical analysis of concentrations during the different generations are summarised in Table A-3 and presented in detail in Table A-4.

Table A-3. Summary of results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate

		Nominal concentration of tamoxifen citrate [$\mu\text{g/L}$]				
		0.16	0.50	1.6	5.0	16
P-generation	[$\mu\text{g/L}$]	0.35	0.46	1.2	4.0	14
n=10	[%]	218	92	77	81	85
F₁-generation	[$\mu\text{g/L}$]	0.09*)	0.22	0.77	2.7	11
n=3	[%]	54	44	48	54	68
F₂-generation	[$\mu\text{g/L}$]	0.08*)	0.29	0.76	2.3	9.4
n=1	[%]	53	58	48	46	59

*) Note: since the analysis was near the limit of quantification, the values were calculated based on the mean recovery of the other treatment levels

A decrease of the tamoxifen citrate concentrations could be observed throughout the study. To stabilize the test concentrations, the stock solutions were adjusted to pH 3 using 1M HCl. The pH of the test medium within the test vessels was not affected and remained between 7.3 and 8.0 throughout the test.

Nevertheless, the concentrations at the end of the test were measured to be between 46 and 59 % of nominal. Thus, the effect concentrations were based on mean measured concentration of tamoxifen citrate. The mean measured concentrations were calculated separately for P-, F₁- and F₂-generation, and thus, the calculation of the effect concentrations was based on the respective mean measured values.

Chapter 8: Supplement A

Table A-4. Detailed results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate

P-generation (n=10 measurements)					
Nominal test concentration		Mean measured test concentration		sd	
		[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
	C	< LOQ	-	-	-
0.16	A	0.16	100	n.d.	n.d.
	B	0.28	172	0.19	69
	C	0.61	381	n.d.	n.d.
	mean	0.35	218	-	-
	sd	0.23	146	-	-
0.50	A	0.40	80	0.23	57
	B	0.50	99	0.25	50
	C	0.48	96	0.33	70
	mean	0.46	92	-	-
	sd	0.05	10	-	-
1.6	A	1.2	73	0.83	71
	B	1.2	73	0.66	57
	C	1.4	86	0.75	54
	mean	1.2	77	-	-
	sd	0.12	7.6	-	-
5.0	A	3.8	76	2.7	70
	B	4.0	80	2.2	55
	C	4.3	85	2.2	52
	mean	4.0	80	-	-
	sd	0.23	4.6	-	-
16	A	13	78	6.6	53
	B	15	97	8.8	57
	C	13	78	5.6	44
	mean	14	84	-	-
	sd	1.7	11	-	-

sd: standard deviation

Table A-4. (continued) Detailed results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate

F₁-generation (n=3 measurements)					
Nominal test concentration		Mean measured test concentration		sd	
[µg/L]	vessel no.	[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
	C	< LOQ	-	-	-
0.16	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
	C	< LOQ	-	-	-
	mean	-	-	-	-
	sd	-	-	-	-
0.50	A	0.21	41	0.01	3.4
	B	0.21	42	0.06	27
	C	0.25	49	0.11	43
	mean	0.22	44	-	-
	sd	0.02	4.4	-	-
1.6	A	0.90	56	0.54	60
	B	0.74	46	0.20	27
	C	0.66	41	0.37	57
	mean	0.77	46	-	-
	sd	0.12	7.7	-	-
5.0	A	3.0	61	1.2	40
	B	2.7	54	0.43	16
	C	2.4	48	1.9	80
	mean	2.7	54	-	-
	sd	0.31	6.2	-	-
16	A	12	72	n.d.	n.d.
	B	11	69	n.d.	n.d.
	C	10	63	1.2	12
	mean	11	68	-	-
	sd	0.74	4.6	-	-

sd: standard deviation

Table A-4. (continued) Detailed results of chemical analysis (measured concentrations) during a two generation test with tamoxifen citrate

F₂-generation (n=1 measurement)			
Nominal test concentration		Mean measured test concentration	
		[µg/L]	[%]
[µg/L]	vessel no.	[µg/L]	[%]
Control	A	< LOQ	-
	B	< LOQ	-
	C	< LOQ	-
0.16	A	< LOQ	-
	B	0.16	100
	C	< LOQ	-
	mean	0.16	100
	sd	-	-
0.50	A	0.38	76
	B	0.18	36
	C	0.31	62
	mean	0.29	58
	sd	0.10	20
1.6	A	0.58	36
	B	0.97	61
	C	0.74	46
	mean	0.76	48
	sd	0.20	12
5.0	A	2.3	46
	B	2.3	47
	C	2.3	46
	mean	2.3	46
	sd	0.03	-
16	A	9.4	59
	mean	9.4	59
	sd	-	-

sd: standard deviation

A-4.2 P-generation

The study was initiated by introduction of 8 adult males and females per test vessel.

Reproduction was recorded for about 20 days. Since a first analysis of the test concentrations after 4 weeks of study duration revealed recovery rates of tamoxifen citrate below 5% of the nominal concentration in the whole test system, the recording of reproduction had to be stopped. After stabilisation and confirmation of the test concentrations the recording of reproduction was initiated again on study day 73.

A-4.2.1 P-generation, Observations

During the reproductive phase of the P-generation no concentration related mortality could be observed. A change in mating behaviour could not be observed in any treatment level.

A-4.2.2 P-generation, Reproduction

To record the parameters fecundity and fertility, eggs were collected from spawning trays on each working day, counted and inspected for early blastula stages. For the final evaluation the egg number per female and day, and the cumulative number of fertilised eggs per treatment was calculated.

Fecundity

The egg number per female and day was not significantly affected (Fig. A-2).

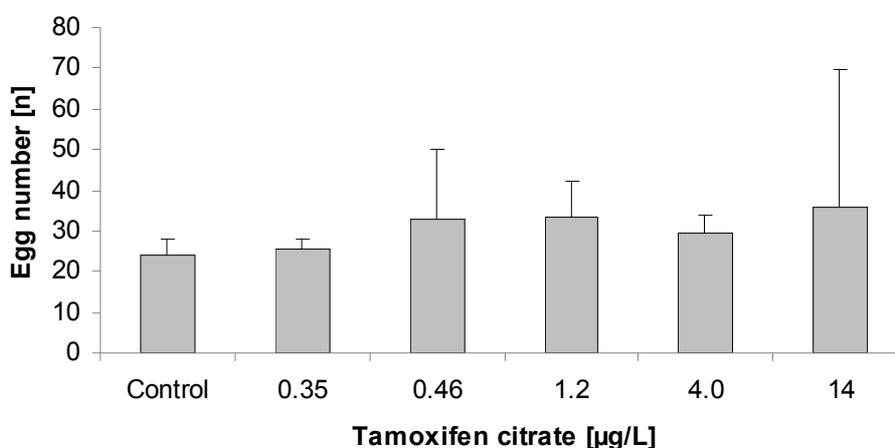


Fig. A- 2. P-generation, Egg numbers per day and female. Means of three replicates and standard deviation. Test concentrations are presented as mean measured concentrations.

Regarding the cumulative number of fertilised eggs, a clear and significant reduction could be observed at the highest measured test concentration of 14 µg tamoxifen citrate/L (Fig. A-3).

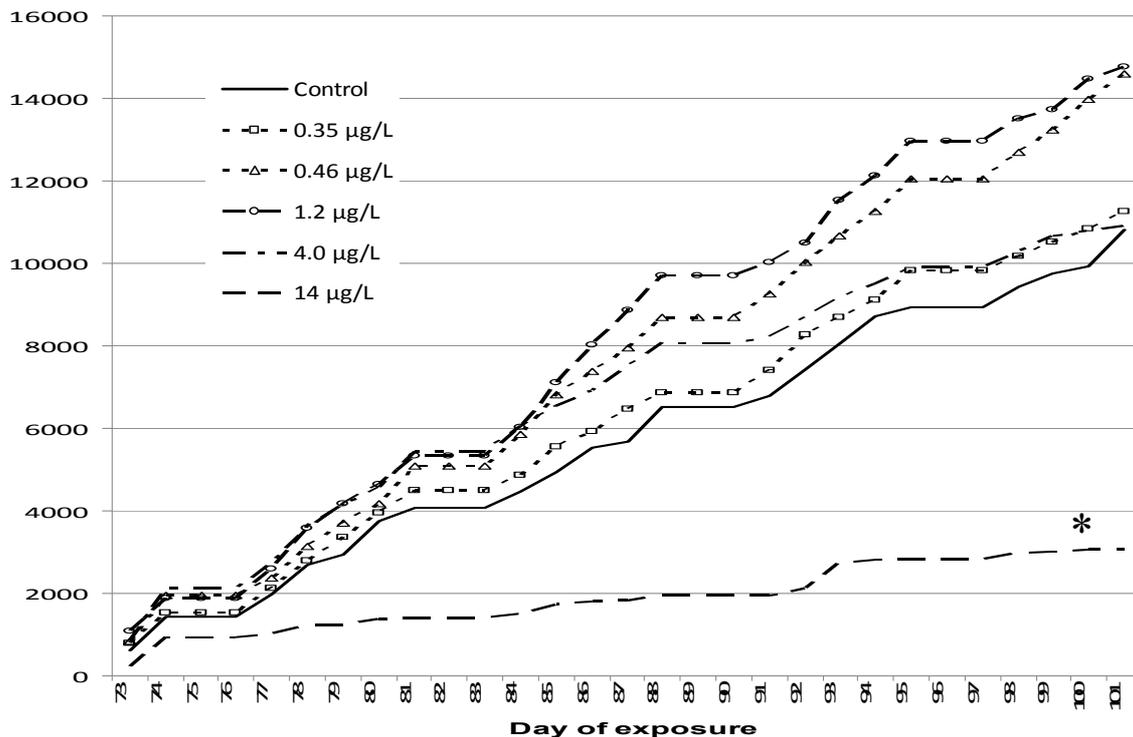


Fig. A- 3. P-generation, Cumulative number of fertilised eggs. Test concentrations of tamoxifen citrate are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

Fertility

Fertilisation rate was significantly reduced at the highest test concentration (Figure A-4).

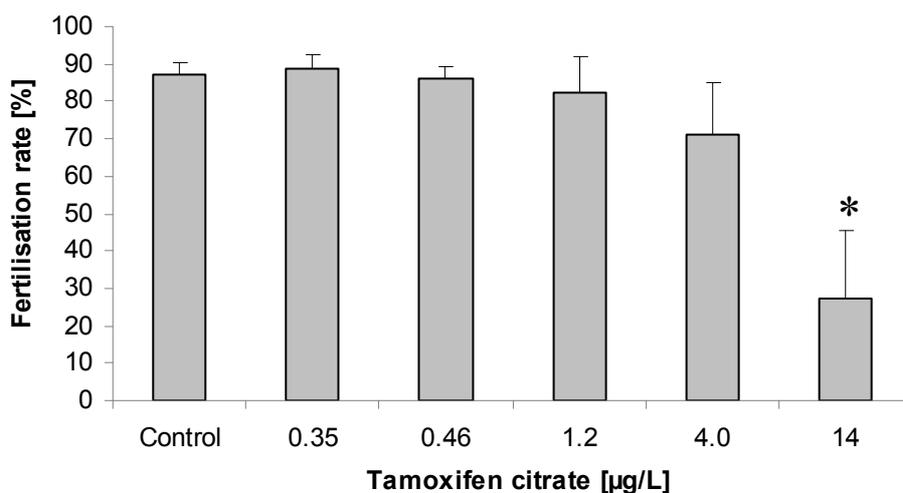


Fig. A- 4. P-generation, Fertilisation rate [%]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

A-4.2.3 P-generation, Biomarker

Blood samples were taken from the adult fish at termination of the P- and the F₁-generation. The bloodplasma was analysed for the content of the egg yolk precursor protein vitellogenin. Additionally, the sex steroid 11-keto testosterone was measured in male bloodplasma.

Vitellogenin

For the determination of the vitellogenin concentration, bloodplasma samples of 15 males and 15 females per treatment were analysed. The VTG concentrations were related to the total protein content. The results showed a significant decrease for both males and females at 4.0 and 14 µg tamoxifen citrate/L (Figure A-5, A-6).

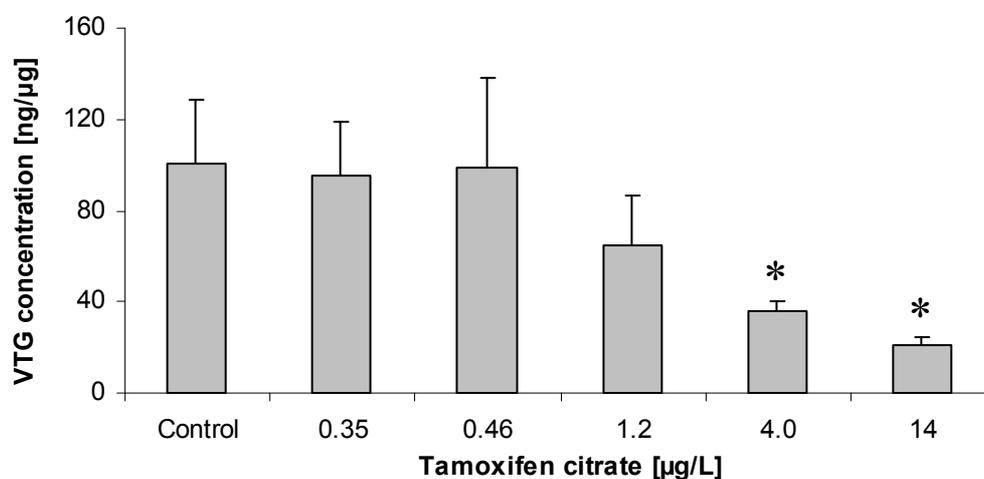


Fig. A- 5. P-generation, VTG concentration in female blood plasma [ng/µg] Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

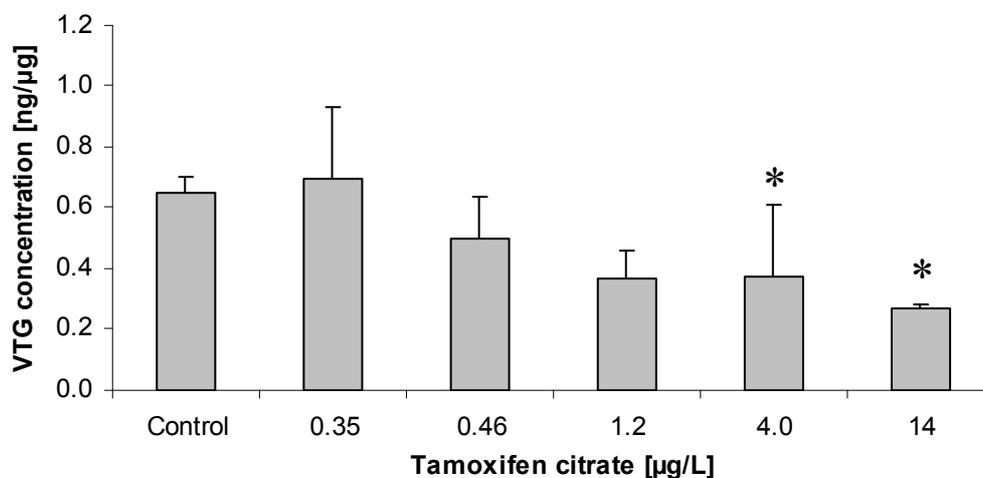


Fig. A- 6. P-generation, VTG concentration in male blood plasma [ng/µg] Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

11-keto testosterone

In addition to vitellogenin, the sex steroid 11-keto testosterone was measured. For the P-generation, a significant increase of the androgen concentration could be observed at the highest test level (Figure A-7).

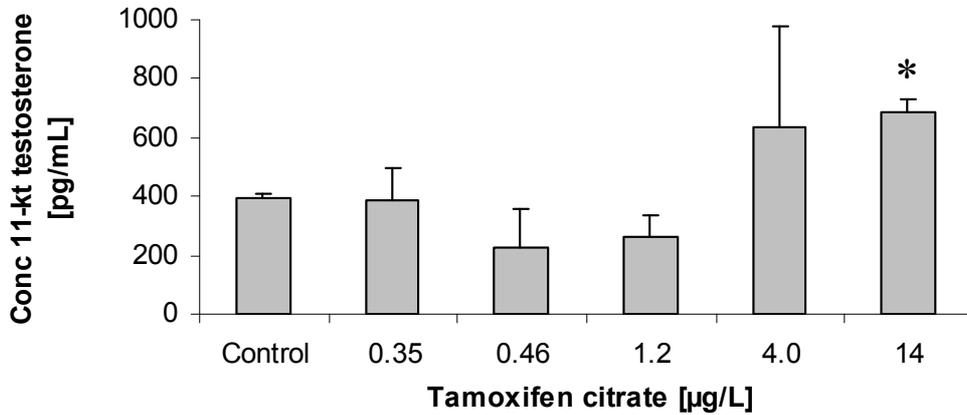


Fig. A- 7. P-generation, 11-keto testosterone concentration in male blood plasma [pg/mL] Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

All results regarding the P-generation are summarised in Table A-5.

Table A-5. P-generation, Summary of all endpoints

	Nominal concentrations tamoxifen citrate [$\mu\text{g/L}$]					
	Control	0.16	0.50	1.6	5.0	16
	Mean measured concentrations [$\mu\text{g/L}$]					
	Control	0.35	0.46	1.2	4.0	14
Egg number /female/day [n]	24 \pm 3.9	25 \pm 2.6	33 \pm 17	33 \pm 9.0	30 \pm 4.5	36 \pm 34
Cumulative number of fertilised eggs [n]	3608 \pm 572	3757 \pm 430	4865 \pm 2672	4920 \pm 1591	3636 \pm 1215	1020 \pm 351 *)
Fertilisation rate [%]	87 \pm 3.1	89 \pm 3.7	86 \pm 2.8	82 \pm 10	71 \pm 14	27 \pm 18*)
Vitellogenin females [ng/ μg]	100 \pm 28.3	95.4 \pm 23.1	99.1 \pm 39.2	64.9 \pm 21.7	35.6 \pm 4.9*)	21.2 \pm 3.3*)
Vitellogenin males [ng/ μg]	0.65 \pm 0.05	0.69 \pm 0.24	0.50 \pm 0.14	0.36 \pm 0.10	0.37 \pm 0.24*)	0.27 \pm 0.01*)
11-keto testosterone [pg/mL]	396 \pm 14.8	389 \pm 107	224 \pm 131	260 \pm 72.9	631 \pm 346	683 \pm 47.1#)

Means of replicate chambers (n=3) \pm standard deviation

*) Significant negative deviation from control (p<0.05)

#) Significant positive deviation from control (p<0.05)

A-4.3 F₁-generation

The Filial 1 (F₁)-generation was initiated by placing 100 fertilised eggs from the P adult fish in fry chambers. In the following, hatch, survival and growth were recorded.

A-4.3.1 F_1 -generation, Observations

During the reproductive phase of the F_1 -generation, no concentration related mortality could be observed. Also, abnormal behaviour (e.g. loss of equilibrium, change in swimming behaviour, change in mating behaviour, etc) could not be observed at any treatment level.

A-4.3.2 F_1 -generation, Hatch/Survival

Hatch

The hatching rate, being the number of hatched larvae in relation to the number of introduced eggs, was determined to be above 80% in the control group, and at 0.22, 0.77 and 2.7 μg tamoxifen citrate/L. Hatch of the F_1 fish larvae was significantly reduced at 11 μg tamoxifen citrate/L (Figure A-8).

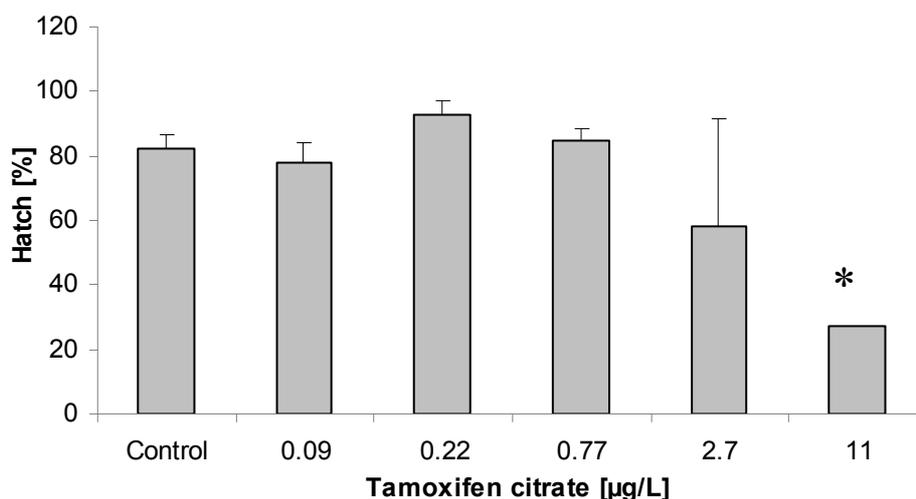


Fig. A- 8. F_1 -generation, Hatch of fish larvae [%]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

Survival

Post hatch survival, being the number of surviving larvae in relation to the number of hatched larvae, was 74% in the control, and thus the quality criterion as given by the respective guideline regarding post hatch survival was fulfilled (Figure A-9).

Growth

Reduced length of the fish larvae could be observed at the highest treatment level. Since only 5 animals remained in one replicate of this treatment level, this group was excluded from statistical evaluation. No effect on length could be observed for the other treatment groups.

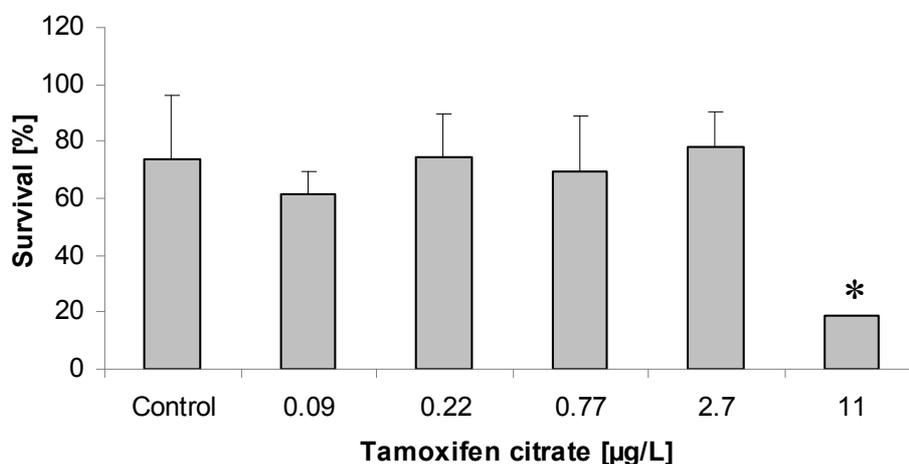


Fig. A- 9. F₁-generation, Survival of fish larvae at day 28pf [%]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control. Note: Treatment 5 only consists of one replicate containing 5 animals, and thus, was excluded from the statistical evaluation

A-4.3.3 F₁-generation, Juvenile Growth

No effect on survival could be observed for the juvenile fish of the F₁-generation.

Length of the F₁ fish was measured at day 28 and day 63. The pseudo specific growth rate was calculated according to the OECD guideline 215 (OECD, 2000). The control and the treatment groups 1-4 showed similar values (Figure A-10). At test concentration 5, a slight increase of the growth rate could be observed. However, this treatment consisted only of one group containing 5 animals. Thus, the effect on the growth rate was related to the low fish density in the respective test vessel.

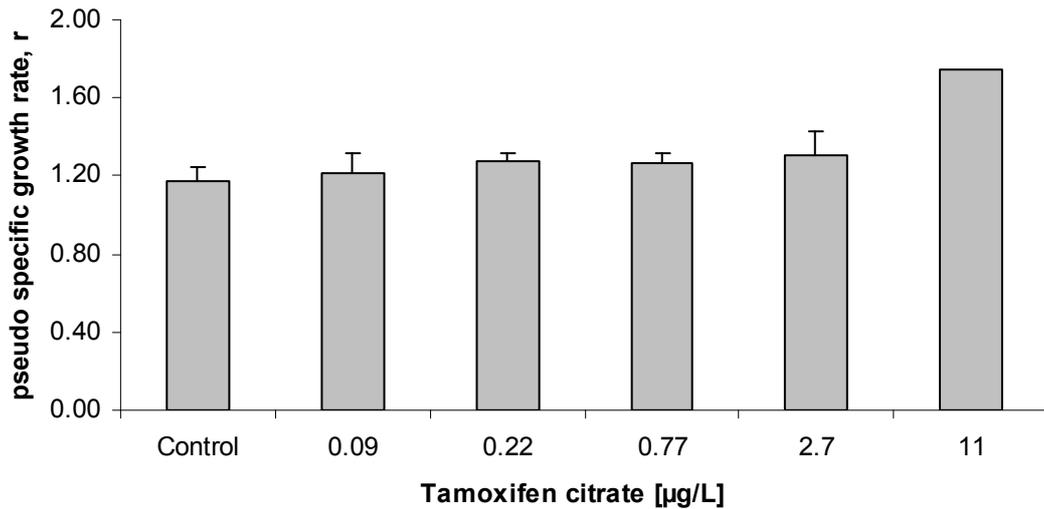


Fig. A- 10. F₁-generation, Pseudo specific growth rate based on length measurements on day 28 and day 63. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control. Note: Due to the reduced fish density, the highest treatment level was excluded from the statistical evaluation of the growth parameters.

A-4.3.4 F₁-generation, Reproduction

Fecundity

The total egg numbers were recorded for a period of 55 days. The total egg numbers in the treatment groups were generally low compared to the control group. As a consequence, the cumulative numbers of fertilised eggs were significantly reduced in all exposure groups (Figure A-12). However, regarding total egg number per day and female, no significant change could be detected (Figure A-11).

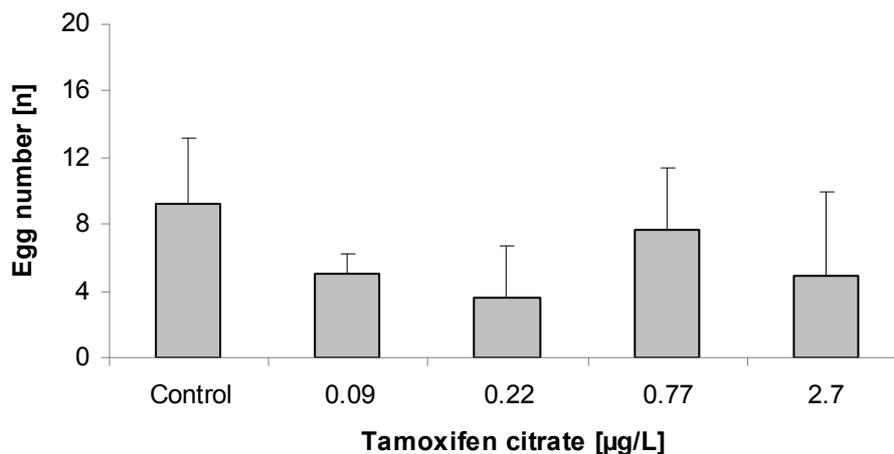


Fig. A- 11. F₁-generation, Egg numbers per day and female [n]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. Note: No eggs were spawned at treatment group 5 (nominal: 16 $\mu\text{g/L}$).

Cumulative number of fertilised eggs

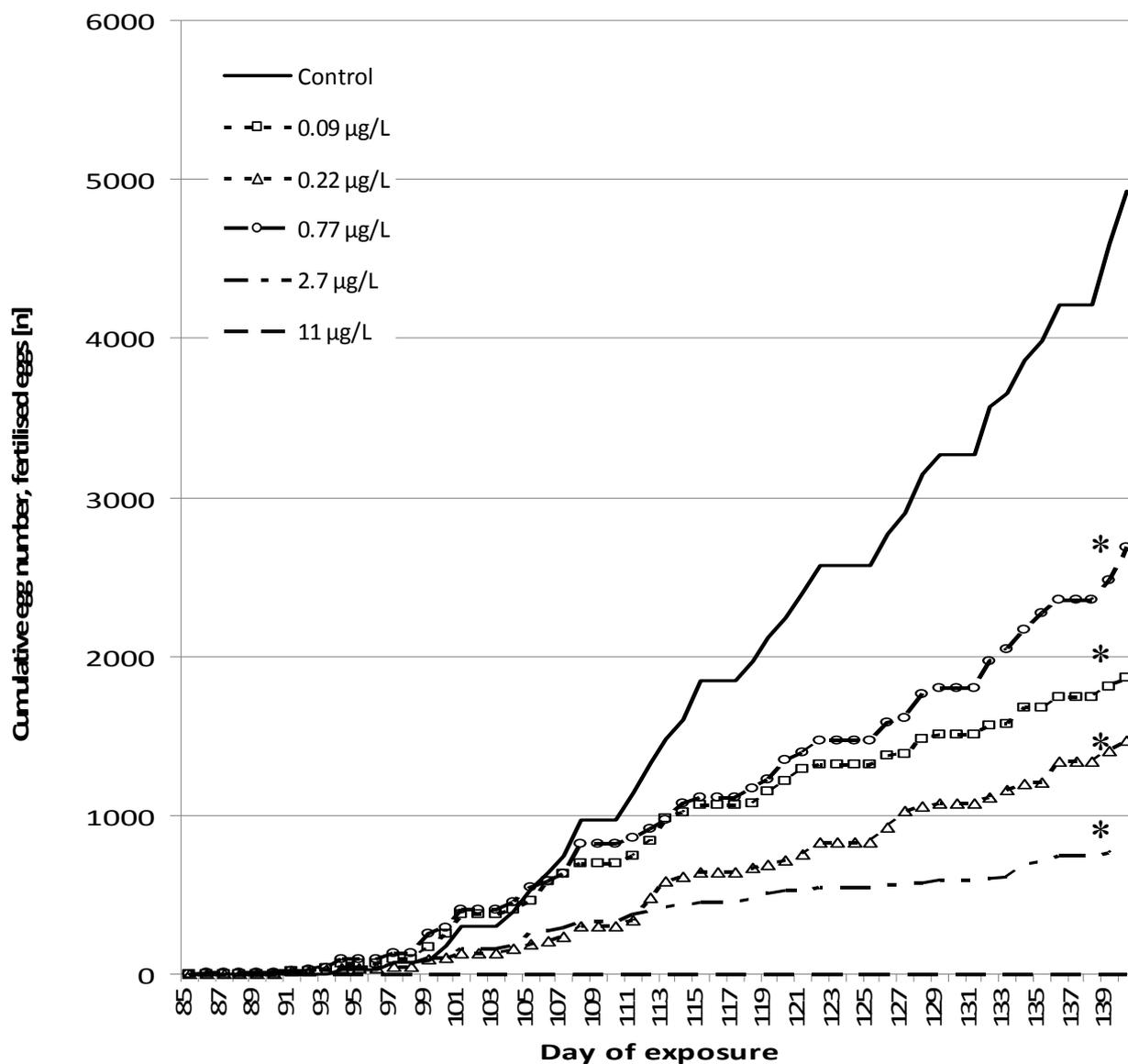


Fig. A- 12. F₁-generation, Cumulative number of fertilised eggs [n]. Test concentrations are presented as mean measured concentrations. *) Significant (p<0.05) compared to the control.

Note: No eggs were spawned at treatment group 5.

Fertility

No effect on the fertilisation rate could be detected (Figure A-13).

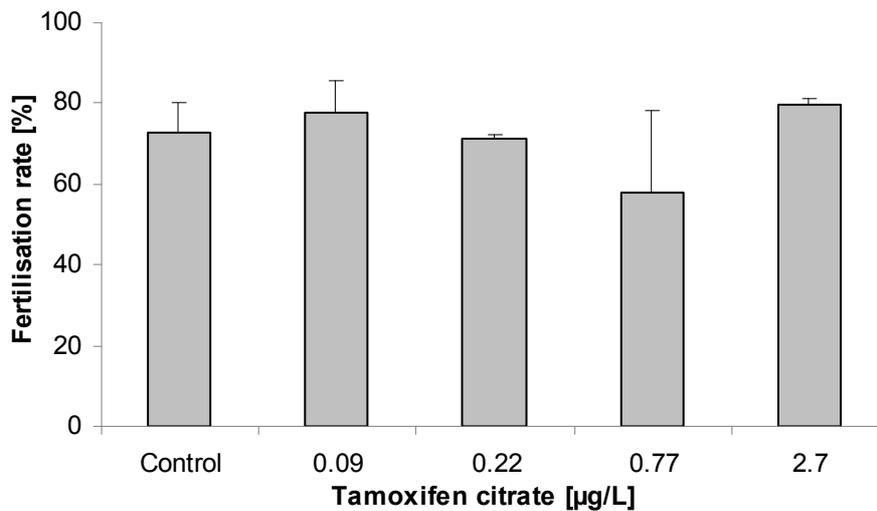


Fig. A- 13. F₁-generation, Fertilisation rate [%]. Mean of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations.

