TEXTE
16/2026

Final report

Development of an extended OECD One-Generation Reproduction Test with Zebrafish (ZEOGRT) for the Identification of Endocrine Disruptors

by:

Dr. Matthias Teigeler, Dr. Kornelija Franzen,
Dr. Elke Eilebrecht, Prof. Dr. Christoph Schäfers

Fraunhofer Institute for Molecular biology and Applied Ecology IME, Schmallenberg, Germany

publisher:
German Environment Agency

German Environment Agency

Umwelt Bundesamt

TEXTE 16/2026

REFOPLAN of the Federal Ministry for the Environment,
Climate Action, Nature Conservation and Nuclear Safety

Project No. (FKZ) 3716 65 419 0
FB001864/ENG

Final report

Development of an extended OECD One-Generation Reproduction Test with Zebrafish (ZEOGRT) for the Identification of Endocrine Disruptors

by
Dr. Matthias Teigeler, Dr. Kornelija Franzen,
Dr. Elke Eilebrecht, Prof. Dr. Christoph Schäfers

Fraunhofer Institute for Molecular biology and Applied
Ecology IME, Schmallenberg, Germany

On behalf of the German Environment Agency

Imprint

Publisher
Umweltbundesamt
Wörlitzer Platz 1
06844 Dessau-Roßlau
Tel: +49 340-2103-0
Fax: +49 340-2103-2285
buergerservice@uba.de
Internet: www.umweltbundesamt.de

Report performed by:
Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie IME
Auf dem Aberg 1
57392 Schmallenberg
Germany

Report completed in:
August 2025

Edited by:
Section Coordination Group Ecotoxicology and Bioaccumulation of Division IV Chemical Safety
Susanne Walter-Rohde, Dr. Gerd Maack (Fachbegleitung)

DOI:
https://doi.org/10.60810/openumwelt-8044

ISSN 1862-4804

Dessau-Roßlau, February 2026

Abstract: Development of a Prolonged OECD One Generation Reproduction test with Zebrafish (*Danio rerio*) for Identification of Endocrine Disruptors

This report summarizes the validation of the Zebrafish extended one generation reproduction test (ZEOGRT). The test design covers a one-generation test approach including an adult parental reproduction phase (F0), a full filial 1 generation (F1), and the hatching period of a second filial generation (F2).

The test aims to detect potential endocrine acting substances by assessing both impact on population relevant endpoints as well as effects on physiological parameters like biomarker concentrations. Histological and histopathological examination can be added to obtain information on the specific mode of action. The test procedure is flexible to cover estrogenic, androgenic modes of action and allows to include further assessments on e.g. gene expression or thyroid endpoints. However, the implementation of these parameters was not part of the validation.

Project 2.59: New Test Guideline on Zebrafish Extended One Generation Reproduction Test (ZEOGRT) was included on the test guidelines workplan by the Working Group of National Coordinators for the OECD Test Guidelines Programme (WNT) in April 2016.

This report presents the results from phase I and phase II of the validation. For phase I, in total four ZEOGRT studies were performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Germany between 2018 and 2020. In these studies, zebrafish were exposed to an estrogen receptor antagonist, an aromatase inhibitor, a synthetic progestin and a glucocorticoid. Within this report, the intra-laboratory variability was assessed. Furthermore, the effect concentrations derived were compared with available data from the literature.

For the second part of the validation process (phase II), which examines inter-laboratory variability, it was possible to identify two laboratories that contributed with own studies. Finally, three additional studies were added to the data pool.

To extend the data set, additional control mean values for the different biological parameters were collected from available industry studies to allow a calculation and evaluation of control variability.

Phase I of the project confirmed the applicability of the protocol, and the results obtained showed consistency with available effect data from the literature.

Phase II confirmed the successful transfer of the protocol to the international laboratories

Within an expert meeting held at the German environment agency (UBA) in June 2024, available results were discussed. Moreover, the discussion included modifications of the test protocol, the development of suitable pre-test strategies and strategies how to implement the new test guideline in the regulatory context.

Entwicklung eines verlängerten OECD Ein Generationen Reproduktionstests mit Zebrabärblingen (ZEOGRT) zur Identifizierung von endokrinen Disruptoren

Dieser Bericht fasst die Ergebnisse eines Validierungsprojekts für einen verlängerten Ein-Generationen Reproduktionstests mit Zebrabärblingen (ZEOGRT) zusammen. Der Testaufbau beinhaltet einen Ein-Generationen Ansatz und deckt eine parentale Reproduktionsphase mit adulten Fischen ab (F0) sowie eine vollständige Filial 1 Generation (F1) und schließlich die Schlupfphase einer zweiten Filialgeneration (F2).

Ziel des Tests ist die Untersuchung und Identifikation von potenziell hormonaktiven Substanzen, indem sowohl populationsrelevante Endpunkte als auch Effekte auf physiologische Parameter wie etwa spezifische Biomarker erfasst werden. Histologische und histopathologische Analysen können hinzugefügt werden, um den zugrunde liegenden Wirkmechanismus zu identifizieren. Die Testprozedur ist geeignet, um potenziell östrogene und androgene Wirkmechanismen zu detektieren, erlaubt aber darüber hinaus weitere Untersuchungen einzubinden, wie etwa Genexpressionsmessungen sowie die Analyse Schilddrüsen-gesteuerter Parameter. Allerdings waren diese Methodiken nicht Bestandteil des Validierungsprozesses.

Das Project 2.59: New Test Guideline on Zebrafish Extended One Generation Reproduction Test (ZEOGRT) wurde im April 2016 in den Arbeitsplan der Arbeitsgruppe der Nationalen Koordinatoren für das OECD-Prüfrichtlinienprogramm (WNT) aufgenommen.

Dieser Bericht präsentiert die Ergebnisse der zwei Validierungsphasen (I und II). Im Rahmen von Phase I wurden zwischen 2018 und 2020 insgesamt vier ZEOGRT Studien am Fraunhofer Institut für Molekularbiologie und Angewandte Oekologie IME durchgeführt. In diesen Studien wurden Zebrabärblinge mit insgesamt vier Substanzen exponiert, die verschiedene Wirkmechanismen repräsentierten: ein Östrogenrezeptor Antagonist, ein Aromatasehemmer, ein synthetisches Progestin sowie einem Glukokortikoid. In diesem Bericht wurde auf Basis der Ergebnisse die Intra-Labor Variabilität untersucht. Darüber hinaus wurden die ermittelten Effektkonzentrationen mit verfügbaren Daten aus der wissenschaftlichen Literatur verglichen.