A-4.3.5 F₁-generation, Biomarker

Blood samples were taken from the adult fish at the end of the exposure period of the F₁-generation. The blood plasma was analysed for the content of the egg yolk precursor protein vitellogenin. Additionally, the sex steroid 11-keto testosterone was measured in male blood plasma.

Vitellogenin

Vitellogenin concentrations were analysed in blood plasma samples of 15 males and 15 females per treatment (5 females, 5 males per replicate), if enough animals were available for sampling. The VTG concentrations were related to the total protein content. The results showed a significant increase of VTG in male blood plasma at 0.77 and 2.7 µg tamoxifen citrate/L (Figure A-14). However, this effect can be considered as not being biologically relevant, since the increases are still within the range of historical control values (ranging from 0.1-1.5 ng/µg, *unpublished data*). Female fish were not significantly affected.

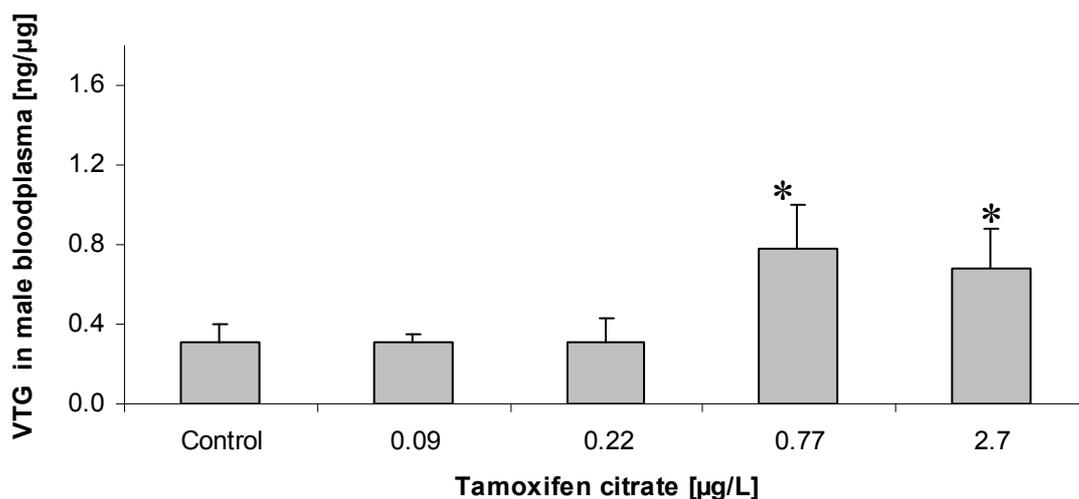


Fig. A- 14. F₁-generation, VTG concentration in male blood plasma [ng/μg]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant (p<0.05) compared to the control.

11-keto testosterone

For the measurement of 11-keto testosterone, the bloodplasma of 5 males per replicate was analysed. The analysis revealed decreased levels at the low test concentrations. At the high treatment levels, the concentrations of 11-keto testosterone increased and reached values as measured in the controls (Figure A-15).

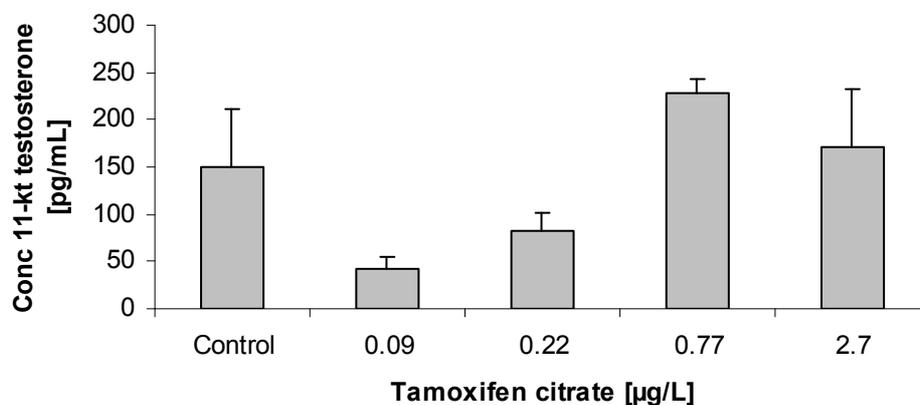


Fig. A- 15. F₁-generation, Concentration of 11-keto testosterone in male blood plasma [pg/mL]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations.

A-4.3.6 *F₁-generation, Sex ratio*

The sex ratio was significantly affected at the three highest concentration levels. A shift towards males could be observed, reaching 100% at the highest test concentration (Figure A-16). The mean ratio of females in controls was above 70%. Historical data showed female ratios in controls between 35 and 65%. However, at the low treatment levels, the female ratios were within the range of the controls.

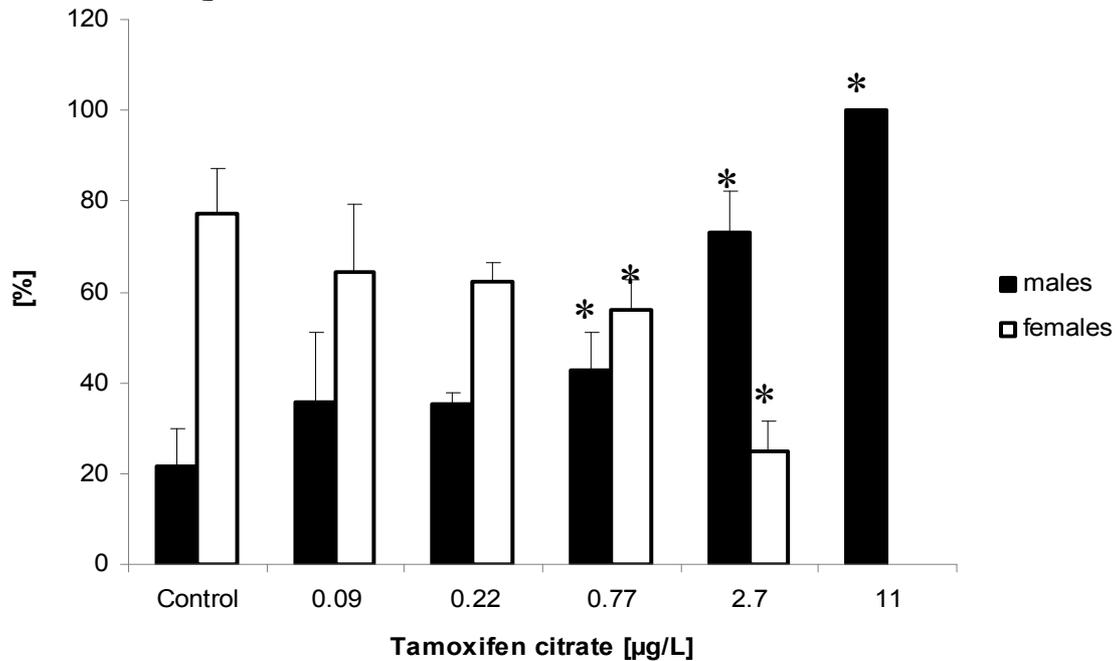


Fig. A- 16. *F₁-generation, Sex ratio of adult fish [%].* Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *) Significant ($p < 0.05$) compared to the control.

A-4.3.7 *F₁-generation, Histopathology*

The overall changes in zebra fish exposed to tamoxifen citrate can be summarized as follows:

- significant dose dependent increase of male fish in the *F₁*-generation;
- bivalent effect on maturity in both sexes: reduced maturity at low and accelerated maturity at higher concentrations;
- increase of atretic immature oocytes, proteinaceous fluid and egg debris, indicating overall reduced egg quality;
- slight dose dependent increase of hyperplasia of Sertoli cells paired in the *F₁*-generation with an increased sperm production in the *F₁*-generation;
- dose-dependent increase of fibrosis in the gonads of both generations.

All endpoints regarding the F₁ fish are summarised in Table A-6 and A-7.

Table A-6. F₁-generation, Early Life Stage, Juvenile Growth and Reproduction, Summary of all endpoints

	Nominal concentration tamoxifen citrate					
	[µg/L]					
	Control	0.16	0.50	1.6	5.0	16
	Mean measured concentration					
	[µg/L]					
Control	0.09	0.22	0.77	2.7	11	
Hatching ELS [%]	82 ± 4.0	78 ± 6.2	93 ± 4.7	85 ± 3.6	58 ± 34	27*)
Post hatch survival ELS, 28dpf [%]	74 ± 23	61 ± 8.2	74 ± 16	69 ± 20	78 ± 12	19*)
Length, day 28pf [cm]	0.92 ± 0.07	0.94 ± 0.03	0.80 ± 0.05	0.81 ± 0.12	0.84 ± 0.14	0.57+)
Pseudo specific growth rate (based on length)	1.18 ± 0.07	1.22 ± 0.09	1.27 ± 0.04	1.26 ± 0.06	1.30 ± 0.13	1.75+)
Egg number /female/day [n]	9 ± 4	5 ± 1	4 ± 3	8 ± 4	5 ± 5	-
Cumulative number of fertilised eggs [n]	4929 ± 1804	1869 ± 274*)	2200 ± 948*)	2689 ± 1386*)	810 ± 685*)	-
Fertilisation rate [%]	73 ± 7.1	78 ± 8.0	71 ± 1.0	58 ± 20	80 ± 1.7	-

Means of replicate chambers (n=3) ± standard deviation

*) Significant (p< 0.05)

+) Due to the reduced number of fish, this group was excluded from statistical evaluation of growth parameters

-) No eggs were found throughout the study

Table A-7. F₁-generation, Sex ratio and Biomarker, Summary of all endpoints

	Nominal concentration tamoxifen citrate [µg/L]					
	Control	0.16	0.50	1.6	5.0	16
	Mean measured concentration [µg/L]					
	Control	0.09	0.22	0.77	2.7	11
Sex ratio, Number of females [%]	77.3 ± 9.7	64.2 ± 15	62.5 ± 4.0	56.1 ± 6.8*)	24.9 ± 6.7*)	-)
Vitellogenin females [ng/µg]	34.7 ± 9.5	35.9 ± 9.9	39.4 ± 22	18.5 ± 9.0	18.2 ± 7.8	#)
Vitellogenin males [ng/µg]	0.31 ± 0.09	0.31 ± 0.04	0.31 ± 0.12	0.78 ± 0.22*)	0.68 ± 0.20*)	#)
11-keto testosterone [pg/mL]	150 ± 60.7	42.2 ± 13.7	81.5 ± 20.8	228 ± 15.5	171 ± 61.9	#)

Means of replicate chambers (n=3) ± standard deviation

*) Significant (p < 0.05)

-) No females were found at this treatment level

#) Due to low number of individuals, this group was excluded from ELISA measurements

A-4.4 F₂-generation

In two vessels with adult F₁ fish, no eggs were found up to the end of the reproductive phase (conc2, replicate1 and conc4, replicate3). For four further groups (conc2, replicate3; conc3, replicate 3; conc 4, replicates 2 and 3), very low egg numbers were observed. In these vessels, only 50 fertilised eggs could be introduced in the fry chambers for initiation of the F₂-generation.

A-4.4.1 F₂-generation, Hatch/Survival/Length

Hatch was above 80% in the controls. No effect on hatching success due to tamoxifen citrate exposure could be observed at any test concentration. The post hatch survival at day 35pf was also not affected (Figure A-17).

Regarding length, a slight increase could be observed at 2.3 μg tamoxifen citrate/L (Figure A-18). Due to reduced spawning, only 50 fertilised eggs could be introduced in the replicates of the highest treatment level. The reduced fish density in the respective fry chambers led to increased growth of the fish.

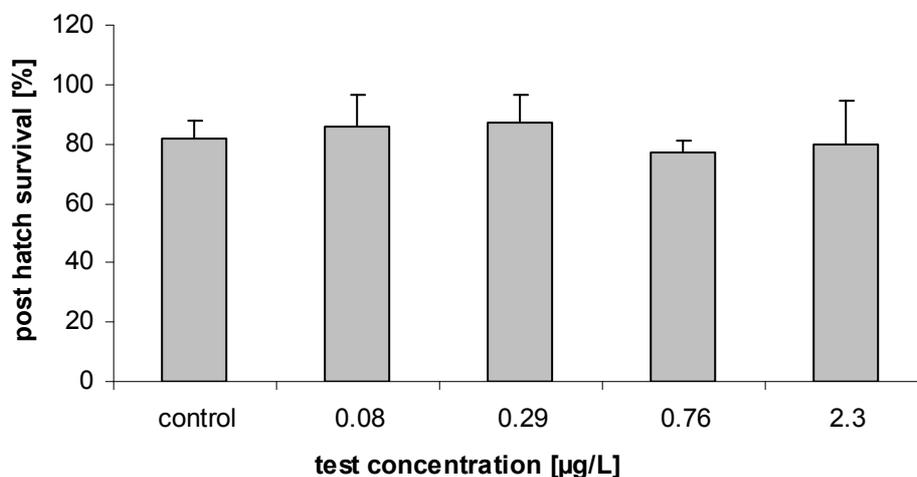


Fig. A- 17. F₂-generation, Survival of fish larvae at day 35pf [%]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations.

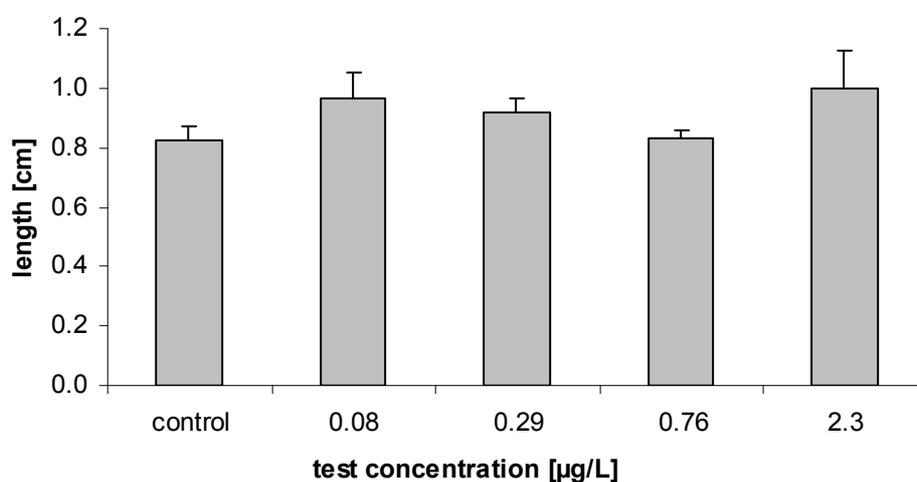


Fig. A- 18. F₂-generation, Length of fish larvae at day 35pf [cm]. Means of three replicates + standard deviation. Test concentrations are presented as mean measured concentrations. *: Significant ($p < 0.05$) compared to the control.

Note: Only 50 eggs (instead of 100) were introduced at the highest treatment level, thus fish density was lower resulting in increased growth.

All endpoints and the respective mean values are presented in Table A-8.

Table A-8. F₂-generation, Early Life Stage, Summary of all endpoints

	Nominal concentration tamoxifen citrate [µg/L]				
	Control	0.16	0.50	1.6	5.0
	Mean measured concentration [µg/L]				
	Control	0.08	0.29	0.76	2.3
Hatch [%]	87 ± 4.5	79 ± 5.0	85 ± 19	88 ± 3.8	86 ± 20
Post hatch survival, 35dpf [%]	82 ± 6.1	86 ± 11	87 ± 9.6	77 ± 3.9	80 ± 15
Length, 35dpf [cm]	0.82 ± 0.05	0.97 ± 0.08	0.92 ± 0.05	0.83 ± 0.03	1.00 ± 0.12*)

Means of replicate chambers (n=3) ± standard deviation

*) Significant (p < 0.05) compared to the control

A-5. Summary and Conclusions

A two generation test with tamoxifen citrate was performed to evaluate and assess the effects after continuous exposure on all life stages of zebrafish.

This includes the parental generation (P-generation), early life stages (life phase directly post hatch), juvenile growth and reproduction of the Filial 1 (F₁)-generation and finally, the early life stage phase of the Filial 2 (F₂)-generation. Furthermore, indicative endpoints were determined including the measurement of molecular biomarkers and histopathology. The recorded endpoints are listed in table 7.

The shift in sex ratio of the F₁ fish towards males was considered to be the most sensitive population relevant endpoint.

A reduced cumulative number of fertilised eggs compared to the control was detected in the F₁-generation in all test concentrations. However, since the total egg number per day and female as well as the fertilisation rate were not significantly affected, this finding was considered as not being biologically relevant.

The measurement of molecular biomarkers revealed a decrease of vitellogenin in both males and females of the P-generation. Compared to the most sensitive population relevant endpoint

(sex ratio of F₁-generation), the VTG response showed similar sensitivity. VTG was found to be increased in the F₁ male fish, but did not exceed historical control levels.

Regarding 11-keto testosterone, a slight, but not significant increase could be observed in male bloodplasma of the P-generation.

The Histopathology evaluation revealed the following results:

- 1) significant dose dependent increase of male fish in the F₁-generation,
- 2) bivalent effect on maturity in both sexes: reduced maturity at low and accelerated maturity at higher concentrations,
- 3) increase of atretic immature oocytes, proteinaceous fluid and egg debris, indicating overall reduced egg quality,
- 4) slight dose dependent increase of hyperplasia of Sertoli cells paired in the F₁-generation with an increased sperm production, and
- 5) dose-dependent increased fibrosis in the gonads in both generations.

In a partial Life Cycle test with tamoxifen, including a Parental-generation of about 21 days and an Early Life Stage Phase of the F₁-generation of about 42 days, van der Veen et al. (2007) observed a reduced hatching rate and reduced growth in weight for the F₁ fish being the most sensitive population relevant endpoints. A reduced fecundity as a consequence of corresponding to reduced vitellogenesis could be observed, but this endpoint was less sensitive than the ones mentioned above. The histopathological analysis revealed an increase of Leydig cells in the gonads of the adult fish.

Williams et al. (2007) performed a Partial Life Cycle Test and a Full Life Cycle Test with tamoxifen citrate. Fathead minnows (*Pimephales promelas*) were used as test organisms. The fish were most sensitive in the juvenile growth phase of the F₁-generation of the Partial Life Cycle Test. The reduction of VTG was found to be more sensitive by two concentration steps. In both study parts, sex ratio and fertilisation rate were not determined.

Table A-9. Two generation test with tamoxifen citrate, Summary of effect data (mean measured values)

		Endpoint		NOEC	LOEC
	Life Phase			[µg/L]	[µg/L]
P-generation	Reproduction	Egg number/day/female		14	>14
		Cumulative number of fertilised eggs		4.0	14
		Fertilisation rate		4.0	14
	Biomarker	Vitellogenin	Females decrease	1.2	4.0
			Males decrease	1.2	4.0
		11-keto testosterone	Males Increase	4.0	14
F₁- generation	Early Life Stage	Hatch		2.7	11
		Survival, day 28		2.7	11
		Growth (Length), day 28		2.7	11
	Juvenile Growth	Survival, day 63		11	>11
		Pseudo spec. growth rate		2.7	11 ¹⁾
	Reproduction	Egg number per day and female		11	>11
		Cumulative number of fertilised eggs		< 0.09	0.09 ²⁾
		Fertilisation rate		11	>11
	Biomarker	Vitellogenin	Females	11	>11
			Males Increase	0.22	0.77 ³⁾
		11-keto testosterone		11	>11
	Sex ratio		Shift towards males	0.22	0.77
F₂	Early Life Stage	Hatch		2.3	>2.3
		Survival day 35		2.3	>2.3
		Growth (Length)		0.76	2.3 ⁴⁾

¹⁾ due to reduced fish density, this group was excluded from statistical evaluation of the growth parameters

²⁾ spawning was poor in all F₁ treatment groups. However, egg number and fertilisation did not show any statistically significant effect

³⁾ considered as not being biologically relevant, since the overall male VTG values were still very low and comparable to historical control values

⁴⁾ effect on growth (length) due to reduced fish density, only 50 instead of 100 eggs were kept for hatching

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Chapter 8: Supplement B

Two Generation Test with zebrafish (*Danio rerio*)

Test substance: 17 β -Trenbolone (17 β -Hydroxyestra-4,9,11-trien-3-on)

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Authors:

Melanie Böttcher¹

Matthias Teigeler²

Christoph Schäfers²

Thomas Braunbeck¹

¹ Ruprecht-Karls-University Heidelberg, Aquatic Ecology and Toxicology Section,
Department of Zoology, Im Neuenheimer Feld 230, D-69120 Heidelberg, Germany

² Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie (IME)
Auf dem Aberg 1, 57392 Schmallenberg, Germany

Content

B-1. Introduction	B-6
B-2. Study conditions	B-6
B-2.1 Test organism and maintenance	B-6
B-2.1.1 Origin of the test organisms	B-6
B-2.1.2 Fish maintenance	B-6
B-2.2 Test substance and test concentrations	B-7
B-2.3 Test system	B-8
B-2.3.1 Test vessels	B-8
B-2.3.2 Flow-through system	B-8
B-3. Performance of the study	B-9
B-3.1 Description of the different life phases	B-9
B-3.1.1 Parental (P)-generation	B-11
B-3.1.2 First filial (F ₁)-generation	B-11
B-3.1.3 Second filial (F ₂)-generation	B-11
B-3.2 Observations	B-12
B-3.3 Specific methods	B-12
B-3.3.1 Collection of eggs	B-12
B-3.3.2 Photography and image analysis	B-13
B-3.3.3 Blood collection and preparation	B-14
B-3.3.4 Measurement of biomarkers using ELISA	B-14
B-3.3.5 Statistical analysis	B-15
B-3.3.6 Histological analyses of gonadal alterations	B-16
B-4. Results	B-17
B-4.1 Chemical analysis	B-17
B-4.2 Parental generation	B-18
B-4.2.1 Macroscopic observations in the parental generation	B-18
B-4.2.2 Reproduction in the parental generation	B-18
B-4.2.3 Molecular biomarkers in the parental generation	B-20
B-4.2.4 Histological alterations in zebrafish of the parental generation exposed to 17β-trenbolone	B-21
B-4.3 F ₁ -Generation	B-24
B-4.3.1 Macroscopic observations in the first filial (F ₁) generation	B-24
B-4.3.2 Hatch and survival in the first filial (F ₁) generation	B-24
B-4.3.3 Juvenile growth in zebrafish of the first filial (F ₁) generation	B-26

B-4.3.4	Sex ratio in zebrafish of the first filial (F ₁) generation	B-27
B-4.3.5	Reproduction in zebrafish of the first filial (F ₁) generation	B-28
B-4.3.6	Molecular biomarkers in the first filial (F ₁) generation	B-29
B-4.3.7	Histological alterations in zebrafish of the first filial generation exposed to 17β-trenbolone	B-30
B-4.4	F ₂ -generation	B-33
B-4.4.1	Macroscopic observations in the second filial (F ₂) generation	B-33
B-4.4.2	Hatch and survival in the second filial (F ₂) generation	B-33
B-4.4.3	Juvenile growth in zebrafish of the second filial (F ₂) generation	B-34
B-5	Summary and conclusions	B-35
B-6	References	B-45

List of tables and figures

Table B-1: Water conditions during exposure to 17 β -trenbolone	B-6
Table B-2: Nominal and measured mean concentrations of 17 β -trenbolone (ng/L).....	B-7
Table B-3: Study plan for the two-generation study on 17 β -trenbolone with zebrafish (<i>Danio rerio</i> ; 5 concentrations + water control, 3 replicates each).....	B-10
Table B-4: Categories of maturation of zebrafish (<i>Danio rerio</i>) gonads following Wolf (2003) and Knörr (2005)	B-15
Table B-5: Classification of histological analyses into primary and secondary diagnoses	B-15
Table B-6: Results of chemical analyses during the two-generation test with 17 β - trenbolone in replicate tanks (R1, R2)	B-16
Table B-7: Summary of histological alterations in the parental generation of zebrafish (<i>Danio rerio</i>) after 62 d exposure to various concentrations of 17 β -trenbolone .	B-20
Table B-8: Summary of histological alterations in the F1 generation of zebrafish (<i>Danio rerio</i>) after 177 d of cumulated exposure to various concentrations of 17 β - trenbolone.....	B-28
Table B-9: Summary of all effects observed in zebrafish during a two-generation test with 17 β -trenbolone	B-34
Table B-10: Summary of relevant effects observed in zebrafish during a two-generation test with 17 β -trenbolone (NOECs and LOECs expressed as nominal concentrations)	B-38
Figure B-1: Breeding chamber	B-7
Figure B-2: Setup of flow-through aquarium system.....	B-8
Figure B-3: After 24 h, fertilized eggs can easily be differentiated from non-fertilized eggs.....	B-12
Figure B-4: Cumulative number of eggs of zebrafish (<i>Danio rerio</i>) exposed to 0, 1, 3, 10, 30 and 90 ng/L 17 β -trenbolone	B-17
Figure B-5: Fertilization rate in the parental generation of zebrafish exposed to 17 β - trenbolone.....	B-17
Figure B-6: Daily fertilization rate in the parental generation of zebrafish (<i>Danio rerio</i>) exposed to various concentrations of 17 β -trenbolone.....	B-18
Figure B-7: Mean vitellogenin (VTG) concentration in blood plasma relative to total protein contents in male (a) and female (b) zebrafish of the parental generation after exposure to 17 β -trenbolone (\pm SD).....	B-18
Figure B-8: Mean concentrations von 11-keto testosterone in blood plasma of male zebrafish of the parental generation exposed to 17 β -trenbolone (\pm SD).....	B-19
Figure B-9: Histology of female gonads of zebrafish of the parental generation exposed to 17 β -trenbolone	B-21
Figure B-10: Paraffin sections of male gonads of zebrafish of the parental generation exposed to 17 β -trenbolone	B-22
Figure B-11: Hatching rate of F1 larvae under exposure to 17 β -trenbolone	B-23

Figure B-12: Mortality of F1 zebrafish after different periods of exposure to 17 β -trenbolone..... B-24

Figure B-14: Mean growth in adult F1 zebrafish after exposure to 17 β -trenbolone based on length and weight measurements B-24

Figure B-15: Sex ratio in F1 zebrafish after exposure to 17 β -trenbolone based on histological observations B-25

Figure B-16: Cumulative number of eggs of the F1 generation of zebrafish (*Danio rerio*) exposed to 0, 1 and 3 ng/L 17 β -trenbolone B-26

Figure B-17: Mean fertilization rates in the F1 generation of zebrafish (*Danio rerio*) exposed to 1 and 3 ng/L 17 β -trenbolone (\pm SD) B-26

Figure B-18: Mean vitellogenin (VTG) concentration in blood plasma relative to total protein contents in male (a) and female (b) zebrafish of the F1 generation after exposure to 17 β -trenbolone (\pm SD) B-27

Figure B-19: Mean concentrations von 11-keto testosterone in blood plasma of male zebrafish of the F1 generation exposed to 17 β -trenbolone (\pm SD) B-27

Figure B-20: Histology of female gonads of zebrafish of the first filial (F1) generation exposed to 17 β -trenbolone B-29

Figure B-21: Paraffin sections of male gonads of zebrafish of the first filial (F1) generation exposed to 17 β -trenbolone B-30

Figure B-22: Hatching rate of F2 larvae under exposure to 17 β -trenbolone B-31

Figure B-23: Mortality of F2 zebrafish after different periods of exposure to 17 β -trenbolone..... B-31

Figure B-24: Mean growth rate based on measurement of length of zebrafish of the F2 generation during exposure top 17 β -trenbolone B-32

Figure B-25: Mean growth in adult F2 zebrafish after exposure to 17 β -trenbolone based on weight measurements B-32

Figure B-26: Concentration-response relationship of percentage of males in the F1 generation of zebrafish (*Danio rerio*) exposed to 17 β -trenbolone..... B-39

B-1. Introduction

A two-generation study with 17 β -trenbolone as test substance was performed at the Dept. of Zoology, University of Heidelberg, Germany. Trenbolone represents the endocrine mode of action of an androgen receptor agonist.

The aim of the study was the evaluation and the assessment of effects after continuous exposure to 17 β -trenbolone during all life stages of zebrafish. This includes the parental generation (P-generation), early life stages (life phase directly post-hatch), juvenile growth and reproduction of the Filial 1 (F₁)-generation, and finally, the early life stage phase of the Filial 2 (F₂)-generation. Furthermore, molecular biomarkers of the P- and F₁-generation were measured, including the yolk protein precursor vitellogenin, and the sex steroid 11-keto testosterone.

B-2. Study conditions

B-2.1 Test organism and maintenance

The zebrafish (*Danio rerio*, Teleostei, Cyprinidae) was used as test organism. This fish is recommended by the OECD TG 210 (1) as test fish for Fish Early-Life-Stage Toxicity (FELS) studies. It is also one of the four test fish species recommended (2) for fish two-generation studies.

B-2.1.1 Origin of the test organisms

The source of the fish was the laboratory culture of the Fraunhofer IME, Schmallenberg, Germany. The origin of the used strain was a commercial fish breeder, namely West Aquarium GmbH in Bad Lauterberg, Germany.

B-2.1.2 Fish maintenance

Until the start of the test, adult zebrafish were kept in communities of approx. 300 individuals (max. age: 6 months) in 150 L glass tanks under flow-through conditions (≥ 90 % O₂ saturation) with a 14 h light/10 h dark photoperiod. Water temperature was kept constant at 26.0 \pm 1 °C. Fish were free from externally visible diseases and not treated with any pharmaceutical (acute or prophylactic) treatment for 6 months prior to the experiment. Fish were fed with commercially available artificial diets (TetraMin™ flakes; Tetra, Melle,

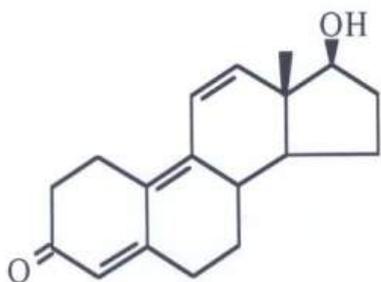
Germany) twice daily, occasionally supplemented with *Artemia nauplii*, or small daphnids of appropriate size obtained from an uncontaminated source. Overfeeding was strictly avoided to ensure optimal water quality; remaining food and feces were removed daily.

Permanent flow-through conditions guaranteed that ammonia, nitrite, and nitrate were kept below detection limits (0 - 5, 0.025 - 1 and 0 - 140 mg/L, respectively; Table B-1). Likewise, PAH, pesticide and heavy metal (exclusive use of V4 stainless steel tubing) concentrations were controlled at 3 months' intervals, but were always below detection limits.

Table B-1. Water conditions during exposure to 17 β -trenbolone

	Control	1 ng/L	3 ng/L	10 ng/L	30 ng/L	90 ng/L
NH ₄ ⁺ (mg/L)	0	0	0	0	0	0
NO ₃ ⁻ (mg/L)	< 10	< 10	< 10	< 10	< 10	< 10
NO ₂ ⁻ (mg/L)	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
Dissolved Oxygen	> 80 %	> 80 %	> 80 %	> 80 %	> 80 %	> 80 %
Carbonate Hardness (°dH)	13.2	13.4	13.6	13.4	12.6	12.2
Total Hardness (°dH)	19.4	19.2	19.4	19.2	16.8	16.8

B-2.2 Test substance and test concentrations



Catalogue Item: 4,9,11-Estratrien-17 β -ol-3-on
 Trivial Name: Trenbolone
 Steraloids Batch: L1668
 Formula: C₁₈H₂₂O₂
 Molecular Weight: 270,37
 Melting Point: 183 - 185 °C
 CAS Number: 10161-33-8

The test substance, 17 β -trenbolone, was provided by Steraloids (London, UK) and tested at nominal concentrations of 1, 3, 10, 30 and 90 ng/L. The substance has been used as a model for androgen receptor agonists; it has been employed as a growth stimulant in the US and Canada (Orlando 2004, Wilson et al. 2002). The strong growth stimulating effect can, most likely, be traced back to both the anabolic properties of an androgen and the anticatabolic characters as an antiglucocorticoid substance (Meyer 2001).

Analytical verification of the 17 β -trenbolone concentrations was carried out by the Fraunhofer-Institute of Molecular Biology and Applied Ecology at Schmallenberg (Table B-2).

Table B-2. Nominal and measured mean concentrations (n = 4; n refers to the number of samplings during the entire exposure period) of 17 β -trenbolone (ng/L).

Nominal concentration	Control	1 ng/L	3 ng/L	10 ng/L	30 ng/L	90 ng/L
Actual concentrations (measured mean values)	0	0.59	1.1	3.6	14.91	31.41

Trenbolone stock solutions were prepared by dissolving 10 mg of 17 β -trenbolone in 10 L dilution water. To achieve adequate concentrations for the use in the dosing system (see below), the stock solutions were further diluted. After a pre-treatment phase of exposure in water for 2 weeks, the dosing of the test substance was started and exposure was initiated by adding freshly fertilized eggs.

B-2.3 Test system

B-2.3.1 Test vessels

Trenbolone concentrations were tested in 22.5 L full glass vessels equipped with a permanent flow-through water supply (water conditions as described above). Fertilized zebrafish eggs were transferred to acryl glass breeding chambers floating in the test solutions

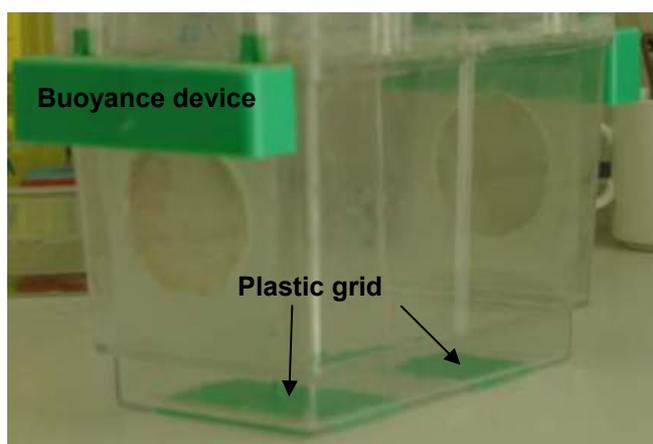


Fig. B-1: Breeding chamber.

(Figure B-1). The bottom of the breeding chambers was replaced by a 260 μ m plastic gauze; likewise, the lateral longitudinal walls were replaced by 250 μ m gauze to ensure optimal water exchange and oxygen supply.

B-2.3.2 Flow-through system

The test substance 17 β -trenbolone was tested in triplicate in five concentrations plus a negative control (dilution water), with a 3-fold water exchange per day. For general water conditions, see above. The flow of the test solutions was adjusted by means of peristaltic pumps (Gilson Minipuls 3; Gilson, Wiesbaden, Germany), the flow rate of the dilution water

was controlled by means of rotameters (Rota Yokogawa, Wehr, Germany). Both peristaltic pumps and rotameters were checked and, if required, re-adjusted on a daily basis. Test solutions and dilution water were mixed in a 12 mm glass tube, which ended approx. 18 cm below the surface of the final test solutions (Figure B-2). In order to optimize O₂ supply, the test solutions were aerated by means of Teflon tubing and glass dispensers. All tubing used was made of premium quality teflon.

In order to adjust the water temperature in the test system, all tanks were placed on heating mats with sensor-driven automatic regulation. Water parameters were controlled on a daily basis (Table B-2).

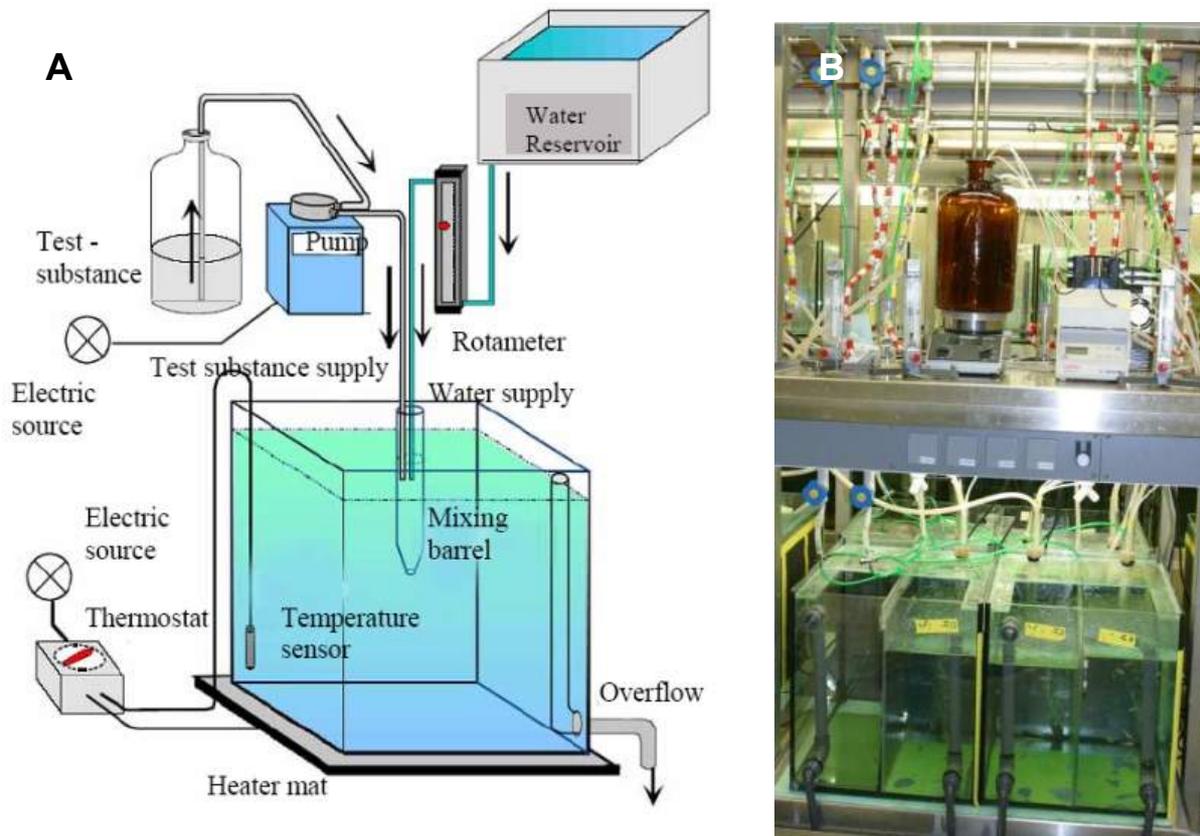


Fig. B-2. Setup of flow-through aquarium system: The mixing barrel is a glass tube with a small opening at the lower end, in which water supplies and test solutions are mixed before flowing into the test tank (A schematic drawing; A picture)

B-3. Performance of the study

B-3.1 Description of the different life phases

A detailed study plan with the durations of the evaluated life phases is given in Table B-3.

Chapter 8: Supplement B

Table B-3. Study plan for the two-generation study on 17 β -trenbolone with zebrafish (*Danio rerio*; 5 concentrations + water control, 3 replicates each)

Day after start of exposure	Phase	Course (related to each of four replicates per concentration)	Endpoints
Pre-treatment phase, at least 7d		Start with 8 males and 8 female fish, reorganization of groups	Reproduction: egg numbers, fertilization rate
0	P	Transfer to exposure tanks (run for 48h)	
21	repro	Sampling of eggs for F ₁ phase	
21	F ₁ ELS	Transfer of 100 fertilized eggs to fry chambers	Daily observation of : Mortality, survival rate, hatching rate, time to 90 % hatch
23	(28 days)	Begin of hatch	
25-42		End of hatch	
49		Transfer to the total volume of the test vessel Randomly reduced to 50 fish Blood/Liver/Gonad sampling of P fish (Sex-Determination of excess fish)	
77	F ₁ Growth	Randomly reduced to 30 fish (Sex-Determination of excess fish)	Weekly observation of Mortality, survival rate, length
120	F ₁ Repro	Sampling of eggs for F ₂ phase	Reproduction: Start, egg numbers, fertilization rate
120	F ₂	Transfer of 50 fertilized eggs to fry chambers	Daily observation of : Mortality, survival rate, hatching rate, time to 90 % hatch
122	ELS (28/35 days)	Begin of hatch	
124		End of hatch	
126			Weekly observation of Mortality, survival rate, length
148/155		Test termination Blood/Liver/Gonad sampling of F ₁ fish (Sex-Determination of excess fish)	Recording of: survival, weight , length, vitellogenin, 11-keto testosterone, gonad histology, sex ratio

B-3.1.1 Parental (P)-generation

The parental generation (P-generation) was generated with 8 males and 8 females in each experimental tank aged more than 150 days. The fish were kept under test conditions for at least three weeks without exposure to 17 β -trenbolone in order to adjust a consistent state of fecundity over all groups, and to re-sort the spawning groups in case of low egg numbers.

When all groups had started regular and continuous spawning on a daily basis, exposure to the test solutions was started. Freshly spawned eggs were collected daily, counted and the number of fertilized eggs was determined over a period of 20 days. The P-generation was sacrificed, when the F₁-generation successfully reached an age of 28 days.

B-3.1.2 First filial (F₁)-generation

For the start of the first filial-generation (F₁-generation), 100 fertilized and randomized eggs spawned by the parental fish of each replicate were placed on nylon nets forming the bottom of breeding chambers fixed at the water surface of each test vessel. Starting from day 6 of age (day 2 after hatch), larvae were fed daily *ad libitum* with breeding food (TetraMin, Babyfutter; Tetra, Melle, Germany) and Nobil Fluid (JBL, Neuhofen, Germany). From day 9 of age, brine shrimp nauplii (*Artemia spec.*; Sanders Brine Shrimp Company, Morgan, Utah, USA) were added *ad libitum*. From day 16 of life, ground TetraMin flake food was added to the daily food *ad libitum*. From day 14, growth was recorded weekly as length measurements by photographic documentation. After 35 days, the fish were randomly reduced to 50 individuals, and released from the fry chambers to the total volume of the test vessels. In parallel, all fish of the parental (P)-generation were sacrificed. After 63 days of age, the fish number was randomly reduced to 30 individuals in order to create identical conditions for reproduction. Reproduction was recorded as described for the P-generation. The F₁-generation was sacrificed when the F₂-generation successfully reached an age of 21 days. Fish were sampled for vitellogenin and 11-keto testosterone, as well as histological analysis.

B-3.1.3 Second filial (F₂)-generation

For the start of the second filial (F₂)-generation, 100 fertilized and randomized eggs spawned by the F₁-fish of each replicate were placed on nylon nets forming the bottom of breeding chambers fixed at the water surface of each test vessel. Larvae were fed daily *ad libitum* as described above for the F₁-generation. From day 14, growth was recorded weekly as length

measurements by photographic documentation. After 35 days, the fish were measured for length and weight, and the test was terminated. Fish were sampled for determination of weight and length, as well as histological analysis.

B-3.2 Observations

Observations of all life stages of fish were recorded daily. Dead eggs, larvae, juvenile and adult fish were recorded and removed as soon as observed. Hatching rates were estimated by daily counting of non-hatched eggs between two days, and the time, when 90 % of the fish had hatched. Abnormal appearance of behaviour was recorded.

Between hatch and 28 and 35 days of age in the F_1 - and F_2 -generations, respectively, larvae/juvenile fish were photographed weekly, and survival rates were estimated. The data were confirmed by counting the fish on day 28 (F_1) and day 35 (F_2), when transferred to the main aquaria (F_1), or sacrificed (F_2). Lengths of F_1 -fish were measured by digital photography after 28 and 63 days of life. For this time interval, the pseudo-specific growth rate based on length was calculated. The time of first spawning, identified as first day at which eggs were found in the spawning trays, was recorded. The reproductive endpoints like egg number and fertilization rate were observed for 20 daily counts.

After successful start of the F_2 -generation, the adult F_1 -fish were sacrificed and sexed by inspection of the gonads. The sex ratio was calculated. The number of females per groups was determined, and used to calculate the number of total and fertilized eggs per female and day. Growth of F_2 -fish until day 35 was measured as individual lengths and weights.

B-3.3 Specific methods

B-3.3.1 Collection of eggs

Spawned eggs were collected daily in all glass spawning trays placed at the bottom of the test vessels. The tray was covered with a lattice (stainless steel), to prevent the eggs from being predated by the adult fish. Artificial plant substrate was placed on the lattice to stimulate spawning into the tray. The onset of lighting (neon lamps with a light intensity of approximately 1000 lux, measured 5 cm above the water surface in the middle of the test vessel) induces mating of fish. Within two hours after spawning, eggs were transferred from the spawning-tray onto a sieve, rinsed with clean water in order to remove faeces and remains

of food, put into glass dishes, and counted as total number and number of fertilized eggs (Figure B-3). Fertility was determined by observing cleavage stages using a binocular.

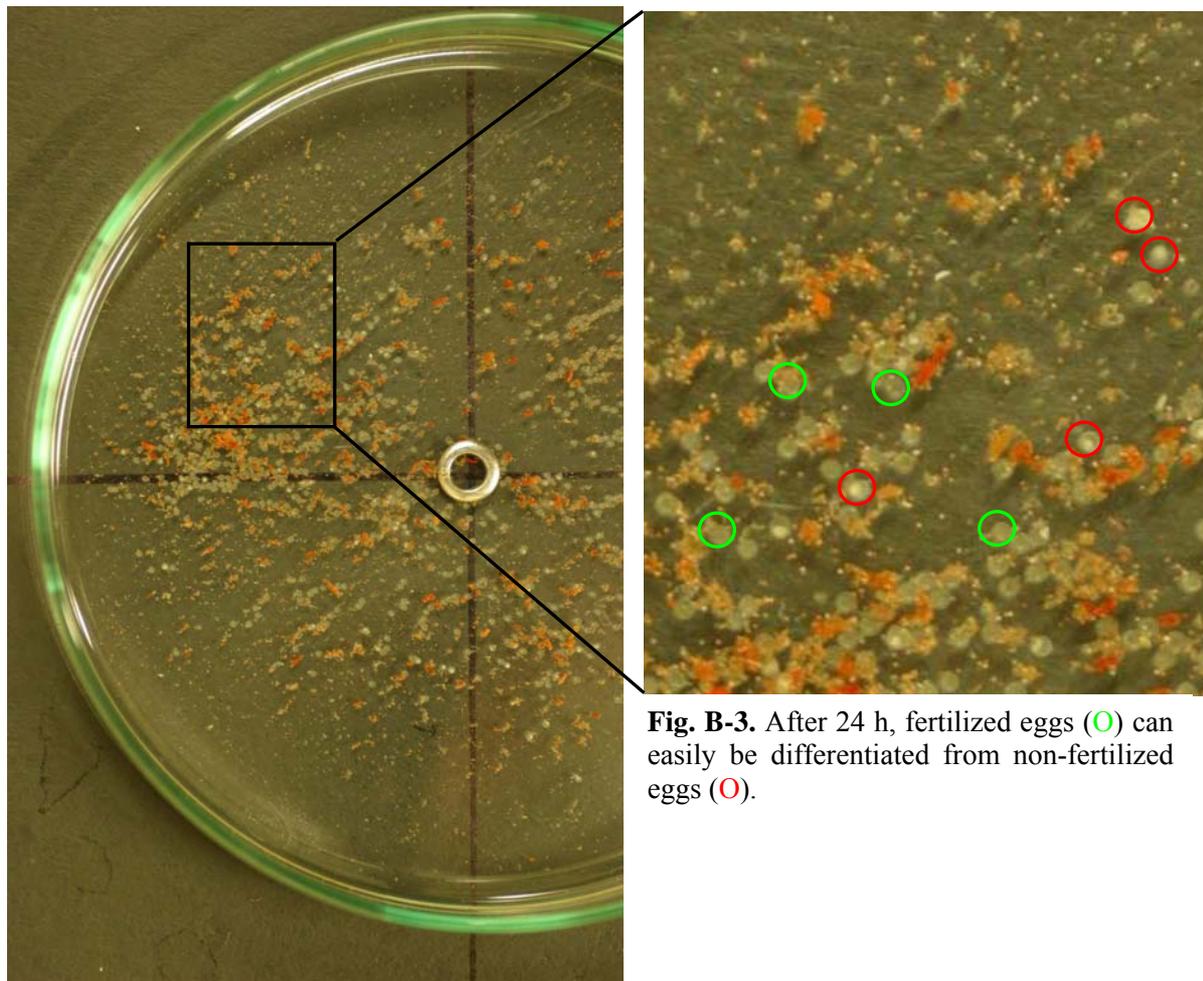


Fig. B-3. After 24 h, fertilized eggs (○) can easily be differentiated from non-fertilized eggs (○).

B-3.3.2 Photography and image analysis

For fry counts and length measurements, photographs were made using a digital camera (Canon G7). Digital image processing were performed by UTHSCSA ImageTool Version 2.0, Alpha 2; University of Texas Health Science Center at San Antonio, 1997. Fish larvae were photographed by putting a vessel underneath a fry chamber to maintain the water level, dislocate the fry chamber with the vessel to the photo device (light plate with additional illumination from above), and re-introducing the fry chamber in the aquarium after photography. Juvenile and adult fish were netted and placed in glass vessels with a low water level in the photo device.

B-3.3.3 Blood collection and preparation

At termination of P- and F₁-generation, blood samples were taken from each fish. Samples were taken by cardiac puncture using syringes with a fixed thin needle. To avoid coagulation of blood and degradation of proteins, the syringes were pre-filled with Phosphate-buffered saline (PBS) containing heparin (1000 units/mL), and the protease inhibitor aprotinin (2 TIU/mL). As ingredients for the buffer, heparin as ammonium-salt (Sigma) and lyophilized aprotinin (Roth, Karlsruhe, Germany) were used.

Before sampling, fish were anaesthetized in a saturated solution of ethyl-4-aminobenzoate (tricaine, Sigma-Aldrich, Deisenhofen, Germany). Blood samples with volumes between 10 and 30 µl were taken. Plasma was separated from the blood by centrifugation (30 min; 5000 rpm; 4°C) and immediately stored at -80°C until further analysis.

B-3.3.4 Measurement of biomarkers using ELISA

Vitellogenin measurement

The measurement of vitellogenin levels was performed using commercially available enzyme-linked immunosorbent assays (ELISA) raised to zebrafish (*Danio rerio*) VTG (homologous ELISA kit) purchased from Biosense, Bergen, Norway.

The VTG-analysis used is based on a sandwich assay utilizing specific binding between antibodies and VTG. The wells of micro-titer plates are coated with a specific capture antibody that binds to VTG in samples added to the wells. Unbound components are washed out, and a different VTG-specific antibody (detecting antibody) is added. Unbound detecting antibody is washed out, and an enzyme-labeled secondary antibody is added. After a last wash, the enzyme activity is determined by adding a substrate being metabolized to a colored product. The enzyme activity (color intensity) measured by a microplate reader is directly proportional to the concentration of VTG in the sample. The assay is calibrated using purified VTG from zebra fish as standard, which was provided by the producer.

Whole protein measurement

In order to minimize variability generated by the blood sampling methods (e.g. by taking up tissue liquid), the measured vitellogenin concentrations were normalized against the blood plasma protein content, expressed as ng VTG/µg protein. Total protein was quantified by using the BCA Protein Assay Reagent Kit (Pierce, Rockford). The method of the BCA Protein Assay combines the reduction of Cu²⁺ to Cu⁺, and allows a selective colorimetric

detection of the cuprous cation (Cu⁺) using a reagent containing bicinchoninic acid. The colored reaction is formed by the chelation of two molecules of BCA with one cuprous ion. This complex shows a strong absorbance at 562 nm, which is almost linear to increasing protein concentration.

11-keto testosterone measurement

The measurement of 11-keto testosterone (11-kT) levels was made using a commercially available enzyme-linked immunoabsorbant assay (ELISA) raised to fish 11-keto testosterone, in general purchased from Cayman Chemicals, Ann Arbor, Michigan, USA.

The assay is based on the competition between 11-kT and an 11-kT-acetylcholinesterase (AChE) conjugate (11-KT tracer) for a limited number of 11-kT -specific rabbit antiserum binding sites. Because the concentration of the 11-kT tracer is held constant, while the concentration of 11-kT varies, the amount of 11-kT is inversely proportional to the concentration of 11-kT in the well. This rabbit antiserum-11-kT complex binds to a mouse monoclonal anti-rabbit IgG antibody that has been previously attached to the well. After a washing step, Ellmann's reagent, which contains the substrate to AChE, is added to the well. The product of this enzymatic reaction shows a strong absorbance at 412 nm. The intensity of this color, determined by spectrophotometry, is proportional to the amount of 11-kT tracer bound to the wells, and inversely proportional to the amount of free 11-kT present in the well during the incubation.

B-3.3.5 Statistical analysis

All biological response data were statistically analyzed and reported separately for males and females. Effects (biomarker endpoints) were compared between treatments versus control group using analysis of variance (ANOVA). If the required assumptions for parametric methods are not met – non-normal distribution or heterogeneous variance, consideration was given to transform the data to homogenize variances prior to performing the ANOVA, or to carrying out a weighted ANOVA. A Dunnett's or Williams' test on multiple pair-wise comparisons (in case of parametric data), or a Welch t-test with Bonferroni adjustment (in case of non-parametric data) was used subsequently (SigmaStat 35).

B-3.3.6 Histological analyses of gonadal alterations

The analysis of histological alterations was carried out according to the protocols by Wolf (2003) and Knörr (2005). The gonads of the fish were categorized into different grades of maturation (Table B-4). In case of abnormal observations in zebrafish gonads, the abbreviation system developed within the OECD standardization procedure for histological analyses of gonadal aberrations was used. Results were classified into primary and secondary effects (Table B-5). Additional observations were recorded as appropriate.

Table B-4. Categories of maturation of zebrafish (*Danio rerio*) gonads following Wolf (2003) and Knörr (2005)

	Females	Males
Stage (Stg) 0 entirely immature	Oogonia (OOA) up to perinucleolar oocytes (PND)	Spermatogonia (SPA), spermatocytes (SPC) and spermatids (SPT)
Stage (Stg) 1 early vitellogenic/ spermatogenic phase	Main part of the germ cells are pre-vitellogenic follicles; mostly perinucleolar oocytes (PND) and alveolar oocytes (CAO)	immature phase is dominating; spermatozoa are present
Stage (Stg) 2 middle vitellogenic/ spermatogenic phase	At least half of the follicles are early (EVO) to late vitellogenic (LVO)	Spermatocytes (SPC), spermatids (SPT) and spermatozoa (SPZ) are present in roughly equal portion
Stage (Stg) 3 late vitellogenic/ spermatogenic phase	The majority of follicles in the ovary are late vitellogenic (LVO) and mature/spawning follicles (MSO).	All maturation grades are present, though the spermatozoa are dominating
Stage (Stg) 4 late vitellogenic/ hydrated	At stage 4, the majority of follicles are late vitellogenic and mature/spawning follicles. Follicles are larger as compared to Stg 3.	-

Table B-5. Classification of histological analyses into primary and secondary diagnoses

	Females	Males
Primary diagnosis	Increased oocyte atresia	Increased proportion of spermatogonia
	Perifollicular cell hyperplasia / hypertrophy	Presence of testis-ova
	Decreased vitellogenesis/ decreased yolk formation	Increased testicular degeneration
	Gonadal staging	Interstitial (Leydig) cells
Secondary diagnosis	Interstitial fibrosis	Decreased proportion of spermatogonia
	Egg debris in the oviduct	Increased proteinaceous fluid within the testicular vessels or interstitium
	Granulomatous inflammation	Asynchronous gonad development
	Decreased post-ovulatory follicles	Altered proportions of spermatozoa or spermatocytes
		Gonadal staging Granulomatous inflammation

B-4. Results

B-4.1 Chemical analysis

Table B-6 summarizes the results of the analytical verification of test concentrations. An accompanying analysis of water samples from the test system was not possible during the study. Therefore, samples were frozen and kept at -20 °C until analysis.

Results in the report refer to nominal values since the number of sampling dates and measurements do not correspond with the recommended number of measurements according to test guidelines.

Table B-6. Results of chemical analyses during the two-generation test with 17 β -trenbolone in replicate tanks (R1, R2, R3)

		09.03.2006	18.05.2006	07.08.2006	06.09.2006	mean ± SD	% of nominal
1 ng/L	R1	1.3	<DL	0.72*	0.58*	0.59 ±	59 ±
	R2	<BG	2.4	0.68*	0.50*	0.69	69
	R3	<BG	<BG	0.51*	0.35*		
3 ng/L	R1	<BG	1.3	1.4	0.95*	1.1 ±	37 ±
	R2	1.5	1.3	1.7	0.87*	0.57	19
	R3	1.2	<DL	1.6	1.4		
10 ng/L	R1	5.8	2.9	2.2	2.4	3.6 ±	36 ±
	R2	5.1	5.1	3.4	3.3	1.2	12
	R3	3.6	3.6	3.6	2.2		
30 ng/L	R1	23	12	12	11	15 ±	50 ±
	R2	23	13	15	15	4.1	14
	R3	12	12	16	16		
90 ng/L	R1	43	20	21	10	31 ±	34 ±
	R2	47	28	41	31	11	12
	R3	41	42	28	26		

* Value below detection limit; calculated on the basis of recovery rates of other treatment levels

B-4.2 Parental generation

B-4.2.1 Macroscopic observations in the parental generation

During the reproductive phase of the P-generation, no concentration related mortality could be observed. In all tanks of the highest concentration (90 ng/L) of 17 β -trenbolone, there was a conspicuous increase in territorial behavior of female fish. Moreover, at the highest concentration of 90 ng/L 17 β -trenbolone, there was an increased amount of food left over, indicating altered feeding behavior of fish.

B-4.2.2 Reproduction in the parental generation

Fecundity (total egg number)

At the two lowest test concentrations of 17 β -trenbolone (1 and 3 ng/L), the cumulative number of eggs as determined over an observation period of 24 days, was significantly reduced under control values (Figure B-4).

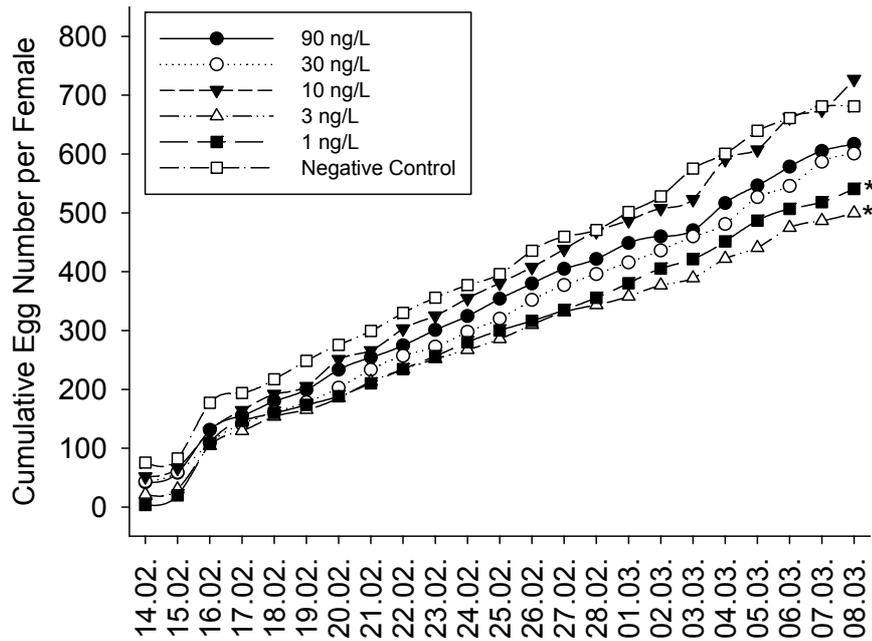


Fig.e B-4. Cumulative number of eggs of zebrafish (*Danio rerio*) exposed to 0, 1, 3, 10, 30 and 90 ng/L 17 β -trenbolone. * Significantly different from controls ($p < 0.05$). Chi square test with Yates correction.

Fertilization rate in the parental generation

At the two lowest test concentrations of 17 β -trenbolone (1 and 3 ng/L), there was a significant increase in the fertilization rate over an observation period of 24 days (Figures B-5, B-6).

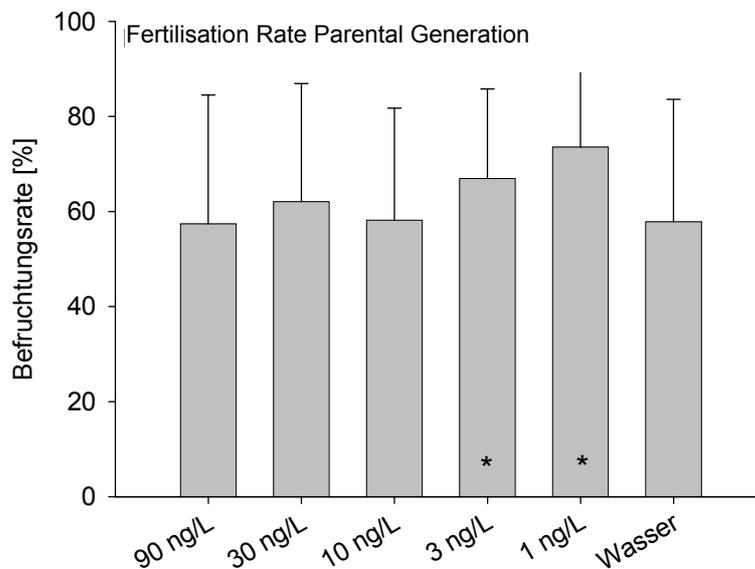


Fig. B-5. Fertilization rate in the parental generation of zebrafish exposed to 17 β -trenbolone. * Significantly different from controls (one-factorial non-parametric analysis of variance with post-hoc Dunn's-Test with $p < 0.001$)

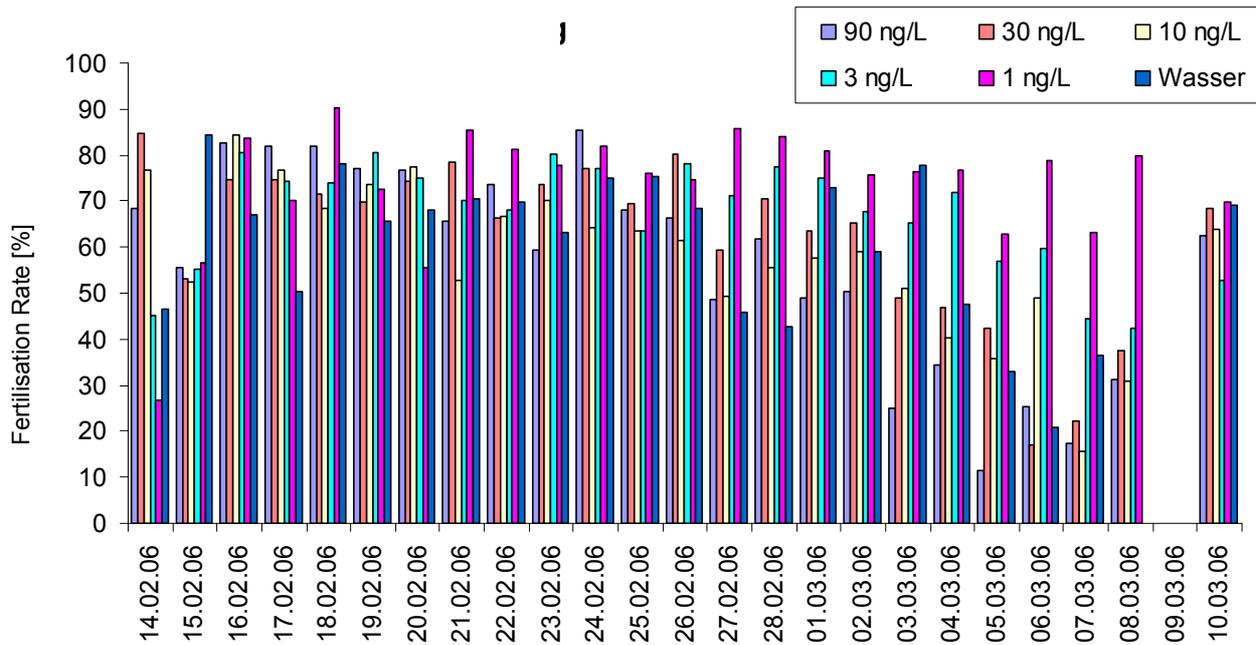


Fig. B-6. Daily fertilization rate in the parental generation of zebrafish (*Danio rerio*) exposed to various concentrations of 17 β -trenbolone. From March 4, 2006, to March 8, 2006, a transient increase in the number of protozoa could be observed, which apparently reduced the fertilization rate in all treatments. The protozoan populations disappeared afterwards without any treatment required

B-4.2.3 Molecular biomarkers in the parental generation

Plasma vitellogenin in the parental generation

Following exposure to 3 ng/L 17 β -trenbolone, there was a significant decrease of plasma vitellogenin in male zebrafish (Figure B-7a); females however, did not show any significant effect of vitellogenin production in any treatment with 17 β -trenbolone (Figure B-7b).