Im zweiten Teil der Validierung (Phase II) wurde in Zusammenarbeit mit externen Laboren eine Vergleichsstudie durchgeführt, um auch hier Daten zur Variabilität der Daten zu generieren. Insgesamt konnten zwei Labore identifiziert werden, die insgesamt drei weitere ZEOGRT Studien durchführten.

Um den Datensatz zu erweitern, wurden aus verfügbaren Industriestudien, die nach dem ZEOGRT Protokoll durchgeführt wurden, die Mittelwerte der Kontrolldaten gesammelt und der Datenauswertung zugeführt.

Die Ergebnisse aus Phase I des Projekts belegten die Anwendbarkeit des Protokolls. Zudem zeigten die ermittelten Effektkonzentrationen gute Übereinstimmung mit den Literaturdaten.

Phase II belegte den erfolgreichen Transfer und die Anwendbarkeit des Protokolls auch in den internationalen Laboren.

Im Rahmen eines Fachgesprächs am Umweltbundesamt in Dessau-Roßlau im Juni 2024 wurden verfügbare Ergebnisse präsentiert und zur Diskussion gestellt. Das Fachgespräch behandelte mögliche Modifikationen des ZEOGRT-Versuchsplans, aber auch die Entwicklung geeigneter Vorteststrategien sowie die Einbindung der neuen Richtlinie in die bestehende regulatorische Bewertung hormonaktiver Substanzen.

Table of content

List of figures

List of tables

List of abbreviations

Abbreviation	Explanation
Anova	Analysis of Variance
(d)pf	(days) post fertilization
DWC	Dilution water control
ELISA	Enzyme linked immunosorbent assay
ELS	Early life stage
F0 generation	Parental generation (F0)
F1 generation	First filial generation (F1)
F2 generation	Second filial generation (F2)
FLCT	Fish life cycle test
FSDT	Fish sexual development test
FSTRA	Fish short term reproduction assay
IME	(Fraunhofer) Institute for Molecular Biology and Applied Ecology
LC MS	Liquid Chromatography - Mass Spectrometry
LOEC	Lowest observed effect concentration
MEOGRT	Medaka extended one generation reproduction test
MoA	Mode of action
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
pf	Post fertilization
RSD	Relative standard deviation
SD	Standard deviation
SOP	Standard operation procedure
SSC	Secondary sex characteristics
TG	Technical guideline
TIU	Trypsin Inhibitor Unit
UBA	Umweltbundesamt (German Environment Agency)
US-EPA	US Environmental Protection Agency
VMGeco	Validation and Management Group for ecotoxicological testing
VTG	Vitellogenin
WNT	Working Party of National Coordinators for the OECD Test Guidelines Programme
ZEOGRT	Zebrafish extended one generation reproduction test

Summary

Introduction and objective

The effect of hormone-active substances to humans and the environment has been described in numerous studies over the past three decades. These substances became known to the public in the 1990s under the term "endocrine disruptors". To assess and identify endocrine-disrupting substances, the Organisation for Economic Co-operation and Development (OECD) has established the OECD Conceptual Framework (CF) for Testing and Assessment of Endocrine Disrupters (ED). The CF lists available standardized methods and methods under development that provide information for ED assessment.

The work presented here aimed to establish a new OECD Test Guideline (TG) covering a multigeneration test with the zebrafish (*Danio rerio*) as a part of the OECD CF for ED.

The development and validation of a protocol considering zebrafish aimed to provide a supplement to the validated test guideline with medaka (medaka extended one generation reproduction test (MEOGRT), TG 240, OECD 2014) and allows to use species-specific advantages. Moreover, the implementation of a further species extends the possibility to use existing data within a regulatory process.

The basic test design of the the Zebrafish extended one generation reproduction test (ZEOGRT) considers a broad range of different life stages which are covered by existing fish test guidelines, i.e. the early life stages (OECD 2013, TG 210), the sexual development (OECD 2011, TG 234) and reproduction (OECD 2012, TG 229). For all of these test protocols, zebrafish is a validated species. Moreover, a broad database of zebrafish studies on population relevant endpoints is available. Both aspects underline the need to establish an extended one generation test (EOGRT) design also for this species.

This report summarizes the validation of theZEOGRT. The test design covers a one-generation test approach including an adult parental reproduction phase (F0), a full filial 1 generation (F1), and the hatching period of a second filial generation (F2). The test aims to detect potential endocrine acting substances by assessing both impact on population relevant endpoints as well as effects on physiological parameters like biomarker concentrations. Histological and histopathological examination can be added to obtain information on the specific mode of action.

The test procedure is flexible to cover estrogenic, androgenic modes of action and allows to include further assessments on e.g. gene expression or thyroid endpoints. However, the implementation of these parameters was not part of the validation.

The project was proposed by Germany and first discussed in October 2015 at the meeting of the OECD Validation Management Group for Ecotoxicity Testing (VMGeco) by the submission of a Standard project submission form (SPSF). The project was included on the test guidelines programme (TGP) workplan by the Working Group of National Coordinators for the OECD TGP (WNT) in April 2016.

This report presents the results from phase I and phase II of the validation. For phase I, in total four ZEOGRT studies were performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Germany between 2018 and 2020. Within this report, the intra-laboratory variability was assessed.

For the second part of the validation process (phase II), which examines inter-laboratory variability, it was possible to identify two laboratories that contributed with own studies between 2021 and 2023. Finally, three additional studies were added to the data pool.

To extend the data set, additional control mean values for the different biological parameters were collected from available industry studies to allow a calculation and evaluation of control variability.

Within an expert meeting held at the Umweltbundesamt (German Environment Agency - UBA) in June 2024, available results were discussed. Moreover, the discussion included modifications of the test protocol and strategies how to implement the new test guideline in the regulatory context.

Validation, phase I

The first ZEOGRT study was initiated in 2018 and conducted at Fraunhofer IME, Germany. The substance tested was tamoxifen-citrate representing an estrogen receptor antagonist. In a second study in 2019, prochloraz was tested as candidate for aromatase inhibitors. As part of another research project for UBA, two further ZEOGRT studies with the synthetic progestin dienogest and the glucocorticoid dexamethasone were conducted between 2019 and 2020, and the results were included in the evaluation of the validity of the ZEOGRT protocol. These studies followed the same protocol as applied for the test with tamoxifen-citrate and prochloraz.

The main goal of this part of the project was to confirm the applicability of the protocol for the test species zebrafish. This included the transfer of the known procedures from the existing fish test guidelines towards a multi generation protocol. And finally, the results from the ZEOGRT studies were compared with data from the Medaka EOGRT validation and from the scientific literature.

Validation, phase II

Within the second part of the project, it was possible to identify two external partner laboratories which were able to conduct own studies according to the study ZEOGRT protocol. The search for further participants of an - at least limited - laboratory ring trail was impeded by either the Covid pandemic, but also by the high effort to implement the ZEOGRT procedure, if not established. For example, financial considerations were the main reason for contract research laboratories refusing to participate, although the importance of the validation was recognized. Moreover, experience with the test species zebrafish was partly not available even at routine fish labs.

Besides the theoretical aspects of the protocol, Fraunhofer IME was able to provide technical knowledge on important procedures including application procedures of the test substances, lab organization for special work steps, and blood sampling of the exposed fish. Moreover, Fraunhofer IME provided data sheets to allow an effective data recording.

Use of control data

To extend the data base, especially to allow an evaluation of the inter-laboratory data variability, additional control data sets were identified from contracted ZEOGRT studies. These were studies awarded to Fraunhofer IME, but also studies conducted in other industry labs could be used. In total, data packages of four studies were provided, while three studies were considered for the final data evaluation. In any case written permissions to use the data were granted. All three studies were conducted with the same strain of zebrafish, originating from the Fraunhofer zebrafish culture.

Expert meeting

An expert meeting was held at UBA, Dessau in June 2024. International experts from academia, industry and authorities were invited to discuss the available results and open points regarding the draft TG.

The discussion made important contributions to open aspects of the study design and the future use of the ZEOGRT. An appropriate concentration setting must ensure clear identification of effects primary caused by endocrine action in contrast to toxic effects. A suitable pre-test strategy should allow a robust estimate of those concentrations. Two options for a pre-test strategy were proposed and discussed. Finally, it was concluded that a combination of a short-term reproduction phase linked with an exposure of early stages can provide the relevant data.

During the meeting, it was discussed whether the implementation of new methodologies should be already included in the draft TG text. It was stated that the currently available methods e.g., *omics* are not validated. However, as these are promising tools also to detect the underlying mode of actions, the guideline should reflect and list them as additional data sources. Moreover, also the detection of thyroid related parameters should be considered by the guideline text.

Finally, there was consensus that the ZEOGRT protocol should be included as a separate part of the OECD TG 240.

Description of the ZEOGRT

The ZEOGRT protocol was developed following the adopted Medaka EOGRT procedure (OECD TG 240). The objective of this test is to assess the effects of continuous exposure to a test chemical on different life stages and life performances of zebrafish (*Danio rerio*) during an extended one generation test. The primary focus is the assessment of the impacts on population relevant endpoints.

The list of relevant life stages includes the spawning period of the parental generation (F0 generation), early life stages, juvenile growth, sexual maturation, and reproduction of the first filial generation (F1 generation), and the early embryonal stage of the second filial generation (F2 generation). Endpoints determined are hatching success, mortalities during different life stages, and juvenile growth (F1 generation, only). The sex ratio of the exposed fish groups is determined either by macroscopic inspection of gonads or can be confirmed by a histological analysis (F1 generation) of the fish. Spawning performance (fecundity) and fertilization rate are recorded for adult fish of F0- and F1-generation and the time to first spawning for the F1-generation.

To obtain mechanistic data for further interpretation of the observed effects the study includes the measurement of biomarkers and a histopathological examination of fish gonads. Blood plasma samples of the adult fish of F0- and F1 generation are taken and measured for vitellogenin (VTG) concentrations. Furthermore, a histopathological examination of the fish gonads of the adult fish of F0- and F1- generation is performed, including maturation stages and pathological lesions.

From the results obtained, the threshold concentrations of the chemical are determined, i.e. the concentration with observed significant sublethal or lethal toxic effects ("LOEC") and the highest concentration tested without any significant effects ("NOEC").

Different test acceptability criteria were defined to assess the test performance. To ensure consistency with available TGs for ED identification in fish, accepted values and ranges for the parameters were considered. This includes test conditions, but also biological endpoints recorded for different generations.

ZEOGRT validation, studies and data sources

The following Table summarizes the studies conducted for both phase I and phase II of the validation as well as the available control data sets.

Table 1: ZEOGRT validation: Studies for both phase I and II, and additional data

Validation	Study number	Test substance
Phase I	1	Tamoxifen-Citrate
	2	Prochloraz
	3	Dienogest
	4	Dexamethasone
Phase II	5	Tamoxifen-Citrate
	6	Prochloraz
	7	Prochloraz
Additional data	8	control data only
	9	control data only
	10	control data only

Materials and methods

Validation, phase I: Studies performed at Fraunhofer IME

All experiments were conducted under flow through conditions. The concentrations of the applied substances were measured in regular intervals by appropriate chemical analysis. Table 2 gives an overview of the test substances applied in phase I. The tests were conducted with five test substance concentrations and a control in four replicates each. Each replicate group was kept in an individual test vessel with 25 L test medium.

Table 2: ZEOGRT validation, phase I: Test substance and test concentrations applied

Study number	Test substance	Mode of action	Nominal test concentrations	
1	Tamoxifen-Citrate	Selective estrogen receptor modulator	[µg/L]	0.20, 0.63, 2.0, 6.3, 20
2	Prochloraz	Aromatase inhibitor	[µg/L]	3.2, 10, 32, 100, 320
3	Dienogest	Progestin	[ng/L]	3.2, 10, 32, 100, 320
4	Dexamethasone	Glucocorticoid	[µg/L]	0.32, 1.0, 3.2, 10, 32, 100

The parental generation (F0) of each study was started with spawning groups of 10 fish (5 males, 5 females). To obtain the F1 generation, 36 embryos from the parental group were kept for hatching. To prepare spawning groups of equal numbers, the F1 groups were reduced to 20 juvenile fish per test vessel at day 35 pf. The F2 generation was prepared of 20 fertilized eggs from the F1 spawning groups.