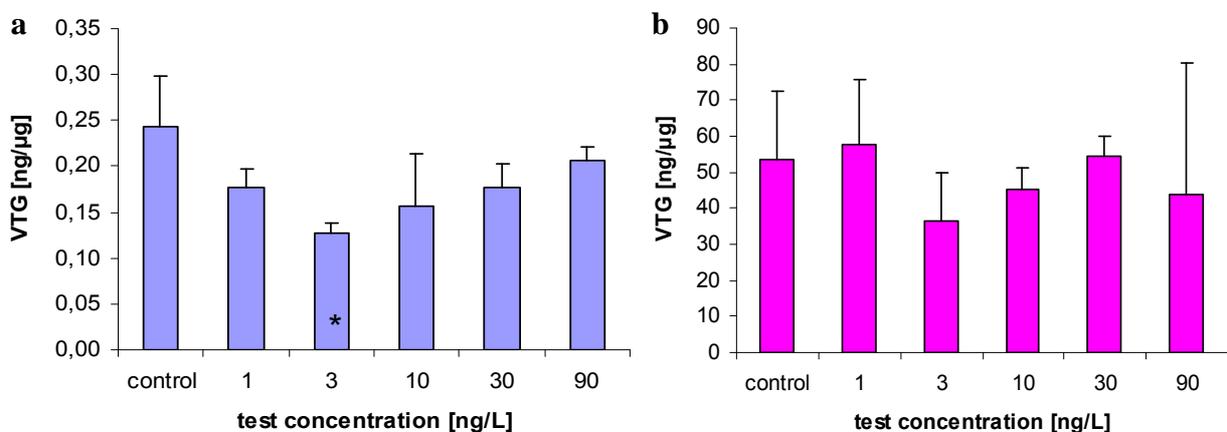


Fig. B-7. Mean vitellogenin (VTG) concentration in blood plasma relative to total protein contents in male (a) and female (b) zebrafish of the parental generation after exposure to 17 β -trenbolone (\pm SD). * Significantly different from control with $p < 0.05$.

11-Keto-testosterone in male zebrafish of the parental generation

At the highest test concentration of 90 ng/L 17 β -trenbolone, the levels of 11-keto testosterone were significantly reduced in male zebrafish of the parental generation (Figure B-8).

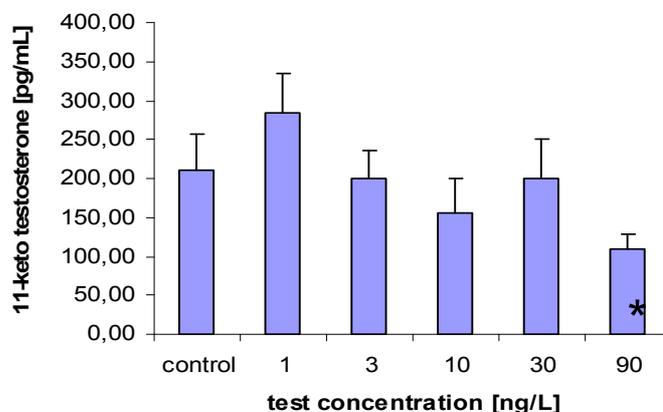


Fig. B-8. Mean concentrations of 11-keto testosterone in blood plasma of male zebrafish of the parental generation exposed to 17 β -trenbolone (\pm SD). * Significantly different from controls with $p < 0.05$.

B-4.2.4 Histological alterations in zebrafish of the parental generation exposed to 17 β -trenbolone

Histological alterations in the gonads of the parental generation of zebrafish exposed to 17 β -trenbolone have been summarized in Table B-7.

Table B-7. Summary of histological alterations in the parental generation of zebrafish (*Danio rerio*) after 62 d exposure to various concentrations of 17β-trenbolone. Data are given as per cent of individuals affected (the total number of individuals in the respective treatment groups are given in brackets). Arrows indicate direction of change (increase, decrease). * Tubuli contained a very small amount of spermatozoa

	Control				1 ng/L Tren				3 ng/L Tren				10 ng/L Tren				30 ng/L Tren				90 ng/L Tren							
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4				
FEMALE																												
Severity Grade																												
Oocyte atresia, immature ↑	10				10				10				10				10				20	10			20	10		
Oocyte atresia, mature ↑	30	20			30	10			20	10	20		10				10	20	20		10	20	20		10	30	10	10
Interstitial fibrosis	20	10	20		10	20	20		10	20	10		40	10	30		10	40			10	40			30	30	10	
Egg debris oviduct													10															
Postovulatory follicles ↑																												
Proteinaceous fluid, interstitial	20				10	10			10	10	10						20				10				10			
Gonadal staging: Stage 2																												
Stage 3	20				40				70				80				40				40				85			
Stage 4	80				60				30				20				60				60				10			
% Females/exposure group	50 % (50)				53.2 % (47)				50 % (48)				53.2 % (47)				48 % (48)				52 % (50)							
MALE																												
Spermatogonia ↑	20	10	10		22				10	10			20				10				10							
Interstitial cells (Leydig cells)					22	33	11		20	20			10	30							30							
Testis-ova																												
Spermatozoa ↑									20																			
Spermatogonia ↓																												
Spermatozoa ↓																					10*							
Asynchron. developed gonads	10																											
Interstitial fibrosis					22																							
Sertoli cells ↑	20				33	11	22		30	20	10		10	40			10	10			20				10	30	10	10
Small testis																												
Blood in the testis	10								10																			
Blood in the peritoneum					10																							
Gonadal staging: Stage 2																												
Stage 3	50				33				100				30				100				100				25			
% Males/exposure group	50 % (50)				46.8 % (47)				50 % (48)				46.8 % (47)				52 % (48)				52 % (50)							

Female parental zebrafish – primary diagnoses

The majority of the gonads of female zebrafish of the parental generation could be classified into late vitellogenic / hydrated. Only one female exposed to 90 ng/L 17 β -trenbolone was in an intermediate to late vitellogenin developmental stage (Figure B-9).

Male parental zebrafish – primary diagnoses

One male parental zebrafish exposed to 30 ng/L displayed oogonia in an otherwise male gonad (testis-ova). The degree of testis maturation in controls could be classified as intermediate spermatogenic in about 50 % of the individuals; the rest of the male individuals were fully mature. In all male zebrafish of the parental generation exposed to 17 β -trenbolone, the testes were classified as late spermatogenic (Figure B-10). At 90 ng/L 17 β -trenbolone, individuals with hypertrophic testis and increased numbers of Sertoli cells could be observed.

Other observations

From 1 ng/L 17 β -trenbolone, the ovaries of the females displayed accumulation of proteinaceous fluid and mild fibrosis, and the oviducts contained moderate amounts of egg debris. In males, testes showed minor shifts in the relative proportions of sperm maturation stages.

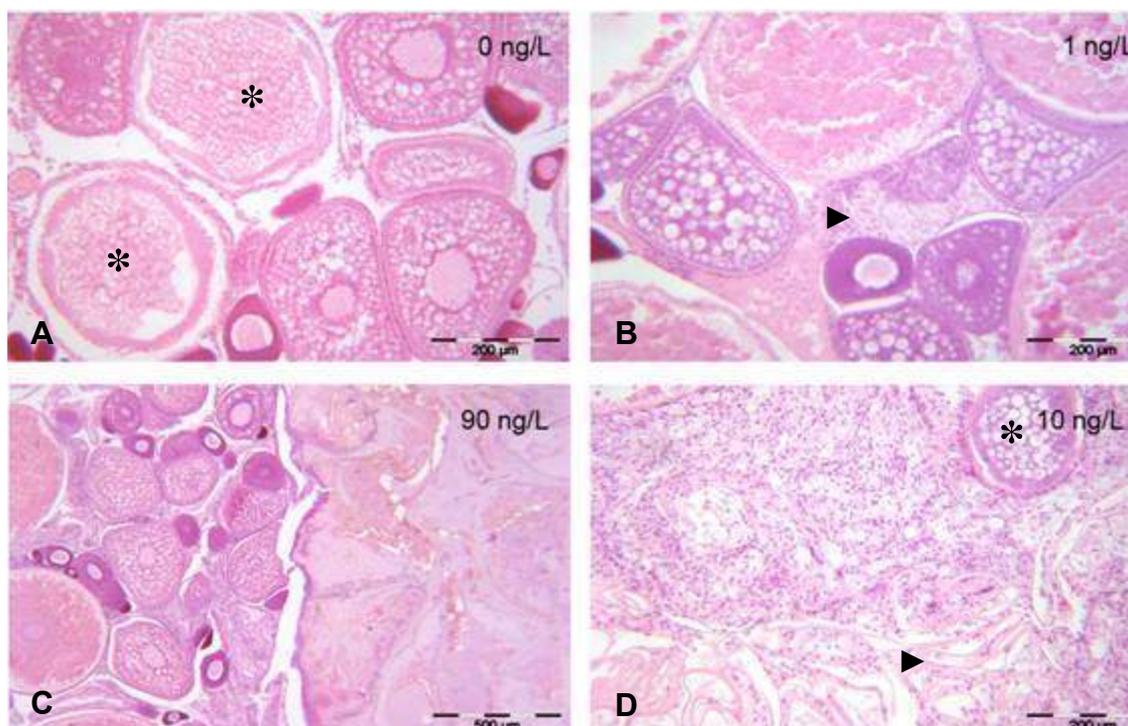


Fig. B-9. Histology of female gonads of zebrafish of the parental generation exposed to 17 β -trenbolone. A – 0 ng/L 17 β -trenbolone: atresia of mature follicles (*). B – 1 ng/L 17 β -trenbolone: proteinaceous fluid (*) and mild fibrosis (▶). C – 90 ng/L 17 β -trenbolone: egg remnants in oviduct (*). D – 10 ng/L 17 β -trenbolone: interstitial fibrosis (▶) and egg debris in oviduct (*). Hematoxylin and Eosin (H&E) staining.

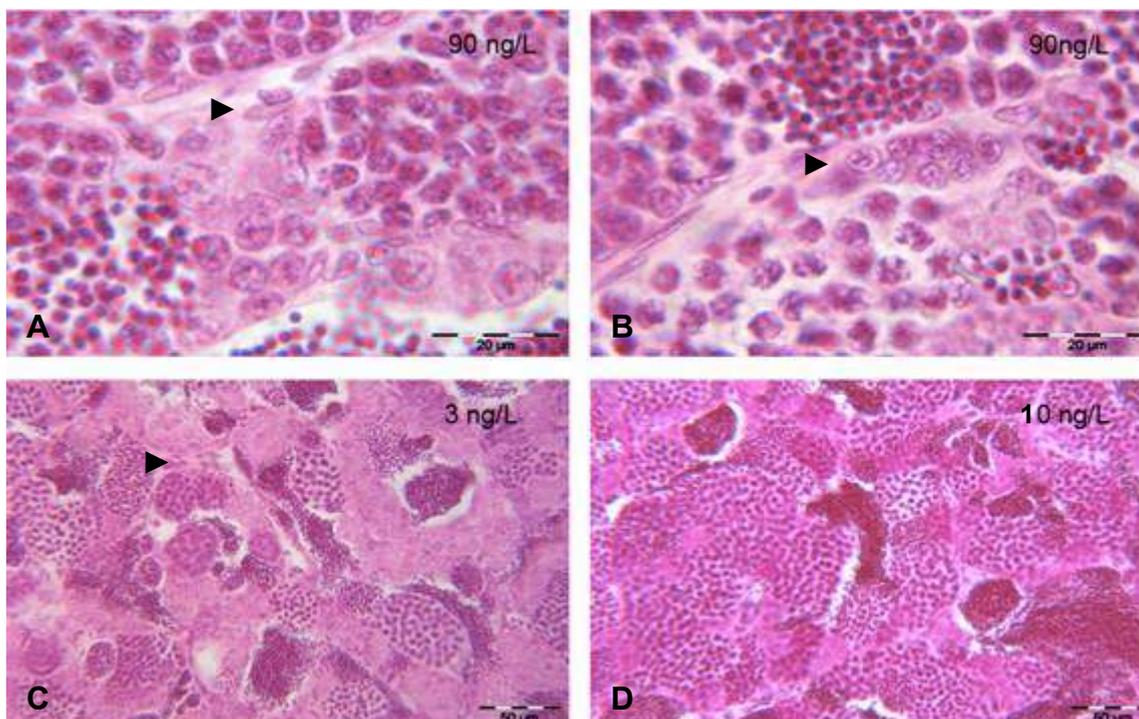


Fig. B-10. Paraffin sections of male gonads of zebrafish of the parental generation exposed to 17 β -trenbolone. A – 90 ng/L 17 β -trenbolone: hypertrophic testis with increased numbers of Sertoli cells (▶). B – 3 ng/L 17 β -trenbolone: high ratios of spermatogonia. C – 90 ng/L 17 β -trenbolone: high proportion of Leydig cells (▶). D – 10 ng/L 17 β -trenbolone: increased levels of spermatids. H&E staining.

B-4.3 F₁-Generation

B-4.3.1 Macroscopic observations in the first filial (F₁) generation

During the entire exposure period of 177 d, no significant macroscopic observations were made in the F₁ generation.

B-4.3.2 Hatch and survival in the first filial (F₁) generation

Hatch

The hatching rate did not show any effect of 17 β -trenbolone (Figure B-11). In all treatment groups, the hatching rate was > 95 %. In the controls, more than 90 % had hatched after 112 h. At this point of time 72, 85, 82 and 79 % of the larvae exposed to 3, 10, 30 and 90 ng/L 17 β -trenbolone, respectively, had hatched.

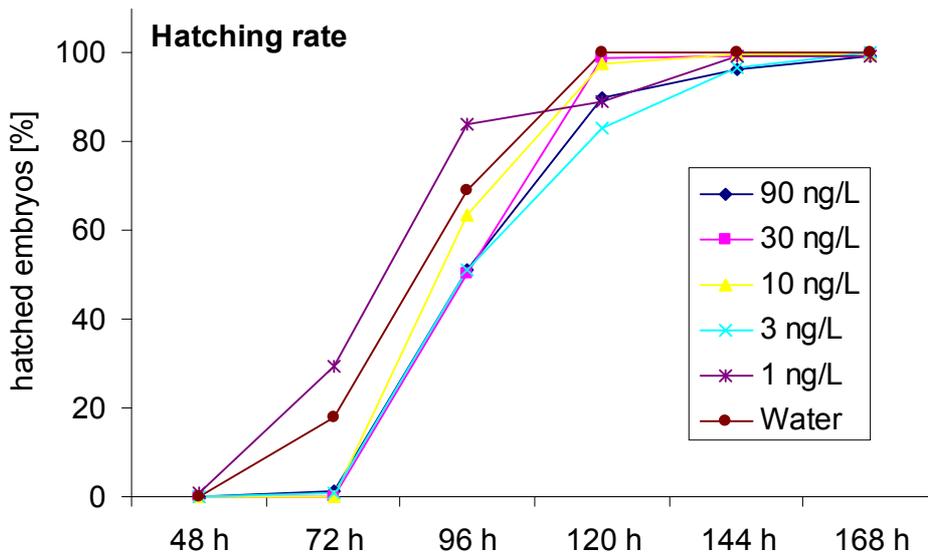


Fig. B-11. Hatching rate of F₁ larvae under exposure to 17β-trenbolone.

Mortality and survival

Mortality was comparatively high in all treatment groups, which was most likely due to the continuous handling stress induced by growth measurement. Nevertheless, after 56 d of exposure to 17β-trenbolone, there was a significant increase in the number of surviving fish at the two lowest concentrations of 1 and 3 ng/L, whereas zebrafish exposed to 30 and 90 ng/L showed a lower survival rate than controls (Figure B-12).

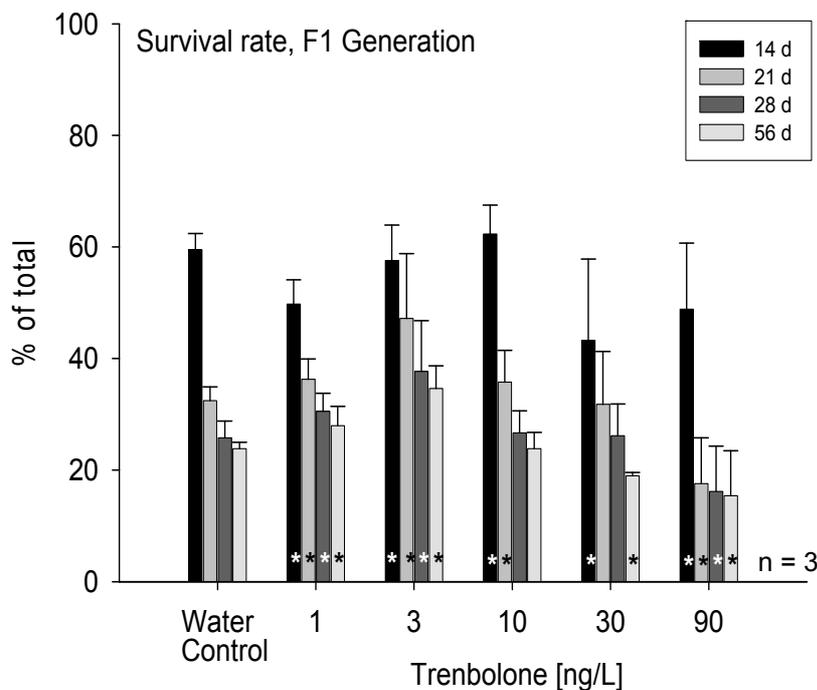


Fig. B-12. Mortality of F₁ zebrafish after different periods of exposure to 17β-trenbolone. * Indicates significant deviation from respective controls (Dunnett's Method with p < 0,001).

B-4.3.3 Juvenile growth in zebrafish of the first filial (F₁) generation

Following 14 d of exposure to 17 β -trenbolone, the zebrafish of the F₁ generation were statistically larger than the corresponding control fish. This effect, however, could no longer be seen after 56 d of exposure.

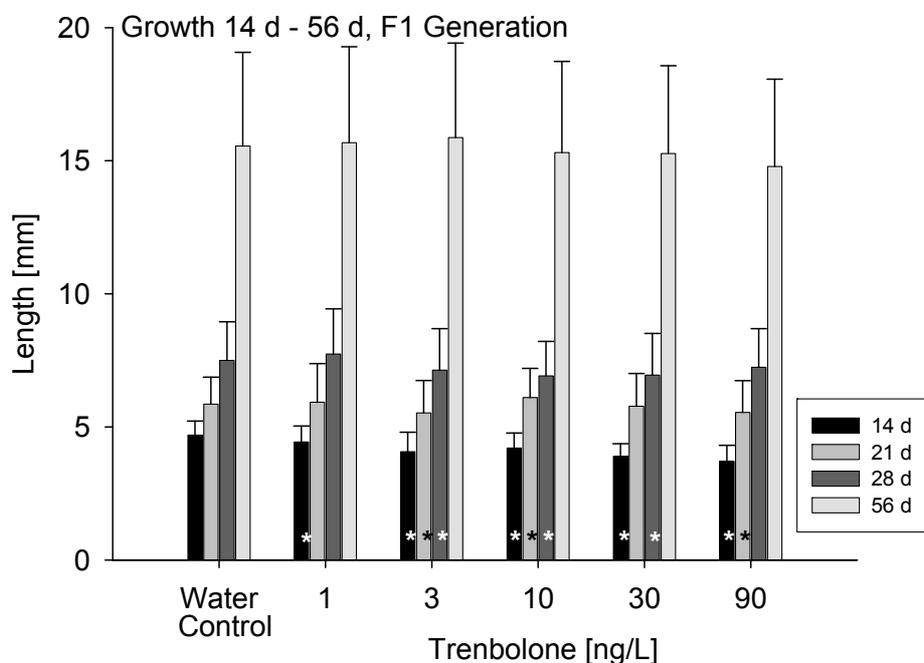


Fig. B-13. Mean growth rate based on measurement of length of zebrafish of the F₁ generation during exposure to 17 β -trenbolone. * Significantly different from controls with $p < 0.05$, according to Dunnett's Method.

In adult F₁ zebrafish, there was a minor tendency to increased weight and length after exposure to 3, 10 and 30 ng/L 17 β -trenbolone, whereas the fish exposed to 1 and 90 ng/L 17 β -trenbolone were slightly smaller (Figure B-14). With exception of the weight at 90 ng/L and length at 3 ng/L, none of these trends, however, were statistically significant.

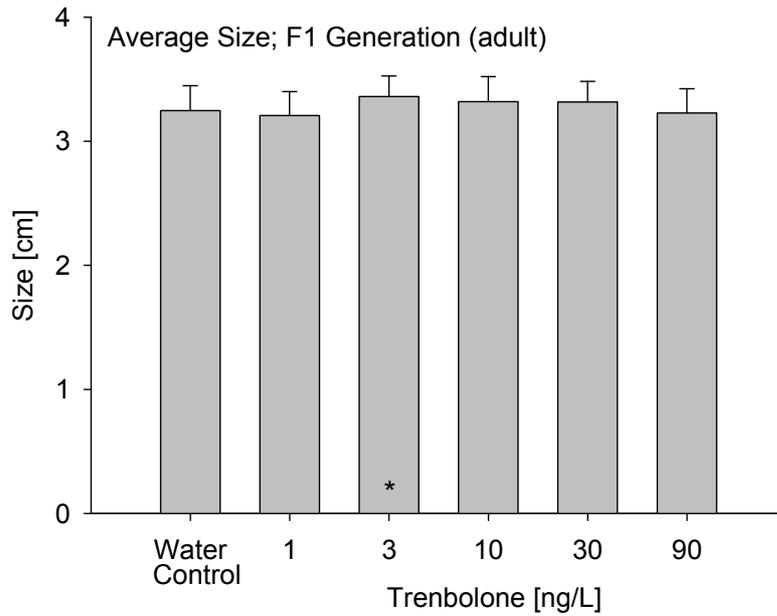


Fig. B-14. Mean growth in adult F₁ zebrafish after exposure to 17β-trenbolone based on length and weight measurements. * Significantly different from controls with $p < 0.05$ according to Dunn's Method.

B-4.3.4 Sex ratio in zebrafish of the first filial (F₁) generation

From ≥ 10 ng/L 17β-trenbolone, the relative proportion of male zebrafish in the F₁ generation was statistically significantly increased (Figure B-15).

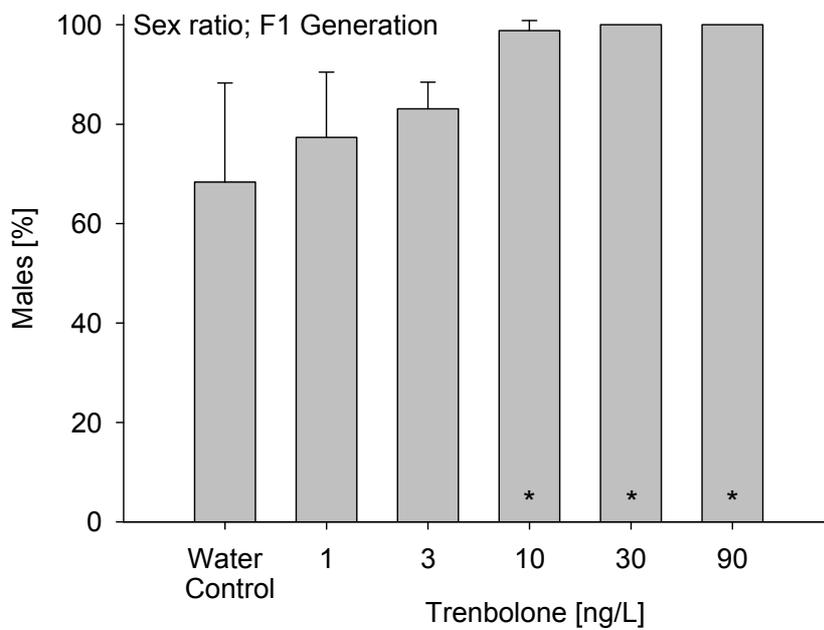


Fig. B-15. Sex ratio in F₁ zebrafish after exposure to 17β-trenbolone based on histological observations. * Significantly different from controls with $p < 0.05$. Multiple Comparisons versus Control Group (Holm-Sidak method).

*B-4.3.5 Reproduction in zebrafish of the first filial (F₁) generation***Cumulated fecundity (total number of eggs) in the F₁ generation**

In the F₁ generation, only controls and the two lowest exposure groups (1 and 3 ng/L 17 β -trenbolone) were able to regularly produce eggs (Figure B-16). At 10 ng/L 17 β -trenbolone, only one female could be found; after exposure to 30 and 90 ng/L 17 β -trenbolone, no female zebrafish could be identified. Total egg numbers produced after exposure to 3 ng/L 17 β -trenbolone were significantly higher than in controls.

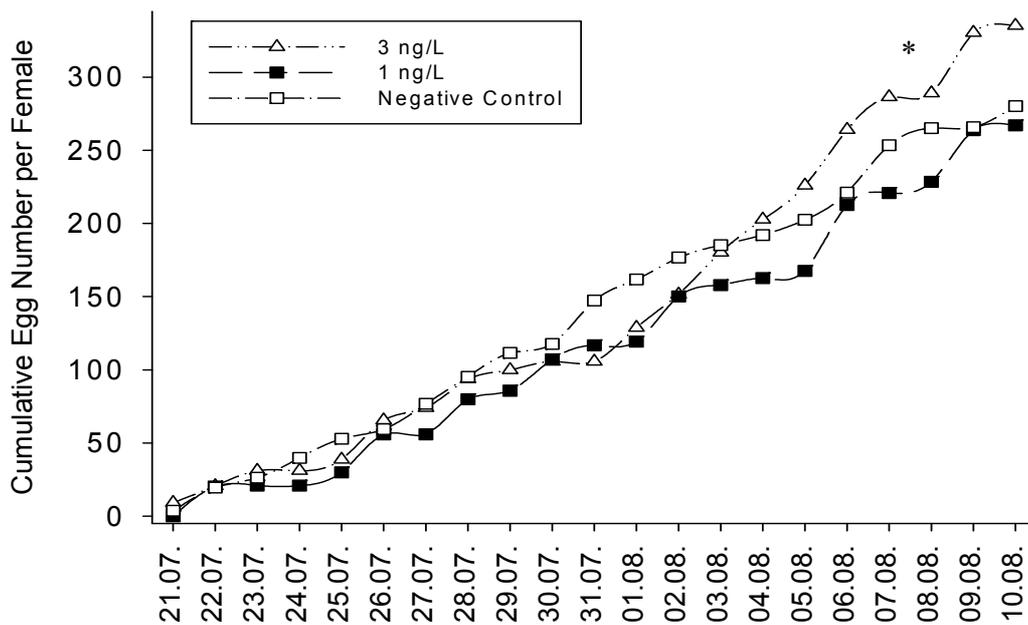


Fig. B-16. Cumulative number of eggs of the F₁ generation of zebrafish (*Danio rerio*) exposed to 0, 1 and 3 ng/L 17 β -trenbolone. * Significantly different from controls ($p < 0.05$). Chi square test with Yates correction.

In the F₁ generation, only controls and the two lowest exposure groups (1 and 3 ng/L 17 β -trenbolone) were able to regularly produce eggs (Figure B-16). At 10 ng/L 17 β -trenbolone, only one female could be found; after exposure to 30 and 90 ng/L 17 β -trenbolone, no female zebrafish could be identified. Total egg numbers produced after exposure to 3 ng/L 17 β -trenbolone were significantly higher than in controls.

Fertilization rate in the F₁ generation

None of the remaining exposure groups (1 and 3 ng/L 17 β -trenbolone) showed any significant change over controls (Figure B-17).

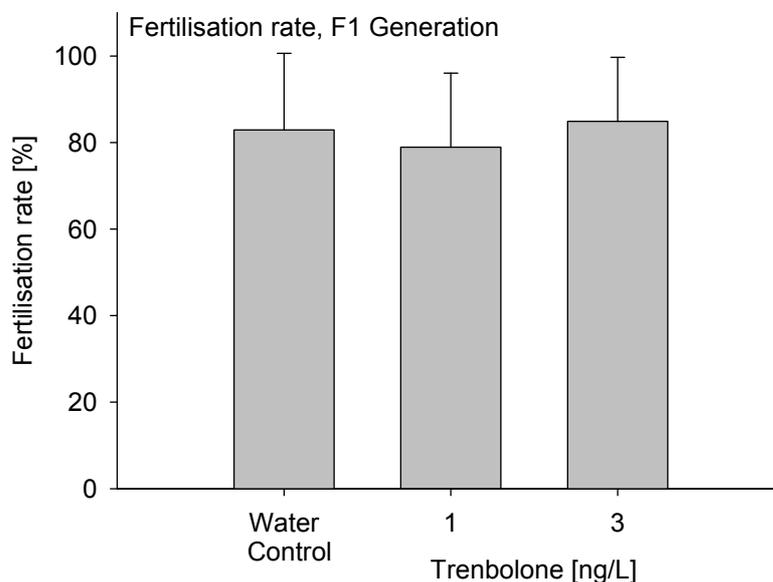


Fig. B-17. Mean fertilization rates in the F₁ generation of zebrafish (*Danio rerio*) exposed to 1 and 3 ng/L 17 β -trenbolone (\pm SD).

B-4.3.6 Molecular biomarkers in the first filial (F₁) generation

Vitellogenin in the F₁ generation

Both in male and female zebrafish of the F₁ generation, no effect on plasma vitellogenin could be detected (Figure B-18).

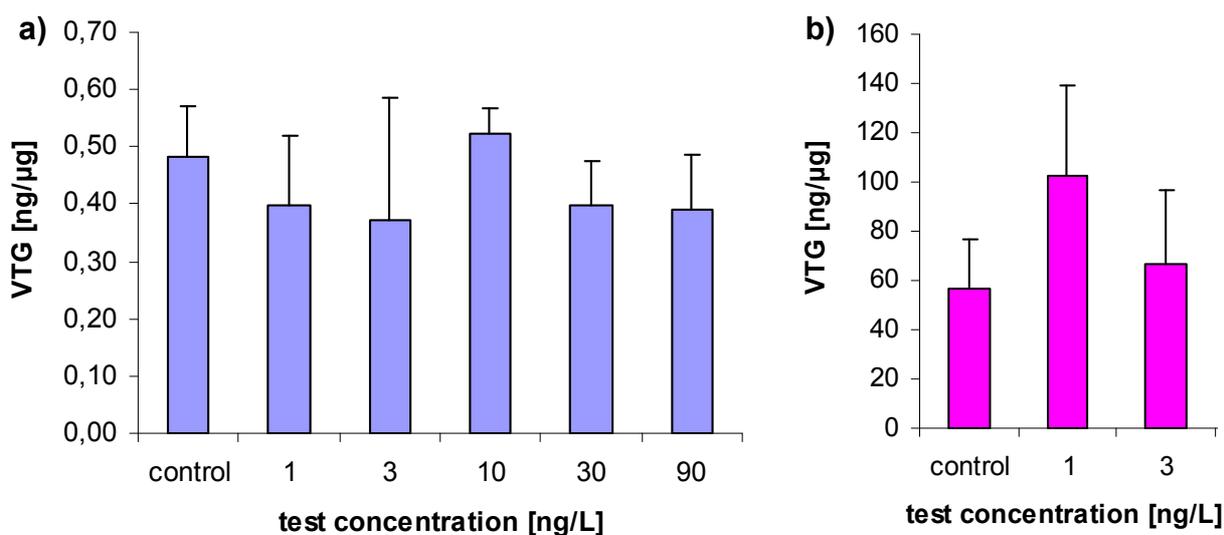


Fig. B-18. Mean vitellogenin (VTG) concentration in blood plasma relative to total protein contents in male (a) and female (b) zebrafish of the F₁ generation after exposure to 17 β -trenbolone (\pm SD).