From the test start onwards, parental fish from F0 generation were exposed during a spawning period of three weeks. In week 4, fertilized eggs were kept to prepare the F1 generation. The F1

fish were monitored over 5 weeks during their early life stage phase, followed by a juvenile growth phase for further 4 weeks. After reaching sexual maturity, start of reproduction and spawning success was observed and recorded over a period of approx. 5-8 weeks. Finally, an F2 generation was prepared by keeping eggs of F1 parental animals and were exposed until hatch.

After the successful start of the filial generations, based on fry survival (F1) or hatching success (F2), the fish of the parental groups were terminated. All fish were anaesthetized using chloro-butanol. A blood sample was taken from each fish via heart puncture to allow a measurement of vitellogenin concentration in blood plasma of the fish.

The phenotypic sex of each fish was determined by macroscopic inspection of fish gonads, i.e. ovary structure for females and testicular tubes for males. Afterwards, individual lengths and weights were measured. Finally, the fish were transferred to an appropriate fixative to allow a histopathological analysis. The phenotypic sex of each fish was confirmed by histological evaluation of fish gonads. Moreover, an analysis of maturation stages was conducted.

For each endpoint recorded, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC)values were determined.

Results and Conclusion

Validity criteria

Within the four ZEOGRT studies performed in phase I, there was a broad compliance with the defined acceptance criteria. The exception was the parameter sex ratio in the F1 generation of the study with tamoxifen-citrate (study 1). For controls a mean ratio of 25.0 and 75.0% was calculated for males and females, respectively.

During the study with dienogest (study 3), there was a timely limited deviation from the recommended range of water temperature. Over a period between day 2 and 10 after test start, single temperatures exceeded the recommended maximum level of 27.5 °C. A maximum of 28.0 °C was not exceeded. High water temperatures were measured at all treatment levels including controls. The results of the fish studies conducted in phase I suggest that the ZEOGRT provides an appropriate setup to detect the relevant apical endpoints. For the test substances applied, the test showed sufficient sensitivity.

The acceptance criteria were also assessed for the three studies performed in the external labs (phase II). For one study (study 7), there were discrepancies regarding the water parameters applied. For single measurements, the water temperatures were outside the range of 26.0 ± 2.0 °C. Moreover, low oxygen concentrations were measured in single cases. In the same study, the F1 post hatch survival of at least 75 % was not met. Moreover, the minimum hatching rate for F2 was not achieved. For study 6, a repeat of F1 preparation was reported, which was necessary to finally meet the validity criterion. To summarize, although there were single outliers from the defined criteria, the laboratories were able to transfer the test protocol including sufficient biological performance of the test animals applied.

Summary of available control data, phase I and II of the validation

To derive information about the variability of intra-laboratory data the mean control values and standard deviations for each endpoint were calculated and assessed.

The obtained fecundity data showed low variability between the studies for both F0 and F1 generation. In this context, it was evident, that the mean total egg numbers per female per day were lower in F1 compared to F0, which is a consequence of the younger age of the fish. The F0 spawning groups are prepared using adult fish taken from a lab fish culture, additionally, there is much emphasis in the setting of appropriate groups. The final setting of 5 males and 5 females

represents an optimized setting to achieve high egg numbers. Typically, the fish already have an age of >6 months, which means a higher grade of maturation (compared to F1). In contrast, the F1 fully originates out of the test setting (=offspring from F0) including shorter time for development. The recording of reproduction success directly starts after the fish reached maturity. Although able to reproduce and to fertilize the eggs, the fish are "just" adult and the ovaries are not at their capacity limit.

Currently, there is no validity criterion for zebrafish fecundity defined in e.g. OECD TG 229, however, for the choice of groups showing appropriate spawning performance the document states: "… but it is relatively common to observe average spawns of >10 eggs/female/day for each species". This criterion was considered to assess the reproductive performance in the ZEOGRT studies for both F0 and F1.

The mean fertility rates obtained were characterized by low standard deviations and were similar for F0 and F1, with relative standard deviations (RSDs) of 4.2 and 4.7%, respectively.

The recording for the "time to first spawning" has to be initiated once the fish achieved sufficient maturity to allow the reproductive output. For two phase I studies, it was found that the groups were already able to spawn from the first day spawning trays were placed in the test vessels. Thus, for that studies it was not possible to define the date of spawning start. Therefore, the final protocol should consider an earlier start of reproduction recording.

The sex ratio represents an important parameter for the assessment of endocrine acting chemicals. Within the ZEOGRT studies performed, the sex ratio showed the highest control variability of all population relevant parameters recorded. From those studies that met the F1 acceptance criterion, sex ratios for male fish were between 43.3 and 60.9 %. The overall variability may be triggered by different factors related to e.g. test conditions or gene distribution in the fish groups. To identify these influencing factors, further investigations will have to be conducted.

The recorded growth parameters for adult fish of both F0 and F1, i.e. total length and wet weight, showed clear sex-specific differences and thus should be evaluated separately. Adequate growth performance can also be used to estimate sufficient maturation. The fish of F1 were generally smaller, but the sex-specific differences of the growth parameters could be identified.

The mean VTG concentration in females were very similar between the control groups, and also between the two generations applied. The mean VTG concentrations in male fish were found to be in the expected low range, however, single outliers induced uncertainty of the data sets, also impacting the statistical evaluation and thus the robust detection of effect concentrations with respect to VTG decrease. By calculating geometric mean concentrations of VTG for the single replicates, the bias induced by the outliers should be reduced.

In a second step of the data evaluation, the control performance for the phase II studies was evaluated. It has to be noted that the laboratories involved in the validation exercise had established own zebrafish strains. Therefore, different growth performances could be observed.

Basically, it can be stressed that all studies expressed a very good performance regarding the reproduction parameters. Notably, all studies were able to identify the "time to first spawning" within the same period of testing. The acceptance criterion for sufficient fecundity of both F0 and F1, i.e. at least 10 eggs per female and day, was met in each case. As also recorded and discussed for the phase I studies, the egg numbers for F0 were usually higher as for F1. The exception was Study 5, where F1 egg numbers were finally higher.

Also, the fertilization rates were at a high level, without major differences for F0 and F1. As described for the phase I data set, the fertilization rates were characterized by a very low variability under control conditions.

As already mentioned, fish strain-specific differences in the growth performance could be observed. However, sex-specific differences could also be determined in these studies.

The mean sex ratios (expressed as % males) were all found to be in line with the given acceptance criteria. However, high variability of control sex ratio was identified and ranged between 30.1 % (study 6) and 69.3 % (study 7, dilution water control). However, the standard deviations within the control replicates were within an acceptable range between 12.1 % (study 6) and 23.1% (study 7, solvent control).

The vitellogenin concentrations found were all characterized by high variation for both females and males. The occurrence of single outliers resulted partly in large standard deviations within the same order of magnitude as the obtained mean values. Moreover, the mean VTG concentrations detected for males in the studies 5 and 6 already displayed high background concentrations, higher than the male VTG concentrations measured in the phase I dataset. However, in any case, a clear sex-specific difference of the mean values was obtained for males and females. Moreover, comparable mean VTG concentrations were detected for both F0 and F1.

To extend the validation data base, especially to allow an evaluation of the protocol specific variables, additional control data sets were identified from contracted ZEOGRT studies. In total, three studies were considered, i.e. presented as studies 8, 9 and 10. Notably, all three studies were conducted with the same strain of zebrafish. The data were transferred from the available study reports.

The control data obtained from studies 9 and 10 are quite close to the mean control values distilled from validation phase I. This refers e.g. to the reproduction endpoints egg numbers and fertilization rates for both F0 and F1. Also, the growth parameters in terms of length and weight measurements for the different life stages showed good compliance. The biological performance regarding hatching and life-stage specific survival was in line with the defined acceptance criteria.

The comparison of VTG concentrations was impeded due to differences in the data sets. Within study 8, VTG concentrations were measured from body homogenates, while VTG levels out of studies 9 and 10 originated from blood samples. However, in study 9, the VTG results were related to total protein and VTG in study 10 was only related to the blood volume. Nevertheless, it is transparent that there are sex-specific differences in the mean value of several orders of magnitude, fulfilling a basic requirement for VTG evaluation.

The control parameters for study 8, however, were characterized by lower growth performance. For example, both length and weight of the F0 do not show sex-specific differences. Compared with the phase I data, especially the female size seems retarded. As a secondary effect, the egg numbers were also lower compared to the other studies. This observation was also made for the F1 with the same range of sizes and reproduction output. However, the fertilization success did not show any limitations.

Comparison of effect concentrations, studies with Tamoxifen-citrate

The available data from this validation project allows the comparison of two studies conducted with Tamoxifen-citrate in the same range of test concentrations. For both studies, a stable application of the test chemical was confirmed. For F0 generation, a sensitive effect on reproduction was observed in the study conducted in phase I of the validation. However, no effect on F0 reproduction was observed in the corresponding study of phase II.

During the F1 generation, both studies showed corresponding effects on the early life stage performance. One study displayed already a reduced hatching success, but for both studies, the post hatch survival rate was found to be reduced at the same concentration range. In study 1 (phase I), an effect on survival was also present in the juvenile phase.

Furthermore, egg numbers were significantly reduced in both studies. However, a concentration dependent delay for the start of first spawning as well as a reduced fertilization capacity was only found in one of the two studies.

The growth performance of the adult fish was characterized differently in both studies. While remaining females expressed enhanced growth in study 1, the males within study 5 (phase II) were retarded in growth. However, both studies clearly identified a sex ratio shift towards an increased number of male fish in the exposed groups. These observations were concentration dependent, and a 100% shift towards males was detected. In the further course, the hatching success in the F2 generation was found to be reduced in study 1, but no effect was found in the corresponding study 5.

Consistent results were identified regarding the measurement of VTG. For both studies, the VTG concentrations for F0 females were significantly reduced. For F1, the decrease of female VTG was confirmed in study 1.

The histopathological analysis of fish gonads was only meaningful for study 1, which identified a concentration dependent increase of maturity stages of both males and female gonads.

Comparison of effect concentrations, studies with Prochloraz

During the validation project, three studies were conducted with Prochloraz in the same range of test concentrations. For all studies, a stable application of the test chemical was confirmed. For F0 generation, a sensitive effect on reproduction was only observed in one study conducted in phase II of the validation. However, no effect on F0 reproduction was observed in the corresponding studies of phase I (study 2) and phase II (study 7). Notably, both studies conducted in phase II revealed increasing growth performance for the adult F0 fish.

The early life stages of the F1 generation were found to be affected in all three studies. In one study, the hatching rate was already reduced, but most prominent was a decreased post hatch survival. Although the effect was identified in each study, the related effect concentrations differed. Two of the studies displayed an additional growth retardation of the fish larvae, in terms of a reduced length. The juvenile stages were not impacted in any of the studies.

No effect on F1 reproduction was recorded in any of the studies. Furthermore, with exception of an increased male length in study 2, no growth effects on the adult fish occurred. The most prominent effect derived consistently in all studies was a significant shift in sex ratio towards an increased number of male fish. At the top concentration, 100 % male fish were identified in all three studies.

The final evaluation of the F2 generation revealed no significant effect on hatching in all available studies.

The measurement of VTG revealed a significant decrease of VTG concentrations for both F0 and F1 for two of the studies. In the third study, no effect was detected. Interestingly, in that study increased male gonad maturity was identified, while in the other studies no effects on the fish gonads were found.

Zusammenfassung

Die Wirkung hormonaktiver Stoffe auf Mensch und Umwelt wurde in den letzten drei Jahrzehnten in zahlreichen Studien beschrieben. In der Öffentlichkeit wurden diese Stoffe in den 1990er Jahren unter dem Begriff „endokrine Disruptoren“ bekannt. Zur Bewertung und Identifizierung von endokrin wirksamen Substanzen hat die OECD ein Rahmenprogramm aufgelegt, welches die verfügbaren standardisierten Methoden listet, die Informationen für die Bewertung von Umwelthormonen liefern.

Das hier vorgestellte Projekt hatte zum Ziel, eine neue OECD-Prüfrichtlinie (TG) zu erstellen, die einen Mehrgenerationen-Test mit dem Zebrabärbling (*Danio rerio*) als Teil des OECD-Rahmenprogramms für ED abdeckt.

Die Entwicklung und Validierung eines Protokolls unter Berücksichtigung des Zebrabärblings soll die validierte Prüfrichtlinie mit Medaka (Medaka Erweiterter Ein-Generationen Reproduktionsttest (MEOGRT), TG 240, OECD 2014) ergänzen und ermöglicht es, artspezifische Vorteile zu nutzen. Darüber hinaus erweitert die Implementierung einer weiteren Spezies die Möglichkeit, vorhandene Daten im Rahmen eines regulatorischen Prozesses zu nutzen.