11-keto-Testosterone in male zebrafish of the F₁ generation

At 30 ng/L 17 β -trenbolone, the plasma levels of 11-keto testosterone were statistically significantly increased in male F₁ zebrafish (Figure B-19).

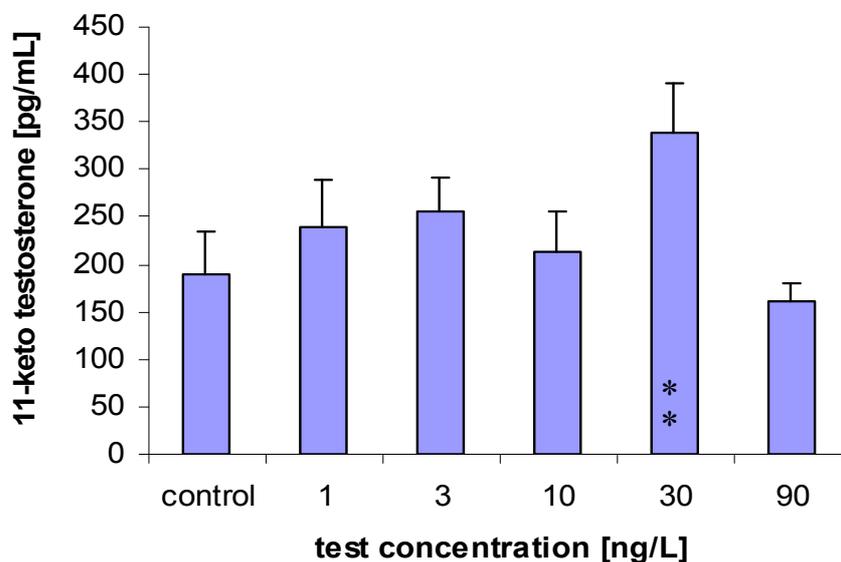


Fig. B-19. Mean concentrations of 11-keto testosterone in blood plasma of male zebrafish of the F₁ generation exposed to 17 β -trenbolone (\pm SD). ** Significantly different from controls with $p < 0.01$.

B-4.3.7 Histological alterations in zebrafish of the first filial generation exposed to 17 β -trenbolone

Female first filial zebrafish – primary diagnoses

All individuals collected from the two highest concentrations of 17 β -trenbolone were 100% male, or masculinized. Among the 3 replicates of the 10 ng/L group, one single female individual could be found, the ovary of which could be staged late vitellogenic to hydrated (stage 3 – 4). The gonads of the female zebrafish in the 1 ng/L exposure group could be categorized into intermediate stages of maturation. If compared to the controls, the relative proportion of females with late vitellogenic to hydrated ovaries increased in a dose-dependent fashion.

Male first filial zebrafish – primary diagnoses

Following exposure to 3, 30 and 90 ng/L 17 β -trenbolone, the gonads of male F₁ zebrafish displayed an increase in Leydig cells (≤ 60 %). Moreover, occasional testis-ova could be observed from 1 ng/L 17 β -trenbolone (Figure B-21).

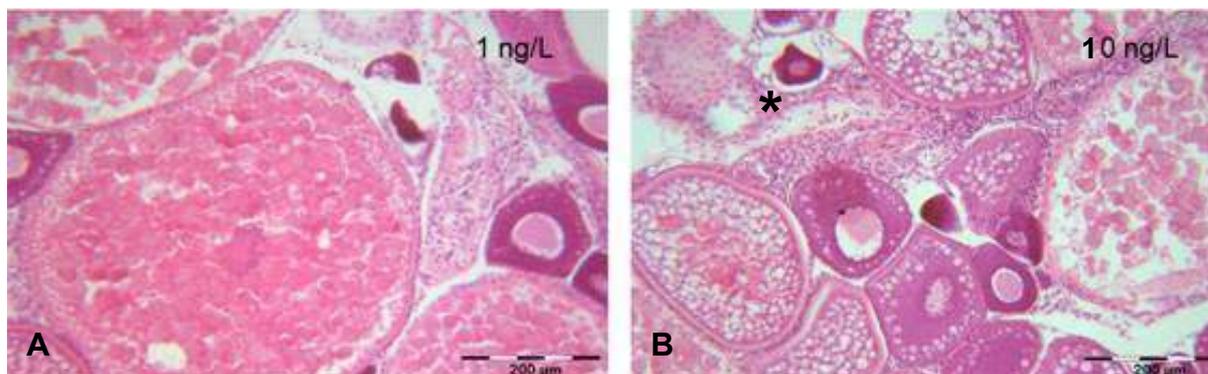


Fig. B-20. Histology of female gonads of zebrafish of the first filial (F_1) generation exposed to 17β -trenbolone. A – 1 ng/L 17β -trenbolone: between mature and perinucleolar oocytes, there are first signs of egg debris in the oviduct. B – 10 ng/L 17β -trenbolone: mild fibrosis (*). H&E-staining.

Other observations

From 1 ng/L 17β -trenbolone, an accumulation of egg debris could be seen in oviducts, as well as formation of fibrosis. At higher 17β -trenbolone concentrations, males displayed a significant increase in Leydig cells.

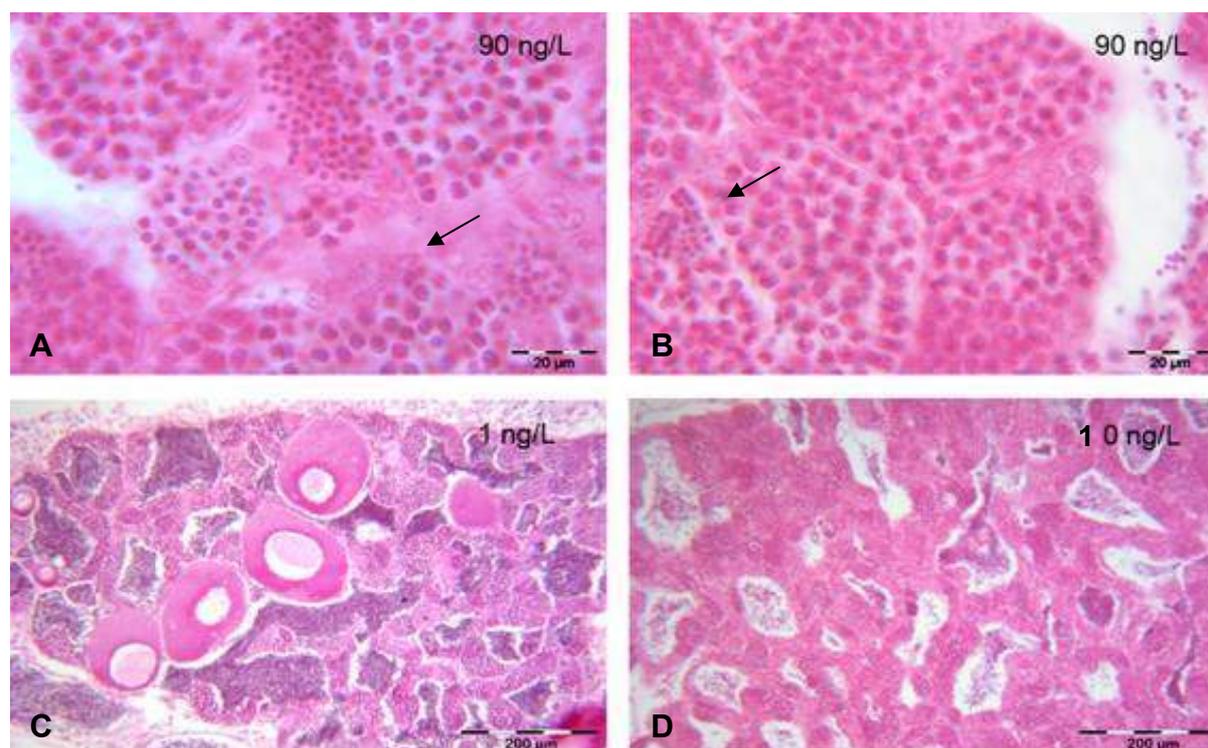


Fig. B-21. Paraffin sections of male gonads of zebrafish of the first filial (F_1) generation exposed to 17β -trenbolone. A – 90 ng/L 17β -trenbolone: Sertoli cells (→) surrounded by spermatogonia. B – 90 ng/L 17β -trenbolone: highly increased numbers of Leydig cells (→), most of which are surrounded by spermatids. C – 1 ng/L 17β -trenbolone: testis-ova. D – 10 ng/L 17β -trenbolone: increased numbers of spermatogonia. H&E-staining.

Table B-8. Summary of histological alterations in the F1 generation of zebrafish (*Danio rerio*) after 177 d of cumulated exposure to various concentrations of 17β-trenbolone. Data are given as per cent of individuals affected (the total number of individuals in the respective treatment groups are given in brackets). Arrows indicate direction of change (increase, decrease). * Tubules contained relatively few numbers of spermatozoa.

Trenbolone (Tren)	Control				1 ng/L Tren				3 ng/L Tren				10 ng/L Tren				30 ng/L Tren				90 ng/L Tren							
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4				
FEMALE																												
Severity Grade	33				11				10	10	20		10				10				100							
Oocyte atresia, immature ↑																												
Oocyte atresia, mature ↑					22	11			10	20			30				100											
Interstitial fibrosis	22								10	10			10				100											
Egg debris oviduct									10				10															
Postovulatory follicles ↑						11			10	10			10				100											
Perinucleolar Oocytes ↑									10	10			10															
Proteinaceous fluid, interstitial					11				10	10			10	10			100											
Gonadal staging: Stage 2									10								100											
Stage 3					67				40				40															
Stage 4					33				50				60															
% Females/exposure group	32.7 % (91)				22.7 % (85)				17 % (88)				1.2 % (64)				0 % (68)				0 % (80)							
MALE																												
Spermatogonia ↑					10								10				20	20	20		20	10			10	30	40	
Interstitial cells (Leydig cells)					10				40				20	20	20		20	10			20	10			10	30	40	
Testis-ova									10																			
Spermatozoa ↓																												
Spermatocytes ↑					10				20	10	10		10				20				20							
Asynchron. developed gonads													10															
Interstitial fibrosis									20	30			20								10				10	20		
Sertoli cells ↑	10	10							10	10	10		20	20	40		30				10	20	30		20	30	10	
Blood in the testis					10	10			10	10							10											
Blood in the peritoneum																												
Gonadal staging: Stage 1					10																							
Stage 2					30				60				5				30										25	
Stage 3					60				40				95				70				100						75	
% Males/exposure group	68.3 % (91)				77.3 % (85)				83 % (88)				98.8 % (64)				100 % (68)				100 % (80)							

B-4.4 F₂-generation

B-4.4.1 Macroscopic observations in the second filial (F₂) generation

During the entire exposure period of 30 d, no significant macroscopic observations were made in the F₂ generation.

B-4.4.2 Hatch and survival in the second filial (F₂) generation

Hatching rate in the second filial (F₂) generation

Hatching could only be observed in the 1 and 3 ng/L exposure groups as there was no egg production in the F₁ at the other concentrations. The hatching rate did not show any effect of 17β-trenbolone (Figure B-22) and was > 95 % in both concentrations. In the F₂ controls, more than 90 % had hatched after 105 h. At this point of time, 88 and 65 % of the larvae exposed to 1 and 3 ng/L 17β-trenbolone had hatched, respectively. The delay in hatch of the F₂ zebrafish exposed to 3 ng/L 17β-trenbolone was statistically significant ($p < 0.01$).

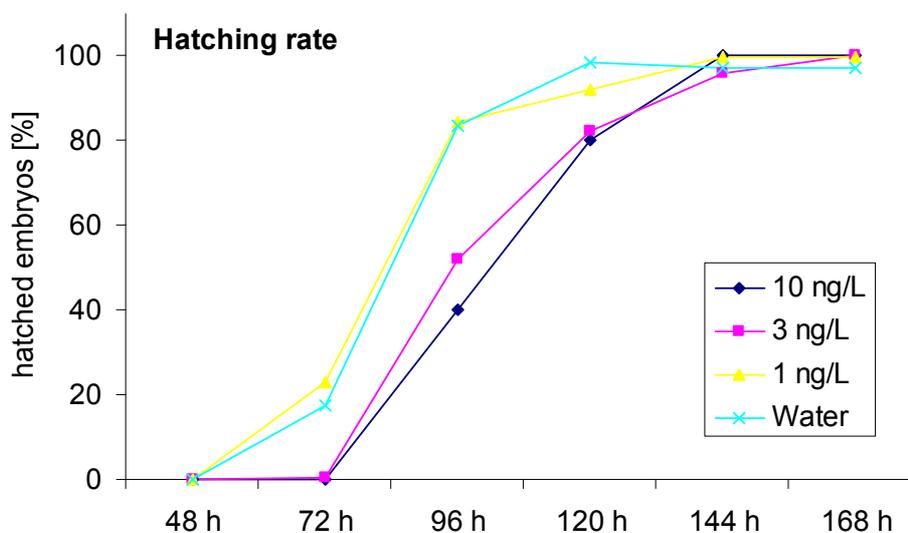


Fig. B-22. Hatching rate of F₂ larvae under exposure to 17β-trenbolone.

Mortality and survival rate in the second filial (F₂) generation

In the F₂ control and the group exposed to 1 ng/L 17β-trenbolone, the survival rate exceeded 60 %, whereas in F₂ zebrafish exposed to 3 ng/L 17β-trenbolone, the survival rate was significantly reduced (Figure B-23).

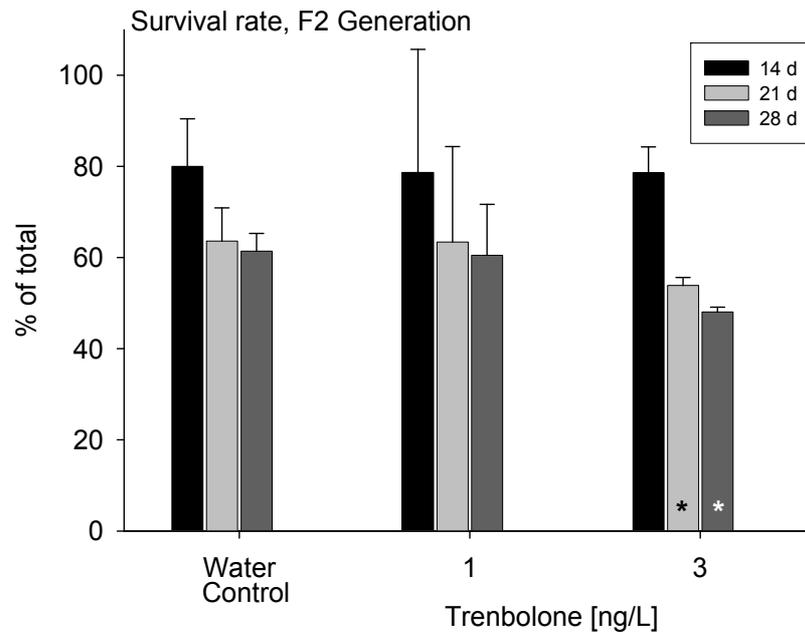


Fig. B-23. Mortality of F₂ zebrafish after different periods of exposure to 17 β -trenbolone. Dunnett's Test with $p < 0.05$.

B-4.4.3 Juvenile growth in zebrafish of the second filial (F₂) generation

Following 30 d of exposure to 17 β -trenbolone, the zebrafish of the F₂ generation exposed to 1 ng/L 17 β -trenbolone were statistically larger than the corresponding control fish (Figure B-24). In contrast, the fish of the 3 ng/L exposure group were significantly reduced in length. In either group, there was a minor increase in weight compared to F₂ controls.

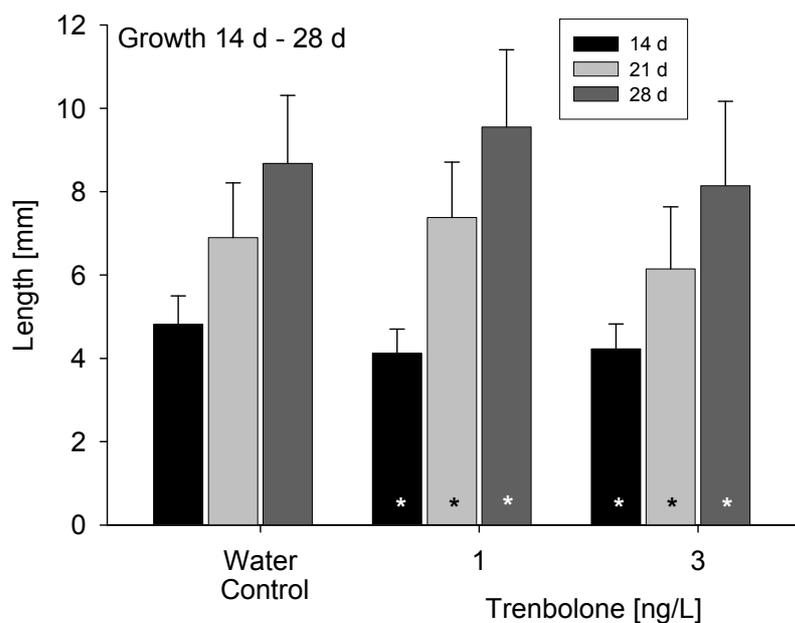


Fig. B-24. Mean growth rate based on measurement of length of zebrafish of the F₂ generation during exposure to 17 β -trenbolone. * Significantly different from controls with $p < 0.05$. Dunnett's Test with $p < 0.05$.

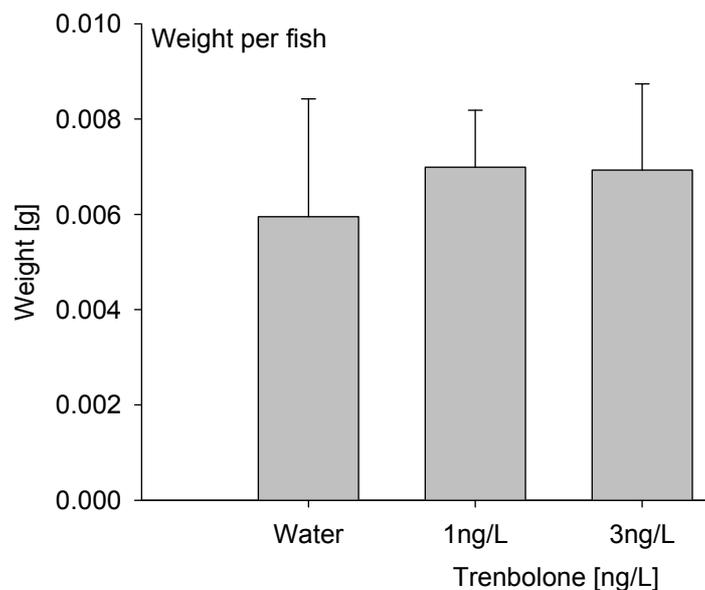


Fig. B-25. Mean growth in adult F_2 zebrafish after exposure to 17β -trenbolone based on weight measurements.

B-5 Summary and conclusions

Within a two-generation study with zebrafish, parental, first and second filial generations (P , F_1 , F_2) were exposed in triplicate to 1, 3, 10, 30 and 90 ng/L 17β -trenbolone under flow-through conditions. After 21 days of exposure, parental fish were allowed to spawn, and egg production, fertilization and hatching rates were determined. In the subsequent F_1 generation, juvenile growth and survival were recorded until maturity. As for the parental generation, F_1 zebrafish were allowed to reproduce, and the following F_2 generation was studied during its early life-stage development. In both P and F_1 generations, vitellogenin production and plasma levels of 11-keto testosterone were measured as biochemical markers of sexual steroid metabolism. Finally, after reproduction, histopathology of gonads was analyzed in P and F_1 zebrafish.

Results document that the design selected for the two-generation test was principally suitable to detect the effects of an androgen agonist such as 17β -trenbolone from concentrations in the lower ng/L range. For many endpoints, however, the dose-response relationship was not linear; rather, U-shaped or inverted U-shaped trends could be observed, with 3 ng/L 17β -trenbolone being frequently the most effective concentration. Table B-9 summarizes all observations and measurements made in zebrafish exposed to 17β -trenbolone over two generations.

The biologically most significant effect was the complete masculinization of zebrafish in the F₁ generation observed starting from ≥ 10 ng/L 17 β -trenbolone. However, other endpoints such as egg production and fertilization rates (P, F₁), vitellogenin (P, F₁), and timely hatching (not in absolute numbers) in the F₂ generation were affected from as low as 1 ng/L 17 β -trenbolone. Likewise, histopathological analyses revealed changes starting from 1 ng/L 17 β -trenbolone, however, not for primary diagnoses, and usually for a limited number of individuals only.

As mentioned, for many endpoints, the dose-response relationship was not linear. U-shaped or inverted U-shaped trends could be observed, with 3 ng/L of 17 β -trenbolone being frequently the most effective concentration.

In the F₁ generation, survival was increased following exposure to 1 and 3 ng/L 17 β -trenbolone, whereas at higher concentrations survival rates were reduced, thus potentially documenting a hormesis-like effect of 17 β -trenbolone, which correlated with an increased fertilization rate in the parental generation after exposure to 1 and 3 ng/L 17 β -trenbolone.

In the second filial (F₂) generation, mortality was increased starting from 3 ng/L 17 β -trenbolone. Juvenile growth only showed an effect at 1 ng/L in the F₁ generation. Parameters, which did not show any change with exposure to 17 β -trenbolone, were apical endpoints such as survival/mortality and growth.

Among the molecular biomarkers, vitellogenin in females was not affected (cf. similar observations in other fish species, Ankley et al. 2003, Seki et al. 2006). In males, an effect could only be documented in parental fish exposed to 3 ng/L 17 β -trenbolone. The marker for androgenic activity, 11-keto testosterone, only gave statistically significant effects at 30 and 90 ng/L 17 β -trenbolone in the parental and first filial generations, respectively.

Table B-9. Summary of all effects observed in zebrafish during a two-generation test with 17 β -trenbolone

Generation	Life Phase	Endpoint	NOEC (ng/L)	LOEC (ng/L)	
Parental (P)	Reproduction	Female territoriality	30	90	
		Cumulative number of fertilized eggs, fertilisation rate	< 1	1, 3*	
	Biomarker	Vitellogenin		3 (trend)	
			female decrease	1	3*
			male decrease	30	90
	11-keto testosterone	male decrease	< 1	1	
	Histopathology ***	female	1	3	
		male	1	3	
1 st filial (F ₁)	Early life-stage	Hatch		> 90	
		Survival day 56	< 1	1**	
	Juvenile growth	Growth (length) day 14	< 1	1	
		Survival day 56	< 1	1**	
		Growth (length) day 56	90	> 90	
		Sex ratio	3	10	
		Cumulative number of fertilized eggs	1	3	
	Reproduction	Fertilization rate		> 3	
		Vitellogenin	female increase	< 1	1 (trend)
			male decrease		Trend
		11-keto testosterone	male increase	10	30
	Histopathology ***	female/male	< 1	1	
2 nd filial (F ₂)	Early life-stage	Hatch (delay only)	1	3	
		Survival day 35	1	3	
		Growth (length)	< 1	1	

* No effect at higher concentrations of 17 β -trenbolone; ** increase at 1 and 3 ng/L 17 β -trenbolone, decrease at 10, 30 and 90 ng/L 17 β -trenbolone (see comments below); *** no statistical evaluation; partly single findings (additional evidence).

For the identification and interpretation of the most sensitive endpoint, the result summary as given in Table B-9 can be classified into five major aspects:

- (1) *masculinization* in the F₁ generation (10 ng/L and higher);
- (2) *low-dose* effects at 1 ng/L;
- (3) *non-linear concentration-response relationships* (U-shaped or inverted U-shaped trends with 3 ng/L being frequently the most effective concentration);
- (4) *hormesis* in the F₁ generation (increased survival at 1 and 3 ng/L, correlating with an increased fertilization rate in the P generation);
- (5) *increasing mortality* of the F₂ generation early life-stages at 3 ng/L.

Evaluation of the results from a regulatory point of view

The effect assessment should be based on at least one of the following arguments:

- statistically significant deviation from controls with reasonable variability between replicates;
- clear concentration-response relationship (sigmoid, when based on normal distribution of sensitivity towards the effect);
- evidence for deviating concentration-response relationships (e.g. hormesis or U/inverted U-shape responses), either by mechanistic knowledge or supporting data from different endpoints.

Moreover, the data should be generated in a valid test, i.e., with acceptable performance of the test fish (survival, growth, reproduction) and application of the state-of-the art methodologies in endpoints measurements and statistical evaluations. In this study the supporting analytical chemistry was not in compliance with regulatory requirements, i.e. the number of sampling points and measurements were not sufficient.

A fish two-generation-test is a complex and long-lasting non-standard study with many possibilities for mistakes, effects of stochastic performance variability, and with high probability of accidental effects by aging of the technical facilities. There are validity criteria for standard tests covered by the two-generation study, which, however should not be applied too strictly, especially to filial generations. Since a considerable body of data is generated by

a two-generation study, there are possibilities to obtain a full picture of effects and to interpret them by weight-of-evidence of all data, also including data from other studies.

The trenbolone two-generation test described in this report suffers from some shortcomings: First of all, analytical verification of the test concentrations was only performed four times within six months. The means of the four measurements varied between 35 and 59 % of nominal. The relative standard deviation of the mean was about 120 % for the lowest concentration, 50 % for the second concentration and 30% for the higher three concentrations. From these data, real effect concentrations can only be estimated as roughly 50 % of the nominal concentrations. In the following passages, if not specifically mentioned, concentrations are expressed as nominal concentrations (as throughout this report).

With respect to the measurement of reproduction endpoints, due to a lack of manpower and spawning trays, only one replicate per concentration could be measured simultaneously per day¹. As a consequence, the mating cue of the spawning tray was supplied to each spawning group only every third day, and eggs spawned on the remaining two days were not counted. Replicate statistics are, therefore, not possible. Evaluation as cumulative numbers of spawned eggs over an extended period of time may be a matter of discussion, but can at least be a potential surrogate parameter to true spawning data.

For unknown reasons, the fertilization rate was unusually low (55 - 60 %) in controls and the three higher treatments. At 1 and 3 ng/L, the rates ranged between 65 and 75 %. The validity criterion for fertilization rates in parental groups of two-generation tests according to the OECD draft proposal, however, is 80 % (pre-exposure). Taken together, the weakness in measuring total egg production, and the low fertilization rates of the controls, are not sufficient for the generation of evidence for hormesis or low-dose effects regarding reproduction. However, although small differences between concentrations should not be over-interpreted, a potential trend to fertilization effects at lower concentrations cannot be completely excluded. In this context, it appears noteworthy that Miller et al. (2007) exposed fathead minnow (*Pimephales promelas*) to 3, 10, 30 and 100 ng/L 17 β -trenbolone, and found a significant decline in fecundity at 10 ng/L and higher concentrations.

Early life stages of the F₁ generation showed a high mortality during their most sensitive phase of starting external feeding, which could not be related to the test substance. The survival rates of the control replicates varied between 20 and 30 % after 28 days, similar to

¹ Since permanent (daily) addition and removal of the spawning trays may also induce considerable handling stress in the fish, this procedure can be discussed from different points of view.

those at 10 and 30 ng/L. At 1 and 3 ng/L, the means were between 30 and 40 %, whereas at the highest test concentration, the mean was below 20 %. The validity criterion for OECD 210 fish early life stage tests is 70 % post-hatch survival, which should mean at least 63 % total survival (hatching rate of 90 %).

It should be noted that exposure of female (parental) rainbow trout (*Oncorhynchus mykiss*) to 35 ng/L 17 β -trenbolone for 60 to 77 days did not alter embryo survival (Schultz et al. 2008). However, in the embryos from 17 β -trenbolone-exposed females, a noticeable delay in developmental progress was observed: On day 19, when eye development is normally complete, the majority of the embryos either lacked eyes or displayed under-developed eyes, in contrast to control embryos. From this finding it was concluded that steroidal androgen exposure in sexually maturing female rainbow trout might impact developmental timing of F₁ offspring (Schultz et al. 2008).

In a model combining a Leslie population projection matrix and the logistic equation, fathead minnow populations occurring at carrying capacity and subsequently exposed to 27 ng/L of 17 β -trenbolone exhibited a 51 % projected decrease in average population size after 2 years of exposure (Miller & Ankley 2004). Populations at carrying capacity exposed to concentrations of ≥ 266 ng/L 17 β -trenbolone exhibited a 93 % projected decrease in average population size after 2 years of exposure. Overall, fathead minnow populations exposed to continued concentrations of 17 β -trenbolone ≥ 27 ng/L (1 nM) were projected to have average equilibrium population sizes that approached zero.

The high mortality in the actual study compromises the results of the entire F₁ generation, since selective mortality of fish age classes more or less susceptible to the test item cannot be excluded. Additionally, at those low survival rates, a difference of 10 - 15 % results in a doubling of fish densities and difficulties when comparing growth rates. Thus, beside statistically significant deviations from controls, a concentration-response relationship is crucial for the interpretation of results. For the trenbolone study, this means that the low survival rates of the controls do not provide sufficient evidence for hormesis or low-dose effects regarding enhanced survival or reproduction, nor for U-shape characteristics of growth.

Histological findings have not been evaluated by replicate statistics. As an inherent trait of non-quantitative histological evaluation, LOECs and NOECs have to be based on observations in individual fish, since individual variability, which becomes particularly prominent in the histological appearance of different organ systems, increases with lower

concentrations (Braunbeck 1993, Braunbeck & Strmac 2001, Segner & Braunbeck 1998). Whether or not, and to what extent, such individual findings can be used for regulatory purposes (e.g., determination of LOECs and NOECs), is under discussion. Nonetheless, histological data can be used for the identification of sublethal stress, for the localization of active modes of action, as well as for confirmation and interpretation of apical effects.

At least part of the typical **biomarker responses** are of adaptive nature and may, therefore, show quite unusual dose-response relationships (Braunbeck & Völkl 1993, Segner and Braunbeck 1998). In order to be able to identify clear trends, however, statistically significant deviations from controls are required, if clear concentration-response relationships (be it linear or U-shaped) are not evident and supported by other data. If there is a significant deviation in one treatment concentration only, interpretation is very difficult without further evidence (e.g., decreased vitellogenin in parental males at 3 ng/L, and increased 11-keto testosterone in F₁ males at 30 ng/L trenbolone). Decreasing vitellogenin levels in males can be used as indicators for physiological thresholds, as there is no need for compensation. However, they are not population-relevant by themselves.

In sheepshead minnow (*Cyprinodon variegatus*), Hemmer et al. (2008) also found effects on vitellogenin synthesis, however, only at very high concentrations of 5 µg/L 17β-trenbolone. Exposure to 17β-trenbolone caused lesser fecundity of Japanese medaka (*Oryzias latipes*), and down-regulated transcription of vitellogenin and choriogenin gene expression in the liver of females (Zhang et al. 2008a, b). Similarly high concentrations (3 µg/L) were tested in a zebrafish DNA microarray application (Wang et al. 2008a, b). In a 60 d exposure of zebrafish and medaka to 50 ng/L 17β-trenbolone, Orn et al. (2006) observed a significant decrease in vitellogenin production in either species. Following a brief water exposure (24 h) of fathead minnow to 50 and 500 ng/L 17β-trenbolone, vitellogenin synthesis was suppressed in female fathead minnow liver (Miracle et al. 2006).

In an inter-laboratory calibration exercise with three-spined stickleback (*Gasterosteus aculeatus*) in a short-term fish screening assay, three participating laboratories did not detect statistically significant increases in vitellogenin in males, but statistically significant increases of spiggin in females after 14 d exposure to nominal concentrations of 5 µg/L trenbolone (Allen et al. 2008). In contrast, in a stickleback kidney cell culture assay for the screening of androgenic and anti-androgenic endocrine disrupting substances, Jolly et al. (2006), 17β-trenbolone was able to stimulate spiggin synthesis in a concentration-dependent manner with

a significant effect starting from a concentration of 0.1 nM (≈ 27 ng/L) to a maximal effect at 10 μ M.

In consequence, among the major aspects of result classification listed above, aspects 2 to 4 (low-dose, hormesis and U-shape responses) are based on only weakly or insufficiently supported data. Different endpoints in different generations were interpreted as either pointing to linear concentration responses most effective at high concentrations (behaviour, histology, 11-keto testosterone in the P generation, sex ratio in the F₁ generation), specific low-dose effects, or hormesis (reproduction in the P generation, survival and growth in the F₁ generation), or highest effects at medium concentrations (VTG in the P generation).

Thus, only the remaining results (Table B-10) can be included in an interpretation with respect to the most sensitive endpoint, and can definitely be compared to data from other studies with 17 β -trenbolone.

Table B-10. Summary of relevant effects observed in zebrafish during a two-generation test with 17 β -trenbolone (EC₁₀, NOECs and LOECs expressed as mean measured concentrations).

Generation	Life Phase	Endpoint		NOEC (ng/L)	LOEC (ng/L)	EC ₁₀ (ng/L)
Parental (P)	Reproduction	Female territoriality		15	31	
	Biomarker	11-keto testosterone	♂ decrease	15	31	
First filial (F ₁)	Maturation	Sex ratio		1.1	3.6	0.59*
Second filial (F ₂)	Early life stages	Hatch (delay), survival d 35, growth (length decrease)		0.59	1.1	

* Probit analysis using maximum likelihood regression was applied to calculate the EC₁₀ value according to Finney (1971). The EC₁₀ value is based on nominal concentrations.

The remaining results can be grouped in parental generation effects in fish exposed as adults, and filial generation effects on fish exposed during sexual maturation and their offspring. The effects on the parental generation were changes in female behaviour and a decrease in male 11-keto testosterone, most likely due to a high androgen level and down-regulation of 11-keto testosterone by feedback inhibition. Both effects only occurred at the highest test concentration (NOEC 30 ng/L), and should not be regarded as population-relevant, since reproduction was apparently not affected.

Both changes in female behaviour and the decrease in male 11-keto testosterone levels were not observed in a 21 d fish screening assay performed at the same concentrations (NOEC: 90 ng/L; Schäfers, personal communication). However, this is not necessarily inconsistent, since the behaviour was not looked at and exposure time was shorter and most probably not sufficient to result in a down-regulation of 11-keto testosterone comparable to that in parental zebrafish in the two-generation test.

Despite the high mortality of the F₁ early life-stages and resulting low fish densities differing by a factor of two, the effect on sex ratio was clear, since it showed a linear concentration-response relationship with a 100 % masculinizing effect at the two highest concentrations (\geq 30 ng/L), and a 95 % effect at 10 ng/L. Due to the high variability of replicates (most probably due to the high mortality), a decrease of males at the lower concentrations of 48 % and 31 % were not statistically significant. In fact, in a 60 d exposure of zebrafish and medaka to 50 ng/L 17 β -trenbolone, Orn et al. (2006) observed masculinization in zebrafish.

Two FSDT studies with 17 β -trenbolone recently performed during the OECD FSDT validation process showed 100 % male fish at concentrations \geq 10 ng/L, and 80% male fish at 9 ng/L (measured concentration); the NOEC was at the lowest concentration, measured below the limit of quantification of 5 ng/L (Henrik Holbech, personal communication; Holbech et al. 2006). In contrast, in a comparative 21 day fish screening assay with medaka and fathead minnow, Seki et al. (2006) observed masculinization of secondary sex characteristics in female medaka with an LOEC of 365 ng/L 17 β -trenbolone, and in fathead minnow with an LOEC of 401 ng/L 17 β -trenbolone.

When using regression analysis on mean percentages of males in the F₁ generation (Figure B-26), the EC₅₀ value was calculated to be 2 ng/L, and the EC₁₀ value to be 0.6 ng/L. Taking into account the uncertainty due to the low survival rates and high replicate variability, the threshold concentration for sex reversal effects is rounded to 1 ng/L. The sensitivity to 17 β -trenbolone in the two-generation test is, therefore, two orders of magnitude higher than the sensitivity of the 21 d screening assay, but is obviously comparable to the sensitivity of the FSDT.

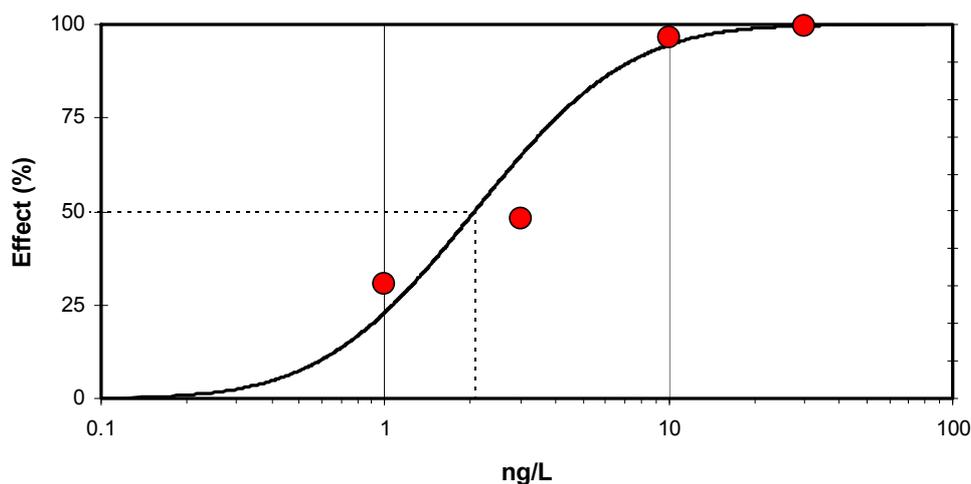


Fig. B-26. Concentration-response relationship of percentage of males in the F₁ generation of zebrafish (*Danio rerio*) exposed to 17 β -trenbolone (controls: 33% males equivalent to 0 % effect).

In terms of LOEC and NOEC for the sex ratio, the early life-stages of the F₂ generation seem to be more sensitive by one concentration step than the sex ratio of the F₁ generation (Tabs. B-9, B-10). However, due to the low replicate variability, the decrease by 15 % survival compared to controls at 3 ng/L 17 β -trenbolone was already statistically significant. At 1 ng/L 17 β -trenbolone, the decrease was only 1 %.

Since in the F₂ generation, the early life-stage growth was also negatively affected at 3 ng/L 17 β -trenbolone, whereas in the F₁ generation early life-stages showed better performance at 3 ng/L 17 β -trenbolone, a maternal transfer effect may be assumed resulting from affected quality of eggs produced by F₁ fish at a concentration that causes sex reversal in sensitive females.

Thus, despite the shortcomings listed above, the two-generation study on effects by 17 β -trenbolone in zebrafish is suited to derive consistent data for a hazard assessment of 17 β -trenbolone. The most sensitive and most relevant endpoint is the sex ratio of the F₁ generation; the most sensitive time window of exposure is obviously the period of sexual maturation.

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Supplement C

Two Generation Test with zebrafish (*Danio rerio*)

Test substance: Flutamide

November 2009

Author:

Matthias Teigeler

Fraunhofer Institut für Molekularbiologie

und Angewandte Oekologie (IME)

Auf dem Aberg 1

57392 Schmallenberg

Germany

Content

C-1. Introduction.....	C-5
C-2. Study conditions.....	C-5
C-2.1 Test organism.....	C-5
C-2.2 Test substance.....	C-6
C-2.3 Test system.....	C-6
C-2.4 Chemical analysis.....	C-7
C-3. Performance of the study.....	C-8
C-3.1 Description of the different life phases.....	C-8
C-3.2 Observations.....	C-10
C-3.3 Specific methods.....	C-10
C-3.3.1 Egg collection.....	C-10
C-3.3.2 Photography and image analysis.....	C-11
C-3.3.3 Blood collection and preparation.....	C-11
C-3.3.4 Measurement of Biomarkers using ELISA.....	C-11
C-3.3.5 Histopathology.....	C-13
C-3.3.6 Statistical Data Analysis.....	C-14
C-4. Results.....	C-15
C-4.1 Chemical analysis.....	C-15
C-4.2 P-generation.....	C-20
C-4.2.1 P-generation, Observations.....	C-20
C-4.2.2 P-generation, Reproduction.....	C-20
C-4.2.3 P-generation, Biomarker.....	C-23
C-4.3 F ₁ -generation.....	C-26
C-4.3.1 F ₁ -generation, Observations.....	C-26
C-4.3.2 F ₁ -generation, Hatch/Survival.....	C-26
C-4.3.3 F ₁ -generation, Juvenile Growth.....	C-27
C-4.3.4 F ₁ -generation, Reproduction.....	C-28
C-4.3.5 F ₁ -generation, Biomarker.....	C-30
C-4.3.6 F ₁ -generation, Sex ratio.....	C-31
C-4.3.7 F ₁ -generation, Histopathology.....	C-32
C-4.4 F ₂ -generation.....	C-35
C-4.4.1 F ₂ -generation, Hatch/Survival/Growth.....	C-35
C-5. Summary and Conclusions.....	C-36
C-6. References.....	C-39

List of tables and figures

Table C- 1: Fish two generation test with zebrafish, important endpoints of the study	C-9
Table C- 2: Categories of maturation of zebrafish (<i>Danio rerio</i>) gonads	C-14
Table C- 3: Summary of results of chemical analysis during a two generation test with flutamide	C-15
Table C- 4: Detailed results of chemical analysis during a two generation test with flutamide	C-16
Table C- 5: P-generation: summary of all endpoints	C-25
Table C- 6: F ₁ -generation, Early Life Stage, Juvenile growth and Reproduction, Summary of all endpoints	C-33
Table C- 7: F ₁ -generation, Sex ratio and Biomarker, Summary of all endpoints	C-34
Table C- 8: F ₂ -generation, Early Life Stage, Summary of all endpoints	C-36
Table C- 9: Summary of all endpoints, recorded during a two generation test with flutamide	C-38
Figure C- 1: Flutamide, Chemical structure	C-6
Figure C- 2: P-generation: egg number per day and female [n]; eggnumber based on 20 counting days (day 0- 20 of exposure)	C-21
Figure C- 3: P-generation: egg numbers per day and female [n]; egg number based on 9 counting days (day 12-20 of exposure)	C-22
Figure C- 4: P-generation: cumulative number of fertilised eggs [n]	C-22
Figure C- 5: P-generation: fertilisation rate [%]	C-23
Figure C- 6: F ₁ -generation: survival of fish larvae at day 35 pf [%]	C-27
Figure C- 7: F ₁ -generation: pseudo specific growth rate of the juvenile fish based on the measurement of lengths on day 35 and day 70.	C-27
Figure C- 8: F ₁ -generation: egg numbers per day and female [n]	C-28
Figure C- 9: F ₁ -generation: cumulative number of fertilised eggs [n].	C-29
Figure C- 10: F ₁ -generation: fertilisation rate [%]	C-30
Figure C- 11: F ₁ -generation: vitellogenin concentration in female blood plasma [ng/μg]	C-31
Figure C- 12: F ₁ -generation: concentration of 11-keto testosterone in male blood plasma [pg/mL]	C-31
Figure C- 13: F ₂ -generation: group weights of fish larvae at day 35 pf	C-35

List of abbreviations

11 kT	11-keto testosterone
AR	androgen receptor
ELS	Early Life Stage
ER	estrogen receptor
F ₁	Filial 1 generation
F ₂	Filial 2 generation
LOEC	Lowest observed effect concentration
MOA	Mode of Action
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
P	Parental generation
pf	post fertilisation
UBA	German Federal Environment Agency (Umweltbundesamt)
VTG	Vitellogenin

C-1. Introduction

From June 17, 2005 to February 24, 2006, a two generation study with flutamide as test substance was performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Schmallenberg, Germany.

Flutamide represents the endocrine mode of action androgen receptor antagonist. The aim of the study was the evaluation and the assessment of effects after continuous exposure to flutamide on all life stages of zebrafish. This includes the parental generation (P-generation), early life stages (life phase directly post hatch), juvenile growth and reproduction of the Filial 1 (F₁)-generation, and finally, the early life stage (ELS) phase of the Filial 2 (F₂)-generation. Furthermore, molecular biomarkers of the P- and F₁-generation were measured, including the yolk protein precursor vitellogenin and the sex steroid 11-keto testosterone.

C-2. Study conditions

C-2.1 Test organism

The zebrafish (*Danio rerio*, Teleostei, Cyprinidae) was used as test organism. This fish is recommended by the OECD TG 210 (OECD, 1993) test fish for Fish Early Life Stage Toxicity (FELS) studies. It is also one of the four test fish species recommended (Anonymous, 2002) for fish two generation studies.

The source of the fish was the laboratory culture of the Fraunhofer IME, Schmallenberg, Germany. The origin of the used strain was a commercial fish breeder, namely the West Aquarium GmbH in Bad Lauterberg, Germany.

Up to the test start, the adult zebrafish were kept in communities of approximately 300 individuals in 150 L tanks under flow-through conditions with a 12 h light/12 h dark photoperiod. The temperature of the water was kept constant at $25.0 \pm 1^\circ\text{C}$.

Fish were fed *ad libitum* a commercially available artificial diet (TetraMin™ flakes; Tetra, Melle, Germany), and nauplii from *Artemia* sp. (Great Salt Lake *Artemia* Cysts, Sanders Brine Shrimp Company, Utah, USA) twice a day.

Purified drinking water was used in the rearing tanks. The purification includes filtration with activated charcoal, passage through a lime-stone column, and aeration until oxygen saturation. Carbonate hardness of the water was nearly 90 mg/L CaCO₃, pH was in the range of 7.5-8.5.

The animals used in the two generation test did not exceed the age of 6 months.

C-2.2 Test substance

Flutamide was used as a test substance. It is a representative of the androgen receptor antagonists. Flutamide (purity $\geq 99\%$; Sigma Aldrich, St. Louis, MO, USA) is used as a therapeutic drug for the treatment of prostate cancer.

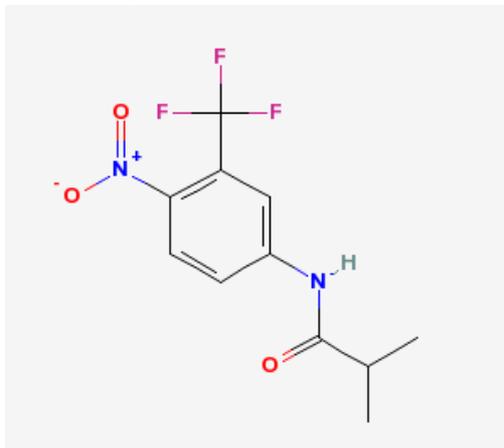


Fig. C- 1. Flutamide, Chemical structure (Source: PubChem Database)

C-2.3 Test system

For the performance of the test, a flow-through system was implemented. Four replicates per treatment were arranged. After two weeks of exposure, a decline of test concentrations was observed, which was assumed to be the result of increased microbiological activity. The amount of test substance in the stock solutions was increased to maintain the nominal concentrations in the test tanks. Furthermore, all pumping tubes were weekly changed and cleaned. To meet the increasing effort of time and costs, the number of replicates was reduced from four to two vessels per concentration before initiation of the F₁-generation.

Full glass aquaria of 42 x 28 x 28 cm (L x W x H) with approx. 25 litres of test medium were used. To adjust the water temperature in the test system, the vessels were placed in a water bath. To start the early life stage phases, the fertilised eggs were placed in fry chambers being plastic cages with nylon nets forming the bottom of the cages.

Five concentrations of the test item and the untreated dilution water as control were tested under flow-through conditions at $25\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$, and a light/dark cycle of 12 h/12 h. No additional aeration was introduced. For each treatment group, consisting of two replicate vessels, an individual dosage system consisting of two pumps was used. Dilution water was pumped into a mixing chamber by a water dosage pump (membrane pump, Prominent, Heidelberg, Germany), placed on a magnetic stirrer. An adequate amount of the stock solution was added into the mixing chamber by a stock solution dosage pump (membrane pump with a

stainless steel head, Prominent, Heidelberg, Germany). The prepared test solution entered the test vessels via flexible tubes, distributed to the two replicate vessels by an electronically regulated splitting chamber driven by compressed air. The daily water exchange rate was adjusted to at least 5 volumes per vessel.

The nominal test item concentrations were 35.7, 82.2, 189, 435 and 1000 µg flutamide/L. The primary stock solution was prepared daily by adding 800 mg of flutamide. The primary stock solution was stirred overnight.

To achieve adequate concentrations for the use in the dosing system, the stock solution was further diluted. Three secondary stock solutions were prepared and kept in stainless steel containers. For the three highest test concentrations, undiluted primary stock solution was used. For the two lowest test concentrations, the primary stock solution was diluted by a factor of 4.

After the pre-treatment phase, which included only water dosage into the vessels, exposure was started by starting the stock solution dosage pumps.

The temperature was measured daily, pH-values and oxygen concentrations were measured at least twice a week. The oxygen concentration did not fall below 60% through the whole exposure period.

C-2.4 Chemical analysis

An accompanying analysis of water samples from the test system was performed weekly. Samples of 5mL of all stock solutions and test concentrations were taken and analysed using HPLC with UV detection. For chromatographic separation, a hypersil ODS column was used with reversed phase technology. For quantitative evaluation an external calibration was performed.

At each sampling date, samples of all vessels including the controls (n=12 vessels) were sampled and measured.

C-3. Performance of the study

C-3.1 Description of the different life phases

A detailed study plan with the durations of the evaluated life phases is shown in table C-1.

The **parental generation** (P-generation) was generated with 8 males and 8 females aged at least 150 days. The fish were kept under test conditions for at least three weeks to adjust a consistent state of fecundity over all groups, and to re-sort the spawning groups in case of low egg numbers.

When all groups were spawning daily, exposure was started. Freshly spawned eggs were collected daily, counted and the number of fertilised eggs was determined. The P-generation was sacrificed when the F₁-generation successfully reached an age of 21 days.

For the start of the **F₁-generation**, 100 fertilised and randomised eggs spawned by the parental fish of each replicate were placed on nylon nets forming the bottom of cages fixed at the water surface of each test vessel. Starting from day 6 of age, larvae were fed daily with breeding food (Tetra, AZ 000, Melle, Germany) *ad libitum*. From day 9 of age, brine shrimp nauplii (*Artemia salina*) were added *ad libitum*. From day 16 of life, ground TetraMin flake food (Tetra, Melle, Germany) was added *ad libitum* to the daily food. After 35 days, the fish were randomly reduced to 50 individuals, and transferred from the fry chambers to the main vessels. After 35 and 70 days of age, length of the juvenile fish was measured. After 70 days of age, the fish number was randomly reduced to 30 individuals in order to create identical conditions for reproduction.

Reproduction of the adult F₁ fish was measured in the same way as described for the P-generation. After the recording of F₁ reproduction, an F₂-generation was prepared. The F₁-generation was sacrificed when the F₂-generation successfully reached an age of 21 days.

For the start of the **F₂-generation**, 100 fertilised and randomised eggs spawned by the F₁ fish of each replicate were placed on nylon nets forming the bottom of cages fixed at the water surface of each test vessel. Larvae were fed daily *ad libitum* as described above for the F₁-generation.

After 35 days, the F₂ fish were measured for individual lengths and group weights, and the test was terminated.

Table C- 1. Fish two generation test with zebrafish, important endpoints of the study

Day after start of exposure	Phase	Course (related to each of three replicates per concentration)	Endpoints
Pre-treatment phase, at least 7d		Start with 8 males and 8 female fish, re-organisation of groups	Reproduction: - Egg numbers - Fertilisation rate
0 – 20	P-generation Reproduction	Transfer to exposure tanks (run for 48h) Sampling of eggs for F₁ phase	
21 -56	F₁-generation Early Life Stage (35 days)	Introduction of 100 fertilised eggs in fry chambers Begin of hatch (day 23) End of hatch (day 25)	Daily observation of : - mortality - survival rate - hatching rate - time to 90 % hatch
		Transfer to the total vessel Randomly reduced to 50 (day 56) Blood/Liver/Gonad sampling of P fish (Sex-Determination of excess fish)	Weekly observation of - mortality - survival rate - length Vitellogenin 11-keto testosterone Gonad histology
57 – 84	F₁-generation Juvenile Growth	Randomly reduced to 30 (day 70) (Sex-Determination of excess fish)	Weekly observation of - mortality - survival rate - length
85 – 120	F₁-generation Reproduction	Sampling of eggs for F₂ phase	Reproduction: - Start - Egg numbers - Fertilisation rate
120	F₂-generation Early Life Stage (35 days)	Introduction of 100 fertilised eggs in fry chambers	Daily observation of : - mortality - survival rate - hatching rate - time to 90 % hatch
122		Begin of hatch	
124		End of hatch	
126			Weekly observation of - mortality - survival rate - length
155		Test termination Blood/Liver/Gonad sampling of F₁ fish (Sex-Determination of excess fish)	Recording of: - survivalweight - length Vitellogenin 11-keto testosterone Gonad histology Sex ratio

C-3.2 Observations

Observations on fish of all life stages were recorded daily. Dead eggs, larvae, juvenile and adult fish were recorded, and removed as soon as observed.

Hatching rates were estimated by daily counting of non-hatched eggs in the period between two days of age and the time, when 90 % of the fish had hatched.

Appearance of any change in behaviour (e.g. loss of equilibrium, change in swimming behaviour, change in mating behaviour, etc) compared to the control was recorded.

Between hatch and 35 days of age of the F₁- and F₂-generation, larvae/juvenile fish were photographed weekly and the survival rates were estimated. The data were confirmed by counting the fish on day 35, when transferred to the main aquaria (F₁), or sacrificed (F₂). Lengths of F₁ fish were measured by digital photography after 35 and 70 days of life. For this time interval, the pseudo-specific growth rate based on length was calculated according to the OECD TG 210 (OECD, 2000). The time of first spawning, identified as first day at which eggs were found in the spawning trays, was recorded. The reproductive endpoints like egg number, and fertilisation rate were observed for 20 daily counts. After successful start of the F₂-generation, the adult F₁ fish were sacrificed and sexed by inspection of the gonads. The sex ratio was calculated. The number of females per group was determined, and used to calculate the number of total eggs per female and day. Additionally, the cumulative number of fertilised eggs was determined.

Growth of F₂-fish until day 35 was measured as individual lengths and group weight.

C-3.3 Specific methods

C-3.3.1 Egg collection

Spawned eggs were collected daily in all glass spawning-trays placed at the bottom of the test vessels. The tray was covered with a lattice (stainless steel), to prevent the eggs from being predated by the adult fish. Artificial plant substrate was placed on the lattice to stimulate spawning into the tray. The switching on of lighting (neon lamps with a light intensity of approximately 1000 lux, measured 5 cm above the water surface in the middle of the test vessel) induces mating of fish. Within two hours after spawning, eggs were transferred from the spawning-tray onto a sieve, rinsed with clean water in order to remove faeces and remains of food, put into glass dishes, and counted as total number and number of fertilised eggs.

Fertility was determined by observing cleavage stages using a binocular according to Nagel et al. (1986).

C-3.3.2 Photography and image analysis

For fry counts and length measurements, photographs were made using a digital camera (Canon Snap Shot). Digital image processing was performed by the ImageTool software package (UTHSCSA Version 2.0, Alpha 2; University of Texas Health Science Center at San Antonio, 1997).

Fish larvae were photographed by putting a vessel beneath a fry chamber to maintain the water level, dislocate the fry chamber with the vessel to the photo device (light plate with additional illumination from above), and re-introducing the fry chamber in the aquarium after being photographed. Juvenile and adult fish were netted and placed in glass vessels with a low water level in the photo device.

C-3.3.3 Blood collection and preparation

At termination of P- and F₁-generation, a blood sample was taken from each fish. The samples were taken by cardiac puncture using syringes with a fixed thin needle. To avoid coagulation of blood and degradation of proteins, the syringes were prefilled with Phosphate-buffered saline (PBS) containing heparin (1000 units/mL) and the protease inhibitor aprotinin (2 TIU/mL). As ingredients for the buffer, heparin as ammonium-salt (Sigma-Aldrich, Munich, Germany) and lyophilised aprotinin (Roth, Karlsruhe, Germany) were used.

Before sampling, fish were anaesthetised within a water bath containing chloro-butanol (20 g/L). Blood samples with volume of 10 to 30 µL were taken. Plasma was separated from the blood by centrifugation (30 min; 5000 rpm; 4°C) and immediately stored at -80°C until further analysis.

C-3.3.4 Measurement of Biomarkers using ELISA

Vitellogenin measurement

The measurement of vitellogenin levels was performed using commercially available enzyme-linked immunoabsorbant assays (ELISA) raised to zebra fish (*Danio rerio*) VTG (homologous ELISA kit) purchased from Biosense, Bergen, Norway.

The VTG-analysis used is based on a sandwich assay utilizing specific binding between antibodies and VTG. The wells of micro-titer plates are coated with a specific capture antibody that binds to VTG in samples added to the wells. Unbound components are washed out, and a different VTG-specific antibody (detecting antibody) is added. Unbound detecting antibody is washed out, and an enzyme-labelled secondary antibody is added. After a last wash, the enzyme activity is determined by adding a substrate being metabolised to a coloured product. The enzyme activity (colour intensity) measured by a microplate reader is directly proportional to the concentration of VTG in the sample. The assay is calibrated using purified VTG from zebrafish as standard, which is provided by the producer.

For measurement of VTG levels in plasma it was necessary to dilute the samples to reach the linear part of the respective calibration curve. Each sample was diluted in two steps depending on the sex of the sampled fish. Each dilution step was measured in duplicate, resulting in 4 measurements per fish. Female plasma samples (control fish) were diluted by factors of 100000 and 200000, male samples (control fish) by factor of 150 and 300. The inter-assay variation of the VTG-ELISA was estimated to be between 14 and 18% (Nilsen, 2004). The intra-assay variation was determined to be between 3 and 10%. The intra-assay variation of the measurements performed in this study was based on a comparison of all standard curves and determined to be 9 and 19% and thus was slightly above the reference values.

Whole protein measurement

In order to minimize variability generated by the blood sampling methods (e.g. by taking up tissue liquid), the measured vitellogenin concentrations were normalised against the blood plasma protein content, expressed as ng VTG/ μ g protein. Total protein was quantified by using the BCA Protein Assay Reagent Kit (Pierce, Rockford). The method of the BCA Protein Assay combines the reduction of Cu^{2+} to Cu^{+} , and allows a selective colorimetric detection of the cuprous cation (Cu^{+}) using a reagent containing bicinchoninic acid. The coloured reaction is formed by the chelation of two molecules of BCA with one cuprous ion. This complex shows a strong absorbance at 562 nm, that is nearly linear to increasing protein concentration.

11-keto testosterone measurement

The measurement of 11-keto testosterone (11-kT) levels was made using a commercially available enzyme-linked immunoabsorbant assay (ELISA) raised to fish 11-keto testosterone purchased from Cayman Chemicals (Ann Arbor, Michigan, USA).

The assay is based on the competition between 11-kT and an 11-kT-acetylcholinesterase (AChE) conjugate (11-KT tracer) for a limited number of 11-kT specific rabbit antiserum binding sites. Because the concentration of the 11-kT tracer is held constant while the concentration of 11-kT varies, the amount of 11-kT is inversely proportional to the concentration of 11-kT in the well. This rabbit antiserum-11-kT complex binds to a mouse monoclonal antirabbit IgG antibody that has been previously attached to the well. After a washing step, Ellmann's reagent, which contains the substrate to AChE, is added to the well. The product of this enzymatic reaction shows a strong absorbance at 412 nm. The intensity of this colour, determined by spectrophotometry, is proportional to the amount of 11-kT tracer bound to the wells, and inversely proportional to the amount of free 11-kT present in the well during the incubation.

Only samples of male fish were measured. To meet the linear part of the respective calibration curve it was necessary to dilute the plasma samples. Each sample was diluted in two steps, 100 and 200fold. Each dilution step was measured in duplicate, thus, every sample was measured 4 times. The inter-assay variation was estimated to be 4-12 %, the intra-assay variation was determined to be 10-19 %. The intra-assay variation of the measurements performed in this study, based on a comparison of all standard curves, was determined to be between 4 and 8 % and thus was in line with the given reference values.

C-3.3.5 Histopathology

The analysis of histological alterations was carried out according to the protocols by Wolf (2003) and Knörr (2005). In case of abnormal observations in zebrafish gonads, the abbreviation system developed within the OECD standardisation procedure for histological analyses of gonadal aberrations was used (OECD, 2009).

Table C- 2. Categories of maturation of zebrafish (*Danio rerio*) gonads

	Females	Males
Stage (Stg) 0 entirely immature	Oogonia (OOA) up to perinucleolar oocytes (PND)	Spermatogonia (SPA), spermatocytes (SPC) and spermatids (SPT)
Stage (Stg) 1 early vitellogenic/ spermatogenic phase	Main part of the germ cells are pre-vitellogenic follicles; mostly perinucleolar oocytes (PND) and alveolar oocytes (CAO)	immature phase is dominating; spermatozoa are present
Stage (Stg) 2 middle vitellogenic/ spermatogenic phase	At least half of the follicles are early (EVO) to late vitellogenic (LVO)	Spermatocytes (SPC), spermatids (SPT) and spermatozoa (SPZ) are present in roughly equal portion
Stage (Stg) 3 late vitellogenic/ spermatogenic phase	The majority of follicles in the ovary are late vitellogenic (LVO) and mature/spawning follicles (MSO).	All maturation grades are present, though the spermatozoa are dominating
Stage (Stg) 4 late vitellogenic/ hydrated	At stage 4, the majority of follicles are late vitellogenic and mature/spawning follicles. Follicles are larger as compared to Stg 3.	-

C-3.3.6 Statistical Data Analysis

The evaluation of the effect concentrations was based on nominal test item concentrations. All biological response data were statistically analysed and reported separately for both males and females, except for endpoints of juvenile fish. Each data set was evaluated for normal distribution and homogeneity of variances.

If the prerequisites for parametric methods were met – normal distribution and variance homogeneity - effects were compared between treatments versus control group using analysis of variance (ANOVA). A Dunnett's or Williams' test on multiple pair-wise comparisons was performed subsequently.

If the prerequisites for parametric methods were not met - non-normal distribution or heterogeneous variance - consideration was given to transform the data to achieve homogeneous variances prior to performing the ANOVA. In a few cases, where variance homogeneity could not be achieved, a Welch t-test with Bonferroni adjustment was performed. The significance level was chosen to be $\alpha = 0.05$. All statistical analyses were conducted using a PC based computer program (ToxRat[®] Professional (Aldorf, Germany)).

C-4. Results

C-4.1 Chemical analysis

Water samples of stock solutions and test vessels were taken once per week and measured for flutamide content using HPLC.

After 12 days of exposure, a decline of the test concentrations could be observed (for details see Table C-3). Several measures were taken to prevent the decline of the flutamide concentrations, including increase of the flow-through rate, UV irradiation of the primary stock solution, as well as weekly exchange of the pumping tubes. Due to the increased substance demand, it was decided to reduce the number of replicates from four to two.

The results of the chemical analysis were reported for each of the different life stages. The mean recovery rates are summarised in Table C-3.

Table C- 3. Summary of results of chemical analysis during a two generation test with flutamide

		Nominal concentration of flutamide [$\mu\text{g/L}$]				
		35.7	82.2	189	435	1000
P-generation	[$\mu\text{g/L}$]	29.0	80.4	159	392	927
day 0-day 11	[%]	81	98	84	90	93
P-generation *)	[$\mu\text{g/L}$]	21.9	36.3	81.4	109	303
day 12 - day 21	[%]	61	44	43	25	30
F₁-generation	[$\mu\text{g/L}$]	44.0	79.2	165	447	892
	[%]	123	96	87	103	89
F₂-generation	[$\mu\text{g/L}$]	41.8	68.0	165	394	928
	[%]	117	83	87	91	93

*) note: after 12 days of exposure, a decline of test substance concentrations could be observed. The mean recoveries up to day 12 are shown.