Das grundlegende Testdesign des ZEOGRT berücksichtigt eine breite Palette verschiedener Lebensstadien, die von bestehenden Prüfrichtlinien mit Fischen abgedeckt werden, d.h. die frühen Lebensstadien (OECD 2013, TG 210), die sexuelle Entwicklung (OECD 2011, TG 234) und die Reproduktion (OECD 2012, TG 229). Für alle diese Testprotokolle ist der Zebrafisch eine validierte Art. Darüber hinaus ist eine breite Datenbank von Zebrafischstudien zu populationsrelevanten Endpunkten verfügbar. Beide Aspekte unterstreichen die Notwendigkeit, ein Erweitertes Ein-Generationen Reproduktionstest- (EOGRT) Design auch für diese Fischart zu entwickeln.

Das Testdesign des ZEOGRT umfasst einen Ein-Generationen-Testansatz, der eine adulte elterliche Reproduktionsphase (F0), eine vollständige Filialgeneration (F1) und den Schlupf einer zweiten Filialgeneration (F2) umfasst. Ziel des Tests ist es, potenziell endokrin wirkende Substanzen zu erkennen, indem sowohl die Effekte auf populationsrelevante Endpunkte als auch die Auswirkungen auf physiologische Parameter wie Biomarkerkonzentrationen bewertet werden. Eine histologische und histopathologische Untersuchung kann hinzugefügt werden, um Informationen über die spezifische Wirkungsweise zu erhalten.

Das Testverfahren ist flexibel, um östrogene und androgene Wirkmechanismen abzudecken, und ermöglicht die Einbeziehung weiterer Bewertungen, z. B. der Genexpression oder von Schilddrüsenendpunkten. Die Implementierung dieser Parameter war jedoch nicht Teil der Validierung.

Das Projekt wurde von Deutschland vorgeschlagen und erstmals im Oktober 2015 auf der Sitzung der OECD Validation Management Group for Ecotoxicity Testing (VMGeco) durch Einreichung eines Standard Project Submission Form (SPSF) diskutiert. Das Projekt wurde von der Arbeitsgruppe der nationalen Koordinatoren für das OECD-Prüfrichtlinienprogramm (WNT) im April 2016 in den Arbeitsplan des Prüfrichtlinienprogramms aufgenommen.

Im vorliegendenBericht werden die Ergebnisse aus Phase I und Phase II der Validierung vorgestellt. In Phase I wurden zwischen 2018 und 2020 insgesamt vier ZEOGRT-Studien am Fraunhofer-Institut für Molekularbiologie und Angewandte Oekologie (IME), Deutschland, durchgeführt.

Für den zweiten Teil des Validierungsprozesses (Phase II), in dem die laborübergreifende Variabilität untersucht wurde, konnten zwei Labore identifiziert werden, die zwischen 2021 und

2023 eigene Studien beisteuerten. Schließlich wurden drei weitere Studien in den Datenpool aufgenommen.

Um den Datensatz zu erweitern, wurden zusätzliche Kontrollmittelwerte für die verschiedenen biologischen Parameter aus verfügbaren Industriestudien gesammelt, um eine Berechnung und Bewertung der Kontrollvariabilität zu ermöglichen.

Im Rahmen eines Expertentreffens beim Umweltbundesamt (UBA) im Juni 2024 wurden die vorliegenden Ergebnisse diskutiert. Darüber hinaus wurden Modifikationen des Prüfprotokolls, aber auch die Entwicklung geeigneter Vorteststrategien und Strategien zur Umsetzung der neuen Prüfrichtlinie im regulatorischen Kontext diskutiert.

Validierung, Phase I

Die erste ZEOGRT-Studie wurde 2018 initiiert und am Fraunhofer IME durchgeführt. Die getestete Substanz war Tamoxifen-Citrat, das einen Östrogenrezeptor-Antagonisten repräsentiert. In einer zweiten Studie im Jahr 2019 wurde Prochloraz als Aromatasehemmer getestet. Im Rahmen eines weiteren Forschungsprojekts für das UBA wurden zwischen 2019 und 2020 zwei weitere ZEOGRT-Studien mit dem synthetischen Gestagen Dienogest und dem Glukokortikoid Dexamethason durchgeführt, deren Ergebnisse in die Bewertung der Validität des ZEOGRT-Protokolls eingeflossen sind. Diese Studien folgten demselben Protokoll, das für den Test mit Tamoxifen-Citrat und Prochloraz angewendet wurde.

Das Hauptziel dieses Teils des Projekts bestand darin, die Anwendbarkeit des Protokolls für die Testart Zebrafisch zu bestätigen. Dazu gehörte die Übertragung der bekannten Verfahren aus den bestehenden OECD TGs mit Fischen auf ein Mehrgenerationenprotokoll. Und schließlich wurden die Ergebnisse der ZEOGRT-Studien mit Daten aus der Medaka-EOGRT-Validierung und aus der wissenschaftlichen Literatur verglichen.

Validierung, Phase II

Im zweiten Teil des Projektes konnten zwei externe Labore identifiziert werden, die in der Lage waren, eigene Untersuchungen nach dem ZEOGRT-Protokoll der Studie durchzuführen. Die Suche nach weiteren Teilnehmern eines - zumindest eingeschränkten - Laborringversuchs wurde sowohl durch die Covid-Pandemie als auch durch den hohen Aufwand für die Umsetzung des ZEOGRT-Verfahrens erschwert, sofern dieses nicht etabliert war. Beispielsweise waren finanzielle Aspekte für potenzielle Auftragsforschungslabore der Hauptgrund eine Teilnahme abzulehnen, obwohl die Bedeutung der Validierung erkannt wurde. Darüber hinaus war die notwendige Erfahrung mit der Testfischart Zebrabärbling teilweise selbst in Routinelabors nicht vorhanden.

Verwendung weiterer Kontrolldaten

Um die Datenbasis zu erweitern, insbesondere um eine Bewertung der Variabilität der Daten zwischen den Laboren zu ermöglichen, wurden zusätzliche Kontrolldatensätze aus beauftragten ZEOGRT-Studien ermittelt. Dabei handelte es sich um Studien, die an das Fraunhofer IME vergeben wurden, aber auch Studien aus anderen Industrielaboren. Insgesamt wurden Datenpakete von vier Studien zur Verfügung gestellt, wobei drei Studien für die endgültige Datenauswertung berücksichtigt wurden. In jedem Fall wurden schriftliche Genehmigungen zur Nutzung der Daten erteilt.

Fachgespräch am Umweltbundesamt

Im Juni 2024 fand im Umweltbundesamt, Dessau ein Fachgespräch statt. Eingeladen waren internationale Experten aus Wissenschaft, Industrie und Behörden, um die vorliegenden Ergebnisse und offenen Punkte des Prüfrichtlinienentwurfs zu diskutieren.

Das Expertengespräch lieferte wichtige Beiträge zu offenen Aspekten des Studiendesigns und der zukünftigen Anwendung der ZEOGRT-Prüfrichtlinie. Eine geeignete Konzentrationseinstellung muss gewährleisten, dass primär durch endokrine Wirkungen induzierte Effekte eindeutig identifiziert werden können, während toxische Effekte ausgeschlossen werden. Eine geeignete Vorversuch-Strategie sollte eine zuverlässige Schätzung dieser Konzentrationen ermöglichen. Es wurden zwei Optionen für eine Vorversuch-Strategie vorgeschlagen und erörtert. Schließlich kamen die Teilnehmenden zu dem Schluss, dass eine Kombination aus einer kurzfristigen Reproduktionsphase in Verbindung mit einer Exposition in frühen Stadien die relevanten Daten liefern kann.

Bei dem Fachgespräch wurde erörtert, ob die Einführung neuer Methoden bereits im Richtlinientext festgelegt werden sollte. Es wurde festgestellt, dass die derzeit verfügbaren Methoden, z. B. die Verwendung von *Omics*, nicht validiert sind. Da es sich hierbei jedoch um vielversprechende Instrumente handelt, mit denen auch die zugrundeliegende Wirkungsweise ermittelt werden kann, sollten sie in der Richtlinie berücksichtigt und als zusätzliche Datenquellen aufgeführt werden. Darüber hinaus sollte auch die Erfassung schilddrüsenbezogener Parameter im Richtlinientext berücksichtigt werden.

Schließlich herrschte Einigkeit darüber, dass das ZEOGRT-Protokoll als eigener Teil in die OECD-TG240 aufgenommen werden sollte.

Beschreibung des ZEOGRT

Das ZEOGRT-Protokoll wurde in Anlehnung an das angenommene Medaka-EOGRT-Verfahren (OECD TG 240) entwickelt. Ziel dieses Tests ist die Bewertung der Auswirkungen einer kontinuierlichen Exposition gegenüber einer Testsubstanz auf verschiedene Lebensstadien und Lebensleistungen von Zebrabärblingen (*Danio rerio*) während eines erweiterten Ein-Generationen-Tests. Das Hauptaugenmerk liegt dabei auf der Bewertung der Auswirkungen auf populationsrelevante Endpunkte.

Die Liste der relevanten Lebensstadien umfasst die Laichzeit der Elterngeneration (F0-Generation), frühe Lebensstadien, juveniles Wachstum, sexuelle Reifung und Fortpflanzung der ersten Filialgeneration (F1 Generation) und das frühe Embryonalstadium der zweiten Filialgeneration (F2 Generation). Die ermittelten Endpunkte sind der Schlupferfolg, die Sterblichkeit während verschiedener Lebensstadien und das Wachstum der Jungfische (nur F1 Generation). Das Geschlechterverhältnis der exponierten Fischgruppen wird entweder durch makroskopische Inspektion der Keimdrüsen bestimmt oder kann durch eine histologische Analyse (F1 Generation) der Fische bestätigt werden. Die Laichleistung (Fekundität) und die Befruchtungsrate werden für adulte Fische der F0 und F1 Generation und die Zeit bis zum ersten Ablaichen für die F1-Generation aufgezeichnet.

Um mechanistische Daten zur weiteren Interpretation der beobachteten Auswirkungen zu erhalten, umfasst die Studie die Messung von Biomarkern und eine histopathologische Untersuchung der Fischgonaden. Den erwachsenen Fischen der F0 und F1 Generation werden Blutplasmaproben entnommen und die Vitellogenin (VTG)-Konzentration gemessen. Außerdem wird eine histopathologische Untersuchung durchgeführt.

Aus den erhaltenen Ergebnissen werden die Schwellenkonzentrationen der Chemikalie bestimmt, d. h. die Konzentration mit beobachteten signifikanten subletalen oder letalen toxischen Wirkungen ("LOEC") und die höchste getestete Konzentration ohne signifikante Wirkungen ("NOEC").

Zur Bewertung der Testleistung wurden verschiedene Testakzeptanzkriterien festgelegt. Um die Übereinstimmung mit den verfügbaren Testrichtlinien für die Identifizierung von ED bei Fischen

zu gewährleisten, wurden akzeptierte Werte und Bereiche für die Parameter aus existierenden OECD Prüfrichtlinien berücksichtigt. Dazu gehören Testbedingungen, aber auch biologische Endpunkte, die für verschiedene Generationen aufgezeichnet wurden.

ZEOGRT Validierung, Studien und Datenquellen

Die folgende Tabelle zeigt die Studien, die in Phase I und II der Validierung durchgeführt wurden. Darüber hinaus sind drei weitere Studien aufgeführt, für die Kontrolldaten ausgewertet wurden.

Table 3: ZEOGRT Validierung: Studien aus Phase I und II, und weitere Datenpakete

Validierung	Studiennummer	Testsubstanz
Phase I	1	Tamoxifen-Citrat
	2	Prochloraz
	3	Dienogest
	4	Dexamethason
Phase II	5	Tamoxifen-Citrat
	6	Prochloraz
	7	Prochloraz
Zusatzdaten	8	Nur Kontrolldaten
	9	Nur Kontrolldaten
	10	Nur Kontrolldaten

Material und Methoden

Validierung, Phase I: Studien am Fraunhofer IME

Alle Versuche wurden unter Durchflussbedingungen durchgeführt. Die Konzentrationen der eingesetzten Substanzen wurden in regelmäßigen Abständen durch entsprechende chemische Analysen gemessen. Tabelle 4 gibt einen Überblick über die in Phase I eingesetzten Testsubstanzen. Die Versuche wurden mit fünf Substanzkonzentrationen und einer Kontrolle in jeweils vier Replikaten durchgeführt. Jedes Replikat wurde in einem eigenen Testbecken mit 25 L Testmedium gehalten.

Table 4: ZEOGRT Validierung, Phase I: Testsubstanzen und untersuchte Testkonzentrationen

Studiennummer	Testsubstanz	Wirkmechanismus	Nominale Testkonzentration	
1	Tamoxifen-Citrat	Selektiver Östrogenrezeptor-Modulator	[µg/L]	0.20, 0.63, 2.0, 6.3, 20
2	Prochloraz	Aromatase inhibitor	[µg/L]	3.2, 10, 32, 100, 320
3	Dienogest	Progestin	[ng/L]	3.2, 10, 32, 100, 320

Studiennummer	Testsubstanz	Wirkmechanismus	Nominale Testkonzentration	
4	Dexamethason	Glucokortikoid	[µg/L]	0.32, 1.0, 3.2, 10, 32, 100

Die Elterngeneration (F0) jeder Studie wurde mit Laichgruppen von 10 Fischen (5 Männchen, 5 Weibchen) begonnen. Um die F1-Generation anzusetzen, wurden 36 Embryonen aus der Elterngruppe zum Schlüpfen aufbewahrt. Um gleich große Laichgruppen zu erhalten, wurden die F1-Gruppen am Tag 35 pf randomisiert auf 20 Jungfische pro Testgefäß reduziert. Die F2-Generation wurde aus 20 befruchteten Eiern der F1-Laichgruppen angesetzt.

Ab Versuchsbeginn wurden die Elternfische der F0-Generation während einer Laichzeit von drei Wochen exponiert. In der vierten Woche wurden befruchtete Eier gesammelt, um die F1-Generation anzusetzen. Die F1-Fische wurden anschließend fünf Wochen lang in ihrer frühen Lebensphase beobachtet, gefolgt von einer vierwöchigen Wachstumsphase als Jungfische. Nach dem Erreichen der Geschlechtsreife wurden der Beginn der Reproduktion und der Laicherfolg über einen Zeitraum von ca. 5-8 Wochen beobachtet und aufgezeichnet. Schließlich wurde eine F2 Generation mit Eiern der F1 Elterntiere angesetzt und der Schlupferfolg untersucht.

Nach dem erfolgreichen Start der Filial-Generationen, der anhand der Anzahl der überlebenden Jungfische (F1) oder dem Schlupferfolg (F2) bewertet wurde, wurden die Fische der Elterngruppen aufgearbeitet. Alle Fische wurden mit Chlorbutanol betäubt. Von jedem Fisch wurde eine Blutprobe mittels Herzpunktion entnommen, um eine Messung der Vitellogeninkonzentration im Blutplasma der Fische zu ermöglichen.

Das phänotypische Geschlecht jedes Fisches wurde durch makroskopische Inspektion der Fischgonaden, d.h. der Eierstockstruktur bei den Weibchen und der Hodenschläuche bei den Männchen, bestimmt. Anschließend wurden die einzelnen Längen und Gewichte vermessen. Schließlich wurden die Fische in ein geeignetes Fixiermittel überführt, um eine histopathologische Analyse zu ermöglichen. Das phänotypische Geschlecht jedes Fisches wurde durch histologische Untersuchung der Fischgonaden bestätigt. Darüber hinaus wurde eine Analyse der Reifestadien durchgeführt.

Ergebnisse und Zusammenfassung

Validitätskriterien

Innerhalb der vier ZEOGRT-Studien, die in Phase I durchgeführt wurden, gab es eine weitgehende Übereinstimmung mit den definierten Akzeptanzkriterien. Die Ausnahme bildete der Parameter Geschlechterverhältnis in der F1 Generation der Studie mit Tamoxifen-Citrat (Studie 1). Für die Kontrollen wurde hier ein mittleres Verhältnis von 25.0 % bzw. 75.0 % für Männchen und Weibchen berechnet.

Während der Studie mit Dienogest (Studie 3) kam es zu einer zeitlich begrenzten Abweichung vom empfohlenen Bereich der Wassertemperatur. Über einen Zeitraum zwischen Tag 2 und 10 nach dem Teststart überstiegen die Einzeltemperaturen den empfohlenen Höchstwert von 27.5 °C. Ein Maximum von 28.0 °C wurde nicht überschritten. Hohe Wassertemperaturen wurden auf allen Behandlungsstufen gemessen, einschließlich der Kontrollen. Aus den Ergebnissen der in Phase I durchgeführten Fischstudien kann abgeleitet werden, dass der ZEOGRT ein geeignetes Setup zur Detektion der relevanten apikalen Endpunkte bietet. Für die verwendeten Testsubstanzen zeigte der Test eine ausreichende Sensitivität.

Die Akzeptanzkriterien wurden auch für die drei Studien bewertet, die in den externen Labors durchgeführt wurden (Phase II). Bei einer Studie (Studie 7) gab es Abweichungen hinsichtlich der angewandten Wasserparameter. Bei Einzelmessungen lagen die Wassertemperaturen

außerhalb des Bereichs von 26.0 ± 2.0 °C. Darüber hinaus wurden in Einzelfällen niedrigere Sauerstoffkonzentrationen gemessen. In der gleichen Studie wurde die F1 Überlebensrate von mindestens 75 % nach dem Schlupf nicht erreicht. Darüber hinaus wurde die minimale Schlupfrate für die F2 nicht erreicht. Für Studie 6 wurde über eine Wiederholung der F1-Vorbereitung berichtet, die notwendig war, um das Akzeptanz-Kriterium schließlich zu erfüllen. Zusammenfassend lässt sich sagen, dass es zwar einzelne Ausreißer von den definierten Akzeptanz-Kriterien gab, die Labore jedoch in der Lage waren, das Testprotokoll zu übertragen.

Zusammenfassung der vorhandenen Kontrolldaten aus Phase I und II der Validierung

Um Informationen über die Variabilität der Intra-Labordaten abzuleiten, wurden die mittleren Kontrollwerte und Standardabweichungen für jeden Endpunkt berechnet und bewertet.

Die erhaltenen Reproduktionsdaten zeigten eine geringe Variabilität zwischen den Studien sowohl für die F0- als auch für die F1 Generation. In diesem Zusammenhang zeigte sich, dass die durchschnittliche Gesamtzahl der Eier pro Weibchen und Tag in der F1 niedriger war als in der F0, was eine Folge des jüngeren Alters der