Table C- 4. Detailed results of chemical analysis during a two generation test with flutamide

P-generation, day 0 – day 11					
Nominal test concentration		Mean measured test concentration		sd	
[µg/L]	vessel no.	[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
	C	< LOQ	-	-	-
	D	< LOQ	-	-	-
35.7	A	36.9	103	7.26	20
	B	36.4	102	3.58	10
	C	23.2	65	12.9	36
	D	19.4	54	18.8	53
	mean	29.0	81	-	-
	sd	9.02	25	-	-
82.2	A	83.8	102	1.05	1.3
	B	81.7	99	0.75	0.9
	C	80.0	97	6.52	7.9
	D	76.1	93	10.2	12
	mean	80.4	98	-	-
	sd	3.27	4.0	-	-
189	A	158	84	9.37	5.0
	B	135	71	45.0	24
	C	179	94	12.9	6.8
	D	164	87	25.5	14
	mean	159	84	-	-
	sd	18.1	9.6	-	-
435	A	394	91	23.5	5.4
	B	399	92	25.1	5.8
	C	399	92	27.8	6.4
	D	374	86	36.9	8.5
	mean	392	90	-	-
	sd	11.8	2.7	-	-
1000	A	974	97	78.3	7.8
	B	982	98	70.3	7.0
	C	907	91	52.7	5.3
	D	845	84	120	12
	mean	927	93	-	-
	sd	64.2	6.4	-	-

sd: standard deviation

Table C-4 (continued). Results of chemical analysis during a two generation test with flutamide

P-generation, day 12 – day 20					
Nominal test concentration		Mean measured test concentration		sd	
		[µg/L]	[%]	[µg/L]	[%]
[µg/L]	vessel no.	[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
	C	< LOQ	-	-	-
	D	< LOQ	-	-	-
35.7	A	27.5	77	12.0	34
	B	26.0	73	10.7	30
	C	16.4	46	11.8	33
	D	18.0	50	12.7	35
	mean	21.9	61	-	-
	sd	5.60	16	-	-
82.2	A	38.8	47	7.42	9.0
	B	38.2	46	7.64	9.3
	C	34.3	42	23.8	29
	D	33.8	41	23.3	28
	mean	36.3	44	-	-
	sd	2.59	3.1	-	-
189	A	89.1	47	2.90	1.5
	B	77.8	41	2.40	1.3
	C	79.6	42	49.7	26
	D	79.1	42	49.4	26
	mean	81.4	43	-	-
	sd	5.18	2.7	-	-
435	A	190	44	54.5	13
	B	169	39	78.3	18
	C	40.6	9.3	16.6	3.8
	D	37.5	8.6	16.5	3.8
	mean	109	25	-	-
	sd	81.6	19	-	-
1000	A	309	31	64.8	6.5
	B	411	41	112	11
	C	291	29	230	23
	D	200	20	115	11
	mean	303	30	-	-
	sd	86.4	8.6	-	-

sd: standard deviation

Table C-4 (continued). Results of chemical analysis during a two generation test with flutamide

F₁-generation					
Nominal test concentration		Mean measured test concentration		sd	
[µg/L]	vessel no.	[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
35.7	A	44.4	124	13.8	39
	B	43.7	122	13.6	38
	mean	44.0	123	-	-
	sd	0.54	1.5	-	-
82.2	A	79.1	96	8.06	9.8
	B	79.3	96	7.67	9.3
	mean	79.2	96	-	-
	sd	0.14	0.2	-	-
189	A	163	86	30.6	16
	B	166	88	33.6	18
	mean	165	87	-	-
	sd	2.77	1.5	-	-
435	A	462	106	80.9	19
	B	432	99	81.5	19
	mean	447	103	-	-
	sd	20.6	4.7	-	-
1000	A	962	96	149	15
	B	822	82	147	15
	mean	892	89	-	-
	sd	98.8	9.9	-	-

sd: standard deviation

Table C-4 (continued). Results of chemical analysis during a two generation test with flutamide

F₂-generation					
Nominal test concentration		Mean measured test concentration		sd	
[µg/L]	vessel no.	[µg/L]	[%]	[µg/L]	[%]
Control	A	< LOQ	-	-	-
	B	< LOQ	-	-	-
35.7	A	42.2	118	3.07	8.6
	B	41.4	116	3.04	8.5
	mean	41.8	117	-	-
	sd	0.57	1.6	-	-
82.2	A	68.5	83	13.3	16
	B	67.5	82	13.9	17
	mean	68.0	83	-	-
	sd	0.69	0.8	-	-
189	A	150	80	16.5	8.7
	B	180	95	26.0	14
	mean	165	87	-	-
	sd	21.1	11	-	-
435	A	405	93	35.8	8.2
	B	384	88	48.2	11
	mean	394	91	-	-
	sd	14.7	3.4	-	-
1000		1029	103	164	16
		827	83	227	23
	mean	928	93	-	-
	sd	143	14	-	-

sd: standard deviation

The overall mean recoveries at the end of the test were measured to be between 89 and 101 % of nominal. Thus, the effect concentrations were based on nominal concentrations of flutamide. As reported, an overall decline of the test concentrations was observed after two weeks of exposure. After completion of the parental reproduction phase, action was taken to ensure stable concentrations in the test vessels for the remaining study. This included the adjustment of an increased exchanging rate of test media in the vessels, and weekly exchange and cleaning of the pumping tubes. The following analytical measurements revealed stable concentrations in the test vessels. The exposure phase with decreased concentrations was limited, and thus, excluded from the overall evaluation. However, due to the increased effort regarding time and costs, the replicate number had to be reduced.

C-4.2 P-generation

Prior to study initiation, 8 adult males and 8 adult females were introduced in each test vessel. At test start, each treatment consisted of 4 replicate groups.

C-4.2.1 P-generation, Observations

During the reproductive phase of the P-generation, no concentration related mortality could be observed in any test vessel. In all treatments and at each observation date, the number of actively spawning males was determined and compared to control fish. Observation of the spawning behavior at every two to three days revealed a reduced activity of the male fish at the highest test concentration (1000 µg flutamide/L). An increased number of males, showing absence of the spawning procedure of the remaining fish, were observed. The chasing of females as well as the competition with other males was missing.

After two weeks of exposure, a decline of the test concentrations could be observed in all test vessels. Corresponding to the test concentration decline, the reduced mating behaviour disappeared and was found to be similar to the control fish.

C-4.2.2 P-generation, Reproduction

To record the parameters fecundity and fertility, eggs were collected from spawning trays on each working day, counted and inspected for early blastula stages. For the final evaluation of

the reproductive phase, the egg number per female and day (fecundity), the cumulative number of fertilised eggs, and the fertilisation rate (fertility) was calculated.

Fecundity

The egg number per female and day for about 20 days of counting was significantly affected at 435 and 1000 μg flutamide/L.

After 12 days of exposure – corresponding to 12 days of egg counting – a decline of test concentrations was detected at all test levels. Up to this date, the egg number per day and female was found to be significantly reduced at 435 and 1000 μg flutamide/L. In the time frame from day 12 to 20, egg numbers of the highest and second highest test concentrations did not differ from the control level (Figure C-2, C-3).

The effect on fecundity was interpreted to be the result of a reduced mating behaviour of the male fish. The observed influence on behaviour was found to be reversible corresponding to the decline of the test substance concentrations.

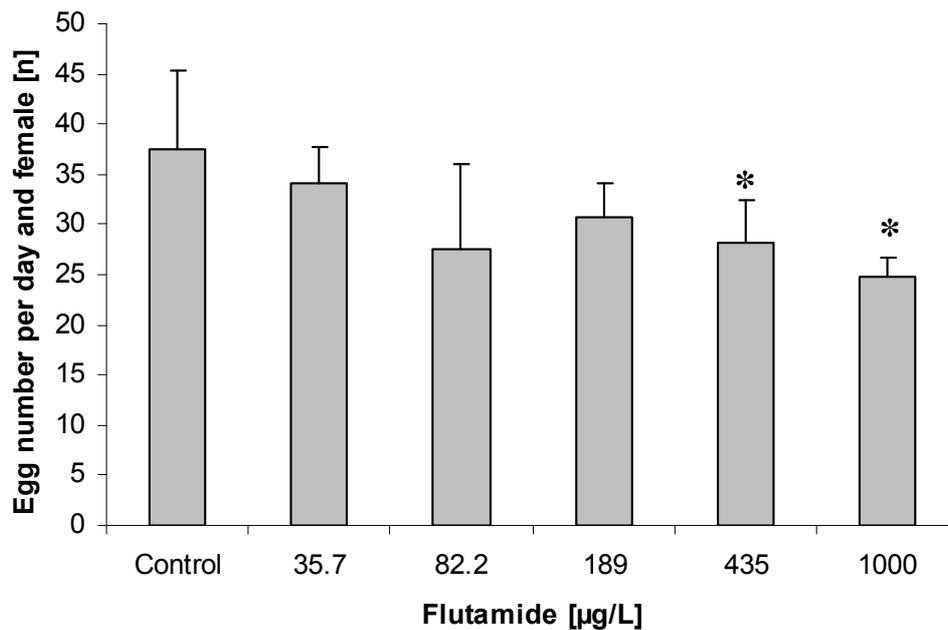


Fig. C- 2. P-generation: egg number per day and female [n]; egg number based on 20 counting days (day 0- 20 of exposure). Means of four replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

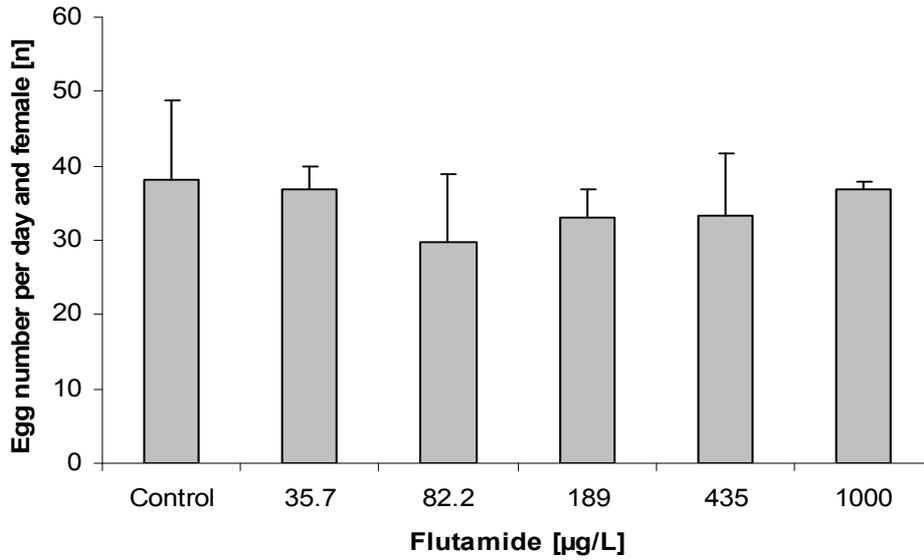


Fig. C- 3. P-generation: egg numbers per day and female [n]; egg number based on 9 counting days (day 12-20 of exposure). Means of four replicates and standard deviations.

Regarding the cumulative number of fertilised eggs, a significant reduction could be observed at 189 µg flutamide/L and above (Figure C-4).

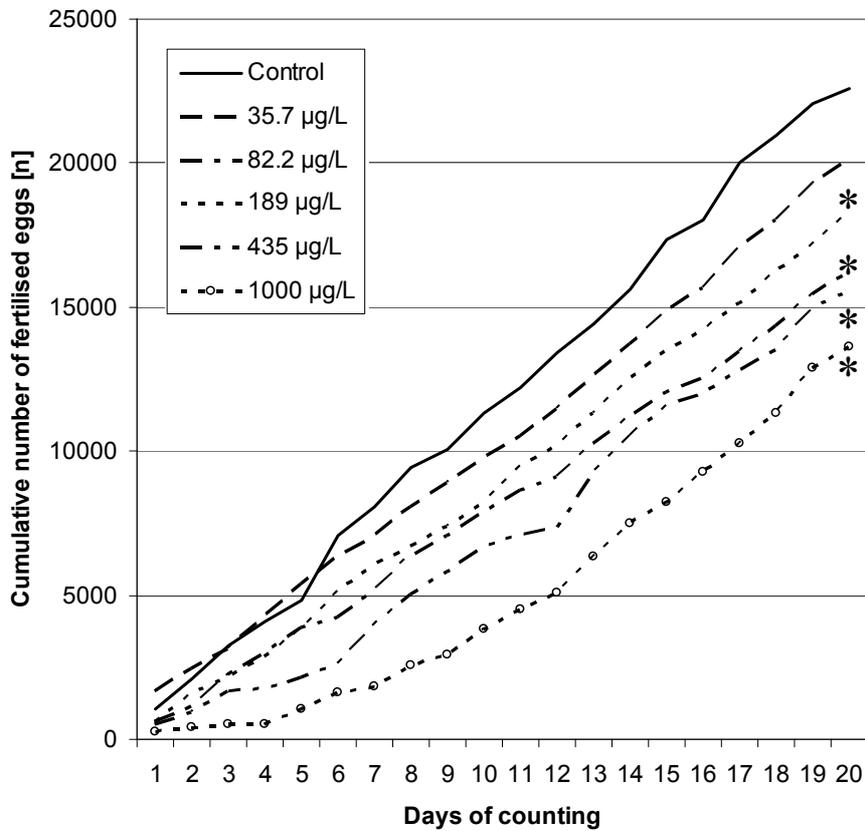


Fig. C- 4. P-generation: cumulative number of fertilised eggs [n]. *) Significant ($p < 0.05$) difference compared to control.

Fertility

Fertilisation rate was slightly, but significantly reduced at the highest test concentration 1000 µg flutamide/L (Figure C-5). However, the mean fertilisation rate was still above 80 %.

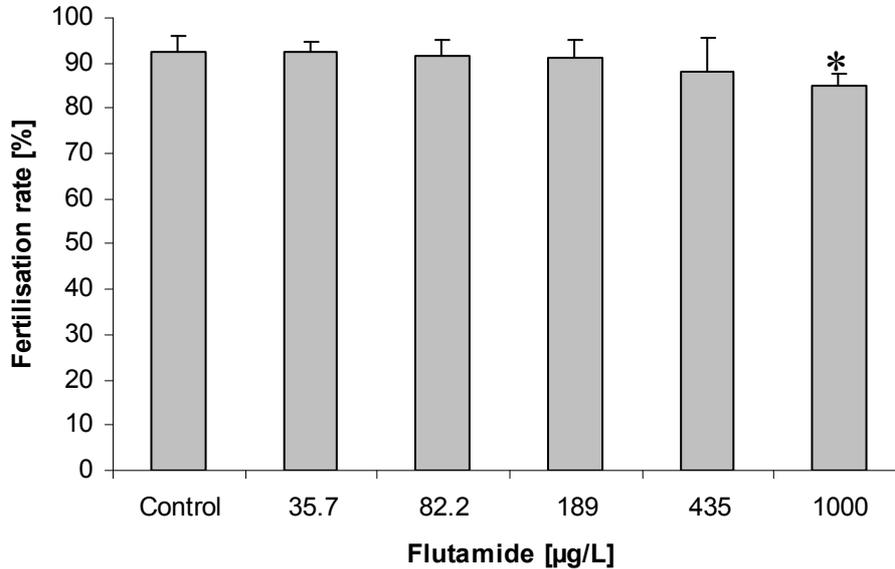


Fig. C- 5. P-generation: fertilisation rate [%]. Means of four replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

C-4.2.3 P-generation, Biomarker

Blood samples were taken from the adult fish at termination of the P-generation. The blood plasma was analysed for the content of the egg yolk precursor protein vitellogenin. Additionally, the sex steroid 11-keto testosterone was measured in blood plasma of the male fish.

Vitellogenin

For the determination of the vitellogenin concentration, blood plasma samples of 5 males and 5 females per treatment were analysed. The VTG concentrations were related to the total protein content for normalisation. The measurement revealed no effect on the VTG levels for both males and females.

11-keto testosterone

The sex steroid 11-keto testosterone was measured in bloodplasma of five males of each replicate. For the P-generation, no significant effect on the bloodplasma concentration could be observed.

Histopathology

Females

All analysed female ovaries from the control group were in maturation stage 3 and 4. For a few animals, slight fibrosis, atresia and an increased number of postovulatory follicles could be detected and proteinaceous fluid could be observed.

Starting with the lowest test concentration (35.7 µg flutamide /L), an increase of egg debris in the oviduct could be observed with increasing severity grade through all higher test concentrations.

At the highest test concentration (1000 µg flutamide/L), all analysed females showed atretic follicles. Regarding the maturation, at least 20 % of the analysed fish were in an immature stage (mid-vitellogenic and below).

Males

Within the control group, all analysed males showed no histopathological changes. All fish were shown to be at maturation stage 3. Only one male showed a slightly increased number of Leydig cells. Corresponding to the test concentrations, an increase of animals with lower maturation grade could be detected. Additionally, a concentration related increase of the number of Leydig cells was observed. The most conspicuous effect at the highest test concentration was the elevated occurrence of interstitial cells, which often were organised in clusters. This effect could be seen in nearly all males.

All endpoints and the respective mean values (\pm standard deviation) are summarised in Table C-5.

Table C- 5: P-generation: summary of all endpoints

	Nominal concentrations flutamide [µg/L]					
	Control	35.7	82.2	189	435	1000
Egg number per female and day [n]	37 ± 7.8	34 ± 3.6	28 ± 7.6	31 ± 2.8	28 ± 4.0 *)	25 ± 2.2 *)
Cumulative number of fertilised eggs [n]	6206 ± 1341	5507 ± 758	4474 ± 1205 *)	5011 ± 525*)	4279 ± 413*)	3751 ± 396*)
Fertilisation rate [%]	93 ± 2.9	91 ± 3.5	92 ± 3.0	91 ± 2.4	85 ± 5.3	85 ± 3.3 *)
Vitellogenin females [ng/µg]	893 ± 154	759 ± 355	780 ± 19	1116 ± 51	717 ± 30	610 ± 123
Vitellogenin males [ng/µg]	0.15 ± 0.08	0.28 ± 0.15	0.08 ± 0.01	0.11 ± 0.05	0.39 ± 0.41	0.22 ± 0.16
11-keto testosterone [pg/mL]	354 ± 67	233 ± 72	168 ± 58	408 ± 28	342 ± 222	365 ± 58

Means of replicate chambers (n=4) ± standard deviation

*) Statistically significant (p<0.05)

C-4.3 F₁-generation

The F₁-generation was initiated by introduction of 100 fertilised eggs from adult fish of the P-generation in fry chambers which were placed at the surface of each test vessel. In the following, hatch, survival and growth of the fish larvae were recorded.

Due to the measured decline of test item concentrations, the replicate number was reduced from four to two vessels.

C-4.3.1 F₁-generation, Observations

The fish larvae of the F₁-generation showed high mortality rates between day 14 and 21 day post fertilisation (pf). Increased mortality was observed in all treatment groups and in the controls and thus was independent from the test substance concentration. For a few replicates, it was not possible to prepare groups of 50 fish at day 35 as it was scheduled. The high mortality rate was considered to be a result of the high flow-through rate of test media leading to an increased stress for the fish larvae. Mortality was highest between week 2 and 3 pf, which includes the process of food adaptation. Increased mortality was not observed for the following juvenile and adult stage.

In one replicate of the second highest test concentration (435 µg flutamide/L), no mating behaviour was observed until test termination. No eggs were spawned.

The macroscopic inspection of the gonads revealed only one male fish in this group.

C-4.3.2 F₁-generation, Hatch/Survival

Hatch

The hatching rate, being the number of hatched larvae in relation to the number of introduced eggs, was determined to be above 80% in all treatment groups and in the control.

Survival

Mortality of fish larvae was found to be increased in all treatment groups. After 35 days, the survival rate of all fish groups, including controls, was below 70%. However, no concentration related effect could be observed.

Growth

Length of the fish larvae after 28 days was found to be slightly, but significantly increased at the two highest test levels.

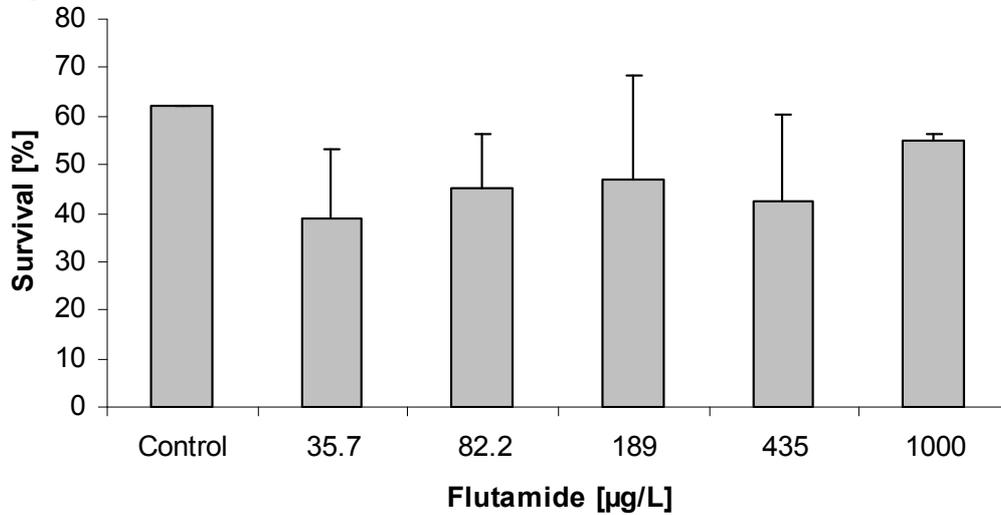


Fig. C- 6. F₁-generation: survival of fish larvae at day 35 pf [%]. Means of two replicates + standard deviation.

C-4.3.3 F₁-generation, Juvenile Growth

Length of the juvenile F₁ fish was measured at day 35 and day 70. The pseudo specific growth rate was calculated based on the length measurements according to the OECD guideline 215 (OECD, 2000). The pseudo specific growth rate was significantly reduced at 435 and 1000 μg flutamide/L (Figure C-7).

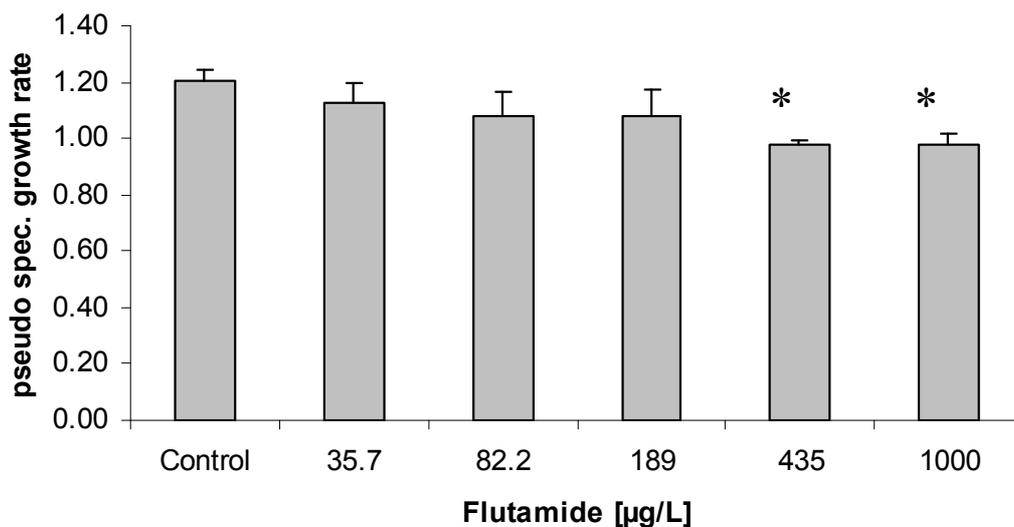


Fig. C- 7. F₁-generation: pseudo specific growth rate of the juvenile fish based on the measurement of lengths on day 35 and day 70. Means of two replicates + standard deviation. *) Significant ($p < 0.05$) difference compared to control

C-4.3.4 F_1 -generation, Reproduction

The reproductive phase was initiated by introduction of the spawning trays into the test vessels. The reproduction was recorded for a period of 69 days. The endpoints time to first spawning, egg number per female and day, cumulative number of fertilised eggs, and fertilisation rate were determined.

In one replicate of the second highest treatment level, no spawning could be observed throughout the total recording period. Thus, this group was excluded from the effect data evaluation.

Time to first spawning

The time to first spawning was not affected. The fish groups of the high treatment levels were found to be the first group in spawning, but no statistically significant difference compared to the control could be detected.

Fecundity

Regarding total egg number per day and female, no significant change could be detected (Figure C-8). The evaluation of the cumulative number of fertilised eggs revealed a decrease at the highest treatment, statistical significance could not be shown.

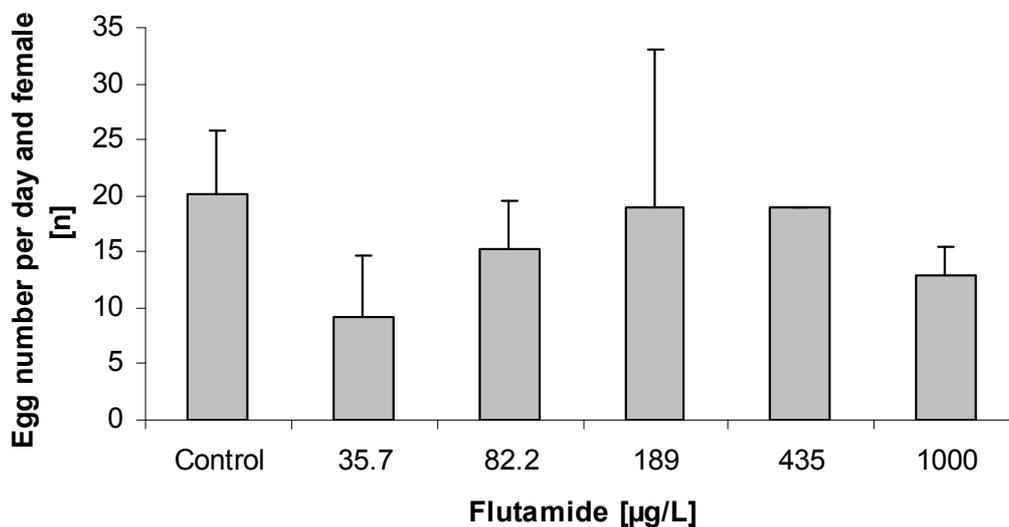


Fig. C- 8. F_1 -generation: egg numbers per day and female [n]. Means of two replicates and standard deviations. In test concentration 4 (435 $\mu\text{g/L}$), spawning was observed only in one replicate.

Cumulative number of fertilised eggs

The evaluation of the cumulative number of fertilised eggs revealed a decrease at the highest treatment; however, statistical significance could not be shown. Due to the early start of spawning, both replicates of the third treatment group (189 μg flutamide/L) showed the highest numbers of fertilised eggs over the recording period.

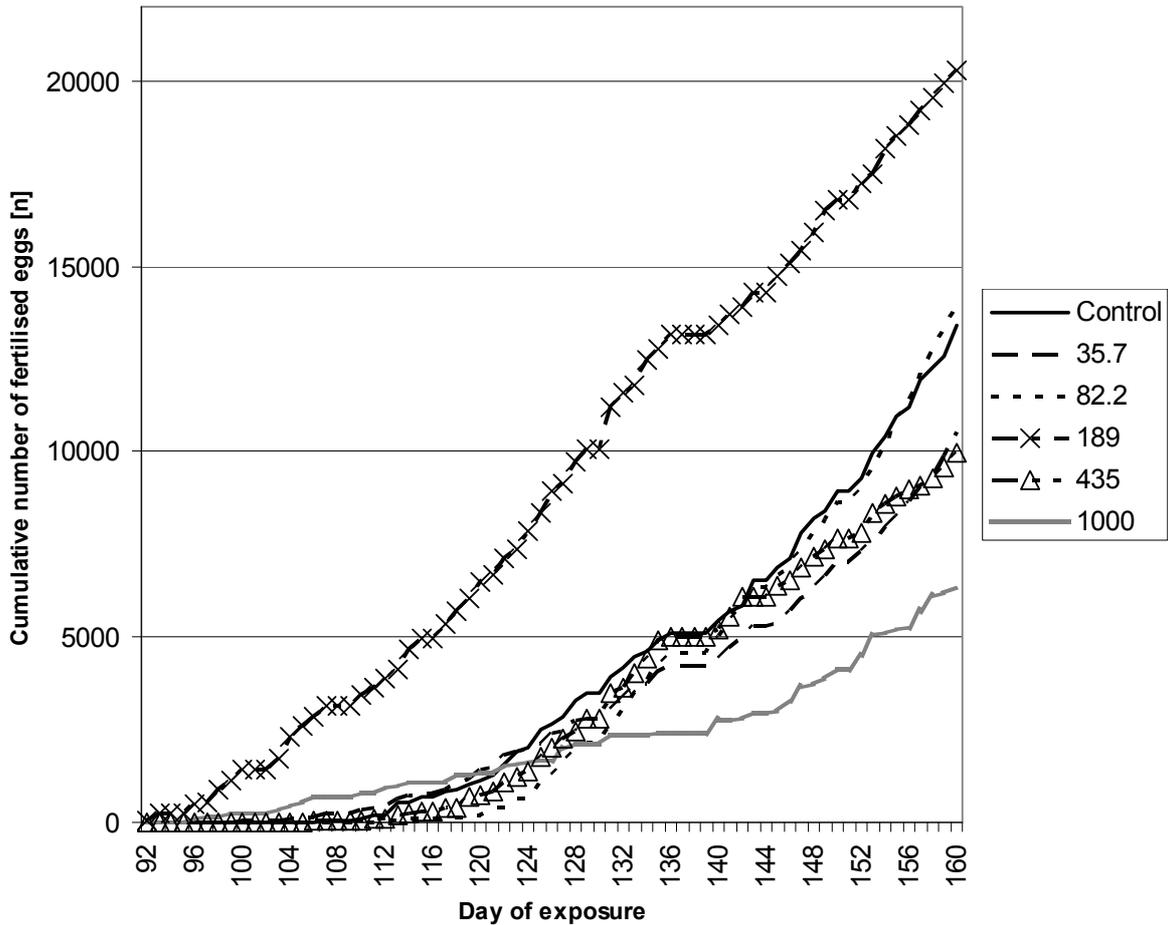


Fig. C- 9. F₁-generation: cumulative number of fertilised eggs [n].

Fertility

The fertilisation rate was found to be significantly reduced at the highest treatment level (Figure C-10).

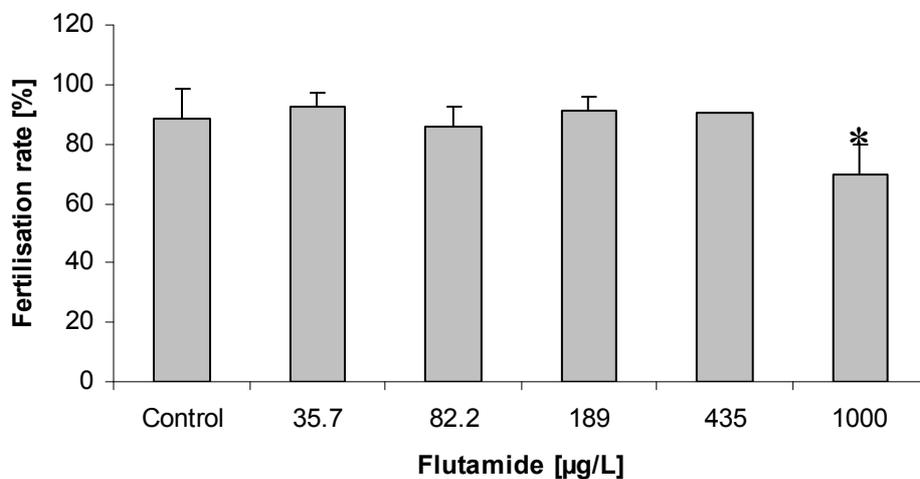


Fig. C- 10. F₁-generation: fertilisation rate [%]. Means of two replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

C-4.3.5 F₁-generation, Biomarker

Blood samples were taken from the adult fish at the end of the exposure period of the F₁-generation. The blood plasma of 5 females and 5 males per test vessel was analysed for the content of the egg yolk precursor protein vitellogenin. Additionally, the sex steroid 11-keto testosterone was measured in blood plasma of 5 male fish per tank.

Vitellogenin

Vitellogenin concentrations were analysed in blood plasma samples of male and female fish, if enough animals were available for sampling. The VTG concentrations were related to the total protein content to reduce variation as a result of the sampling procedure. The measurements revealed a significant decrease of VTG in female blood plasma at 1000 µg flutamide/L (Fig. C-11). The male fish were not affected.

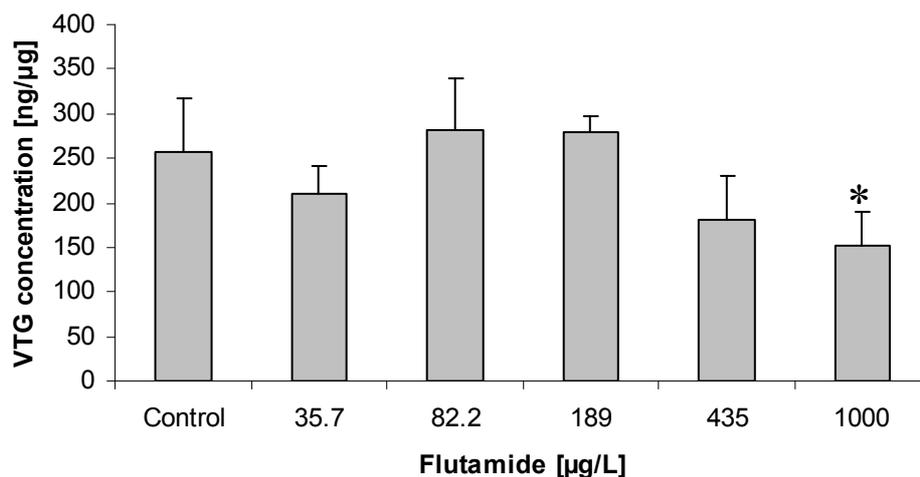


Fig. C- 11. F_1 -generation: vitellogenin concentration in female blood plasma [$\text{ng}/\mu\text{g}$]. Means of two replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

11-keto testosterone

The analysis of 11-keto testosterone in male bloodplasma revealed an increased level of the steroid at the highest test concentration (Figure C-12).

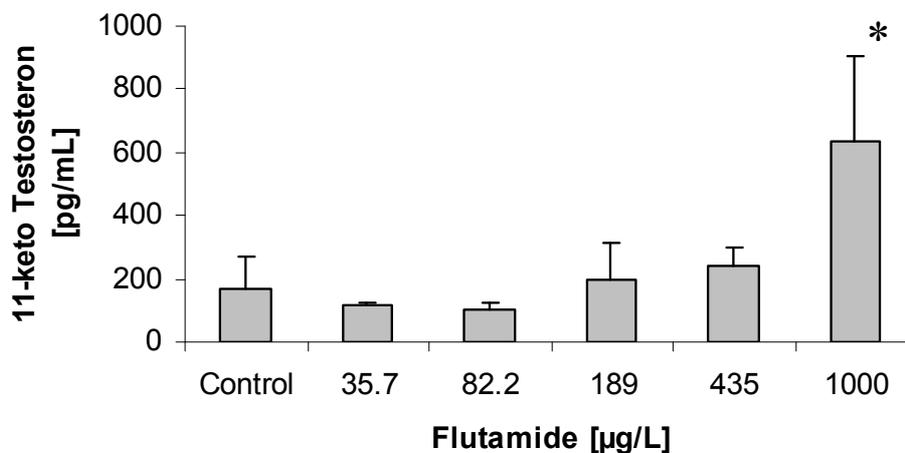


Fig. C- 12. F_1 -generation: concentration of 11-keto testosterone in male blood plasma [pg/mL]. Means of two replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

C-4.3.6 F_1 -generation, Sex ratio

The sex determination of the fish was performed by inspection of the fish gonads at termination of the F_1 -generation. The macroscopic determination was additionally confirmed by gonad inspection during the histopathological evaluation.

The sex ratio in the control groups was 42 % females to 58% males, and thus, in the expected range (40/60% to 60/40%) as observed for zebrafish in former studies. Regarding the other treatment groups, no clear tendency could be observed. In one replicate of the second highest

test concentration (435 µg flutamide/L), a high number of females with only one male fish was detected.

For the high test concentration, an increasing number of only slightly developed gonads could be observed, a sex determination was not possible. Details can be found in the chapter 4.3.7 Histopathology.

C-4.3.7 F₁-generation, Histopathology

The overall changes in zebrafish of the F₁-generation exposed to flutamide can be summarised as follows:

Females

As it was observed for female fish in the controls of the P-generation, a few animals showed effects like interstitial proteinaceous fluid, fibrosis, atresia of mature and immature follicles.

Corresponding to the increase of the test concentrations, the rate of severe egg debris increased. Furthermore, there was a quite high number of immature females at the two highest concentrations.

Males

In the controls, the maturation grade of the testis was quite homogenous. Nearly two third of the analysed fish were in the late spermatogenic phase, one third were mid-spermatogenic. For the high concentrations of flutamide, a reduction of the male number could be observed. All remaining males of these groups showed gonads of the late spermatogenic stage. Except for a slight increase in the number of Leydig cells, no further effect could be observed.

All endpoints regarding the F₁-generation including the respective mean values are summarised in Table C-5.

Table C- 6. F₁-generation, Early Life Stage, Juvenile growth and Reproduction, Summary of all endpoints

	Nominal concentration of flutamide					
	[µg/L]					
	Control	35.7	82.2	189	435	1000
Survival, day 28pf [%]	64 ± 2.8	42 ± 12	46 ± 11	49 ± 23	45 ± 18	57 ± 0.7
Length, day 28pf [cm]	0.67 ± 0.04	0.77 ± 0.05	0.76 ± 0.06	0.77 ± 0.08	0.85 ± 0.02)	0.83 ± 0.02)
Pseudo specific growth rate (based on length)	1.20 ± 0.04	1.12 ± 0.07	1.08 ± 0.09	1.08 ± 0.09	0.98 ± 0.02)	0.98 ± 0.04)
Time to first spawning [d]	119 ± 21	101 ± 4	104 ± 9	93 ± 1	106	95 ± 4
Egg number per female and day [n]	20 ± 6	9 ± 5	15 ± 4	19 ± 14	19	13 ± 3
Cumulative number of fertilised eggs [n]	6698 ± 4101	5266 ± 567	6971 ± 1737	10148 ± 493	9951	3161 ± 1199
Fertilisation rate [%]	89 ± 10	93 ± 4.7	86 ± 6.5	91 ± 4.5	90	70 ± 10 *)

Means of replicate chambers (n=2) ± standard deviation, *) Significant (p < 0.05)

Table C- 7. F₁-generation, Sex ratio and Biomarker, Summary of all endpoints

	Nominal concentrations flutamide [µg/L]					
	Control	35.7	82.2	189	435	1000
Sex ratio, number of females [%]	41 ± 8.6	56 ± 14	56 ± 13	45 ± 31	65 ± 24	35 ± 7.1
Vitellogenin females [ng/µg]	256 ± 62.2	210 ± 30.9	281 ± 58.5	280 ± 16.6	182 ± 47.5	151 ± 38.1 *)
Vitellogenin males [ng/µg]	0.09 ± 0.03	0.16 ± 0.14	0.21 ± 0.04	0.18 ± 0.02	0.11 ± 0.03	0.11 ± 0.03
11-keto testosterone [pg/mL]	171 ± 99.2	115 ± 8.8	101 ± 23.3	201 ± 113	239 ± 63.5	638 ± 270 *)

Means of replicate chambers (n=2) ± standard deviation

*) Significant (p < 0.05)

C-4.4 F₂-generation

The second filial generation was prepared by introduction of 100 fertilised eggs spawned by the F₁ fish. The eggs were transferred to fry chambers which were placed in each test vessel. In one treatment replicate (replicate 1, 435 µg flutamide/L), no eggs were found up to the end of the reproductive phase.

For this group, no F₂-generation could be prepared.

C-4.4.1 F₂-generation, Hatch/Survival/Growth

Hatch was above 80% in the controls. No effect on hatching success due to flutamide exposure could be observed at any test concentration. As observed for the larvae of the F₁-generation, the mortality of the fish larvae was quite high. The survival at day 35pf was below 70% in the controls. However, no concentration related effect on the survival rate could be observed.

After day 35pf, the F₂-generation was terminated, and the fish larvae were measured for individual length and group wet weights. Regarding group weight, a significant decrease could be observed at the highest test concentration (1000 µg flutamide/L) (Figure C-7).

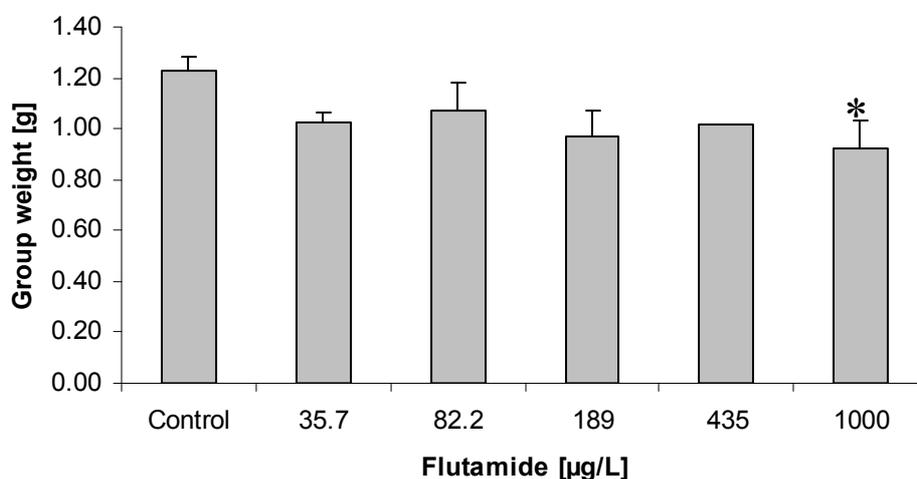


Fig. C- 13. F₂-generation: group weights of fish larvae at day 35 pf. Means of two replicates and standard deviations. *) Significant ($p < 0.05$) difference compared to control.

Table C- 8. F₂-generation, Early Life Stage, Summary of all endpoints

	Nominal concentrations flutamide [$\mu\text{g/L}$]					
	Control	35.7	82.2	189	435	1000
Survival day 35pf [%]	66 \pm 0.7	84 \pm 9.2	81 \pm 20	65 \pm 18	48 #)	61 \pm 0.7
Length [cm]	1.11 \pm 0.06	1.10 \pm 0.04	1.09 \pm 0.08	1.16 \pm 0.08	1.26 #)	1.16 \pm 0.08
Group weight [g]	1.23 \pm 0.06	1.03 \pm 0.04	1.07 \pm 0.11	0.97 \pm 0.10	1.02 #)	0.92 \pm 0.11 *)

Means of two replicates \pm sd

#) Since there was no spawning at the F₁-generation, only one replicate could be prepared

*) Significant ($p < 0.05$)

C.5 Summary and Conclusions

A two generation test with the androgen receptor antagonist flutamide was performed to evaluate and assess the effects on all life stages of zebrafish after continuous exposure. This includes the parental generation (P-generation), early life stages (life phase directly post hatch), juvenile growth and reproduction of the Filial 1 (F₁)-generation and finally, the early life stage phase of the Filial 2 (F₂)-generation. Furthermore, indicative endpoints were determined including the measurement of molecular biomarkers and histopathology.

P-generation

The egg numbers per female and day were significantly reduced at 435 and 1000 μg flutamide/L. The decreased fecundity was interpreted to be the most sensitive population relevant endpoint. The low egg numbers were considered to be the effect of a reduced mating behaviour of the male fish. The effect was interpreted to be reversible as it disappeared with decreasing concentrations of flutamide. Further quantitative studies may be needed to confirm these observations. Besides the reduced egg number, a significantly reduced fertilisation rate could be detected at the highest test concentration.

F₁-generation

Regarding fish larvae of the F₁-generation, hatch and survival was not significantly affected. The length was slightly increased at day 28pf. A reduced pseudo specific growth rate, based on length measurements between day 35 and 70, was detected for the juvenile fish in the F₁-

generation at the highest test concentration. An effect on fecundity, as observed for the P-generation, could not be detected. The time to first spawning of eggs was not affected. The fertilization rate was found to be significantly reduced at the highest test level. In one replicate of the second highest test concentration, no eggs were spawned up to study termination.

The determination of the sex ratio of the F₁-generation did not show a clear effect. The high concentration levels showed an increasing number of females, but this effect could not be statistically confirmed. The histopathological analysis revealed an increasing number of female fish with immature gonads at the two highest concentrations.

F₂-generation

Regarding the early life stage phase of the F₂-generation, hatch and survival of the fish larvae was not affected. A significant reduction of group weights after 35 days was found at the highest test concentration.

Biomarker

The measurement of molecular biomarkers revealed no effect on the vitellogenin concentration in blood plasma of both female and male fish of the P-generation. A slight, but significant, decrease of the Vitellogenin levels could be observed in the F₁-generation at the highest test concentration. Regarding 11-keto testosterone, an increase was detected in blood plasma of male fish of the F₁-generation. An increase of the sex steroid was also detected after 21 day exposure of adult fish in a Fish Screening Assay with flutamide (Teigeler, 2007). All recorded endpoints are listed in Table C-8.

Conclusion

The fecundity of the Parental-generation, characterised by the egg number per female and day, was found to be the most sensitive endpoint with population relevance. The reduced egg number was interpreted to be the result of a reduced mating behaviour of the male fish. This effect was assumed to be reversible since it disappeared with decreasing flutamide concentrations.

The reduced fecundity could not be observed for the F₁-generation. The reduced fertility at the highest test concentration (1000 µg flutamide/L) was a longer lasting effect. Correspondingly, an effect on growth of the early life stages of the F₁ and F₂ larvae could be detected at this treatment level.

Both biomarkers, vitellogenin and 11-keto testosterone, showed similar sensitivity.

Table C- 9. Summary of all endpoints, recorded during a two generation test with flutamide

		Endpoint		NOEC	LOEC	
	Life Phase			[µg/L]	[µg/L]	
P-generation	Reproduction	Egg number per day and female		189	435	
		Cumulative number of fertilised eggs		35.7	82.2	
		Fertilisation rate		435	1000	
	Biomarker	Vitellogenin	Females		1000	>1000
			Males		1000	>1000
		11-keto testosterone	Males		1000	>1000
F₁-generation	Early Life Stage	Hatch		1000	>1000	
		Survival, day 28pf		1000	>1000	
		Growth (Length), day 28pf		189	435	
	Juvenile Growth	Pseudo spec. growth rate		189	435	
	Reproduction	Time to first spawning		1000	>1000	
		Egg number per day and female		1000	>1000	
		Cumulative number of fertilised eggs		1000	>1000	
		Fertilisation rate		435	1000	
	Biomarker	Vitellogenin	Females decrease		435	1000
			Males		1000	>1000
		11-keto testosterone	Males increase		435	1000
		Sex ratio		1000	>1000	
F₂-generation	Early Life Stage	Hatch		1000	>1000	
		Survival day 35		1000	>1000	
		Growth	Group weight		435	1000

Test concentration: control, conc1: 35.7 µg/L, conc2: 82.2 µg/L, conc3: 189 µg/L, conc4: 435 µg/L, conc5: 1000 µg/L (nominal concentration).

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Chapter 9

Conclusion

9.1 Investigations on the genotoxic burden of the River Danube in Germany and the Tietê River in Brazil

9.1.1 What is the genotoxical burden of two different aquatic systems: The Danube River in Germany and the Tietê River in Brazil?

Despite the fact that the water quality of the **River Danube** has improved over the last decades, the numbers of fish caught still follow a downward tendency. The assessment of the genotoxical hazard potential of this area was carried out by a combination of the micronucleus test *in vitro* (with RTL-W1 cells exposed to sediment extracts) and *in situ* (with erythrocytes from European barbels caught in the field) as well as the comet assay *in vitro* (with RTL-W1 cells exposed to sediment extracts) and *in vivo* (with zebrafish exposed to sediment samples). The *in vitro* genotoxicity assays identified several hotspots along the Danube, which could be confirmed by the *in vivo* and *in situ* investigations. All three different test systems showed a good correlation, which indicates a good alignability of the results obtained in the lab to the field.

Chemical analyses of the sampled sites showed no conspicuousness concerning the priority polycyclic aromatic hydrocarbons (PAHs); however the ranking of locations with respect to PAHs resembles that with respect to the bioassays. The substances detected by chemical analyses are only capable of partly explaining the results from the bioassays. In fact it seems that the non-priority pollutants are responsible for the genotoxic burden of the investigated sediments. To confirm the good correlation, a second comparative study on a different ecosystem had to be conducted. For this end, the **Tietê River** in São Paulo, Brazil, was chosen. The Tietê River, crossing São Paulo city (largest city in Brazil by population), is one of the most polluted rivers in the world, due to the insufficiency of proper treatment plants and many industrial sources for a multitude of anthropogenic pollutants.

For the investigation of the genotoxic potential of sediments from the Tietê River the *in vitro* comet assay with RTL-W1 cells was applied, whereas the genotoxical damage in the field was determined by the micronucleus assay with fish erythrocytes.

A strong hazard potential was detected for fish and sediments from the Billings reservoir, and results revealed a major impact of discharges of São Paulo city on the toxic load compared to the other locations, suggesting an improvement of the water quality of the river further downstream. Similar conclusions, based on different parameters, had been obtained in previous studies (Almeida and Rocha, 2006; Fracácio *et al.*, 2003; Mozeto *et al.*, 2004).

Along the course of the Tietê River, differences in the genotoxic potentials were confirmed by both tests, with a high correlation between *in vitro* and *in situ* results. The good correlation of these two tests may be – in terms of weight-of-evidence approaches – an indication for the high ecological relevance of the sediment genotoxicity for the situation in the field.

These results obtained in two different ecosystems point to a high comparability of the applied genotoxicity test performed in the laboratory and in the field. However, additional studies need to be conducted to confirm the suggested correlation.

Taking into consideration the results obtained for the **Upper Danube** in the comet assay from Chapter 2 and Chapter 3 it can be concluded that samples from Rottenacker, Schwarzach, Ehingen and Öpfingen are considered as genotoxic, those from Bad Abbach, Ingolstadt, Jochenstein, Riedlingen and Sigmaringen as moderately genotoxic and the samples from the Lauchert as non genotoxic. For the micronucleus test with RTL-W1 cells similar results were gained, with exception of Öpfingen, which only showed moderate genotoxic activities.

Comparing the results gained in the comet assay from the Upper Danube with those from the **Tietê River**, the sediments from the Billings reservoir are quoted as genotoxic, those from Ponte Nova and Promissão as moderately genotoxic and those from Bariri and Três Irmãos as less genotoxic. Barra Bonita and Tietê River spring are considered to be not genotoxic. Regarding the micronucleus assay with fish erythrocytes, all Billings fish showed an increase in micronucleus formation over specimens from all other locations. Comparing the results from the Billings reservoir with the results from Cavas and Ergene-Gözükkara (2005), who obtained similar induction rates of micronuclei after exposure to cyclophosphamide, it can be concluded that Billings is genotoxic. The micronucleus frequencies in specimens from Promissão and Bariri reservoirs showed no elevated micronucleus rate and are thus considered to be not genotoxic in the micronucleus test.

9.1.2 Does the comet assay *in vitro* correlates with the comet assay *in vivo*?

In the comet assay with RTL-W1 cells, acetonetic sediment extracts from the Danube River were tested (see Chapter 2 and 3). 8 out of 10 sediment extracts displayed genotoxic activity in terms of DNA fragmentation (tail moment), revealing clear dose-response relationships.

Sediment extracts from Rottenacker, Öpfingen and Schwarzach differed significantly from the negative control at all concentrations tested. In contrast, for sediment extracts from the Lauchert tributary no reproducible significant genotoxic effect was observed.

The results obtained for the sediment-contact comet assay with zebrafish (*Danio rerio*) embryos (see Chapter 3) revealed a significantly increased genotoxicity in native sediments even at dilutions of up to 32-fold (Rottenacker). Again, sediments from the Lauchert tributary were free of genotoxicity. A correlation analysis confirmed a high correlation between comet assays with *Danio rerio* exposed to native sediments and comet assays with RTL-W1 cells exposed to acetonic sediment extracts (this study; $r_{\text{Pearson}} = 0.75$ with $p < 0.05$; $R^2 = 0.56$).

Thus, in the scope of this thesis, the comet assay *in vitro* correlates with the comet assay *in vivo*.

9.1.3 Are the comet assay and the micronucleus assay comparable as two alternative genotoxicity / mutagenicity tests in order to characterize the potential genotoxic burden of environmental sediment samples?

In the comet assay as well as in the micronucleus test, the highest genotoxicity was induced by sediment extracts from Rottenacker. The genotoxic activity measured in the comet assay for sediment extracts from Ehingen, Schwarzach and Öpfingen could be classified as also genotoxic in the micronucleus test with exception of Öpfingen, which only caused minor genotoxicity. Little to no genotoxic activity in both tests was shown by the tributary Lauchert. The remaining sediment extracts induced low genotoxic effects. A correlation coefficient of 0.97 ($p < 0.001$; $R^2 = 0.81$), was calculated for the tested sediment extracts in the two *in vitro* test systems indicating that genotoxic potential of analyzed sediment extracts were highly correlated.

In our study both tests were useful and necessary to characterize the potential genotoxic burden of the samples taken. However, due to the different mode of actions which are covered by each of the tests, a general comparability regarding the assessment of the genotoxic burden of environmental samples cannot be concluded.

9.1.4 Are the comet assay and the micronucleus assay with RTL-W1 cells exposed to sediment extracts comparable to results gained in the micronucleus assay with fish caught in the field?

Since only four sites along the Danube River were sampled for *in situ* micronucleus tests (see Chapter 2), the correlation analysis between the micronucleus test *in vitro* and *in situ* is restricted to these four sites and the negative controls. Nevertheless, correlation analysis between these two test systems revealed a correlation coefficient of $r_{\text{Spearman}} = 0.90$ (with $p < 0.1$; $R^2 = 0.61$), indicating high correlation. Likewise, the same correlation coefficient was calculated for the analysis between the comet assay and the micronucleus assay with barbel erythrocytes ($r_{\text{Spearman}} = 0.90$ with $p < 0.1$; $R^2 = 0.61$). Similar studies conducted in the Tietê River with the micronucleus test *in situ* and the comet assay *in vitro* with RTL-W1 cells, also confirmed a high correlation between these two test systems.

Our results indicate that the comet assay and the micronucleus assay with RTL-W1 cells exposed to sediment extracts are comparable to results gained in the micronucleus assay with fish caught in the field. However, the good correlation between *in vitro* and *in situ* genotoxicity bioassays found for the Upper Danube and the Tietê River needs to be confirmed in additional studies. Moreover, the comet assay and the micronucleus assay cover different mode of action and thus a special focus should be laid on their similarities and differences.

9.2 Eligibility of the new/edited protocols for the micronucleus assay

9.2.1 What are the most practicable and suitable methods to assess micronuclei in fixed liver tissue?

When fixing the fish *via* perfusion different organs even of very small fish can be isolated and thus scored separately. Perfusion with fixatives of electron microscopy quality was found crucial for perfect fixation of all tissues for optimal identification of micronuclei and nuclear abnormalities. Fixation and subsequent embedding for electron microscopy are mandatory for a definite identification, since otherwise the sections would be too thick to clearly identify cells from one layer. Once the pre-fixed organs are dissected and immersed in the cooled fixative, they can be stored for several days (for a detailed description of the method, see Chapter 5, Section 5.3.2). This opportunity allows *in situ* sampling with subsequent processing of the samples in the laboratory without any loss of quality. The cells remain in

their original constitution and position within the tissue and a good evaluation of micronucleated cells is possible. However, one has to take into consideration, that especially in juvenile or smaller fish, it might be a challenge to identify an adequate number of cells.

9.2.2 What is a suitable protocol for an application of the micronucleus assay with zebrafish embryos?

Based on the protocol of Kosmehl *et al.* (2007), a modified test protocol for the micronucleus assay with *Danio rerio* was developed. In order to investigate the suitability of this *in vivo* micronucleus assay zebrafish embryos were exposed to Nitroquinolin-*N*-oxide (NQO, see Chapter 5).

Other variations of the micronucleus test *in vivo* have been tested successfully as well (i.e. the micronucleus test with fixed embryos; data not shown). **However the micronucleus test with cell suspensions of *Danio rerio* seemed to be the most practicable.** The eligibility of the micronucleus test with zebrafish embryos exposed to sediments still needs further validation. However, the test results from the comet assay with zebrafish embryos (Kosmehl *et al.*, 2007) suggest a high possibility that the micronucleus assay with zebrafish embryos exposed to sediments could provide an additional approach in sediment testing.

9.3 Investigations on the model androgen trenbolone

9.3.1 Is there a genotoxic potential of trenbolone?

Trenbolone, was tested with the micronucleus assay *in vitro* and *in vivo* as well as the comet assay *in vitro* and *in vivo* (see Chapter 6). The present study clearly demonstrates that there is a genotoxic hazard potential of trenbolone in fish or fish-derived cell suspensions at low concentrations, yet not at the high concentrations tested. The first popular example of this ‘low dose theory’ or the inverted U-shape dose response curve was bisphenol A (BPA). In 1997, one research team, led by Fred vom Saal and Wade Welshons found that even very low levels of BPA could cause harmful effects. Male mice exposed *in utero* to low levels of BPA were shown to have increased prostate weights and decreased daily sperm production. Furthermore, these scientists found that BPA could cause greater effects at low dose levels

than at higher doses (Lyons, 2000). As shown by others, genotoxicity of trenbolone varies very strongly with the cell line used (Schiffmann *et al.*, 1988; Schiffmann *et al.*, 1985).

In the present study, the micronucleus assay *in vivo* showed significant differences in micronucleated cells after exposure to trenbolone. Therefore, the micronucleus assay with zebrafish embryos is capable of detecting the mutagenic potential of trenbolone. However, the metabolism of trenbolone being responsible for these observations in fish and the genotoxic mode of action is only poorly understood. Consequently, it seems suggestive to conduct further genotoxicity tests of trenbolone with various cell lines at a lower concentration range to fully assess the hazard potential of trenbolone to humans and the aquatic environment.

9.3.2 Is the genetic damage caused by trenbolone is partly masked as the molecule is linked to a special tissue or organ in zebrafish and does the chorion acts as a barrier in the case of trenbolone?

The antibody staining after exposure to trenbolone of non-hatched and hatched zebrafish embryos revealed that trenbolone could be found anywhere in the body (see Chapter 7). Thus, in the case of trenbolone, the chorion does not act as a barrier. More lipophilic tissues like yolk seem to accumulate trenbolone, although the partition coefficients of 1040 ($\log K_{OW} = 3$) rather indicates a medium lipophilicity (EPA, 1984). **As trenbolone is ubiquitous in the embryo, no masking of genotoxic effects is expected.**

9.3.3 Which effects were observed after exposure of different life stages of zebrafish (*Danio rerio*) to trenbolone?

Endocrine disruptors interfere with the normal function of the body hormones in controlling growth, metabolism, and body functions. **In the case of trenbolone the major effect observed was the masculinisation in the F₁ generation.** From a concentration ≥ 10 ng/L at least 99 % of the fish were masculinised (see Chapter 8, Supplement B). **Furthermore, the numbers of spawned eggs decreased in the parental and the F₁ generation.** However, the graph for the egg numbers of the parental generation showed a non-monotonic progression. Goetz (1983) suggested that a decrease in egg numbers can be related to an inhibition of the

natural process of endogenous androgens, which probably plays a role in final maturation and/or release of eggs in female fish. The survival rate of the embryos in the F₁ generation and their time to hatch revealed a non-monotonic curve progression as well. This phenomenon of U-shaped dose-response curves was described by Gray *et al.* (2001) as well. They postulated that the observed U-shaped dose responses in mammals caused by androgens and anti-androgens are probably underlying separate mechanisms of action at different dosage levels.

Trenbolone induces a 20 % weight gain with up to 15 % gain in feed efficiency in cattle; therefore, induction of growth had to be expected in fish as well. However, the F₁ generation rather showed a significant decrease in body weight at the highest concentration than any increase. **Only the length increased significantly at the second lowest concentration.**

Induction of vitellogenin (VTG) and 11-keto testosterone (11-KT) are used as a biomarker of exposure of fish to endocrine-active substances. Both field and laboratory studies have shown the value of VTG as a rapidly inducible biomarker for estrogens and antiestrogens in both adult and juvenile fish (Jobling *et al.* 1998; Nilsen *et al.* 2004; Panter *et al.* 2002; Sumpter and Jobling 1995; Thorpe *et al.* 2000). Useful features of VTG induction as a biomarker are the specificity for estrogens, the sensitivity, and the magnitude of the response possible (plasma VTG may increase by up to a millionfold, from nanograms per millilitre to milligrams per millilitre concentrations (Tyler *et al.* 1990)). Assays for VTG are available for a wide range of fish species.

Trenbolone did not induce any changes in the VTG concentration in female zebrafish blood. In males a significant change was assessed at 3 ng/L (see Chapter 8, Supplement B). Other studies revealed that there is no change in the VTG level in males between 10 and 5000 ng/L trenbolone (own data, Ankley *et al.* 2003, OECD, Seki *et al.* 2006). Therefore, the VTG induction follows the previously described U-shaped dose response as well.

As a second biomarker 11-KT was chosen. **The level of the 11-KT concentration decreased at the highest concentration in the parental generation and increased in the second highest in the F1 generation.** A decline in 11-KT in male fish after exposure to trenbolone can also be confirmed by previous studies: Ankley and colleagues (2003) measured significant changes in plasma concentrations of 11-KT in male fathead minnow at 50000 ng/L trenbolone. Furthermore, Jensen and coworkers (2006) exposed fathead minnow to the 17 α -isomer of trenbolone (3 - 100 ng/L). No significant changes could be observed at any

concentration. As shown for female fish, the sensitivity for molecular biomarkers strongly varies among fish species. These results demonstrate that the selected biomarkers are quite sensitive for the detection of endocrine effects of trenbolone, however not in a dose-dependent manner.

9.4 Suitability of the two generation test

9.4.1 Is the two generation test suitable for the detection of effects caused by androgens, anti-androgens and anti-estrogens?

Greater sensitivity to toxicants usually occurs with extended exposure (Birge *et al.*, 1984; Ingersoll *et al.*, 1995; LeBlanc and Surprenant, 1985; Malueg *et al.*, 1984; Spehar *et al.*, 1978). However only a limited amount of freshwater subchronic and chronic toxicity testing has been conducted (Burton, 1991). Early life-stage assays that monitor fecundity and growth are more sensitive than survival studies of the adults (Giesy and Graney, 1989). The sensitivity of many molecular and cellular endpoints is greater than community structure and ecosystem function endpoints; however, determining their ecosystem significance is difficult at this point in time. Therefore, the two-generation test was conducted (see Chapter 8). Beside, morphological (growth, length, weight), reproductive (egg numbers, fertilization rate) and sex ratio changes also levels of molecular biomarkers (VTG, 11-KT) can be assessed. Consequently, this test system serves as a higher tier in *in vivo* testing of chemicals or sediments, by giving a more realistic and holistic reflection of the consequences caused by pollutants in the ecosystem.

In order to elucidate the sensitivity of the two generation test a validation study with flutamide (see Chapter 8 Supplement A), an androgen receptor agonist, trenbolone (see Chapter 8, Supplement B), which already revealed genotoxic effects to zebrafish embryos and tamoxifen citrate (see Chapter 8, Supplement C), an estrogen receptor antagonist was performed.

The advantage of full life-cycle tests is that they are covering all potential sensitivity stages, and that they are suitable for quantitative risk assessments. Their disadvantage is their duration and costs. Life-cycle tests with EDCs have been described for the fathead minnow (e.g., Länge *et al.*, 2001), medaka (e.g., Yokota *et al.*, 2001) and zebrafish (e.g., Fenske *et al.*, 2005; Nash *et al.*, 2004; Segner *et al.*, 2003). Life cycle tests can assess all the end points of the shorter-term developmental and reproduction assays (e.g. the 21-day assay). Furthermore

these tests provide information that can be used for population modelling as well as for diagnosing endocrine MOAs. Moreover, effects may not be evident in the F₀ generation, but may appear only in the F₁ generation, or organizational effects of EDCs induced early in life may become detectable only during later stages (Biggsby *et al.*, 1999; Stahl and Clark, 1998).

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Eidesstattliche Erklärung

Hiermit erkläre ich, Melanie Böttcher, geboren am 01.01.1982 in Mannheim, Deutschland, an Eides statt, dass ich die vorliegende Dissertation selbst verfasst und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Ich, Melanie Böttcher, geboren am 01.01.1982 in Mannheim, Deutschland, erkläre zudem an Eides statt, dass ich an keiner anderen Stelle ein Prüfungsverfahren beantragt habe, dass ich die Dissertation nicht in dieser oder anderer Form bereits anderweitig als Prüfungsarbeit verwendet habe und dass ich sie an keiner anderen Fakultät als Dissertation vorgelegt habe.

Ort/Datum

Melanie Böttcher

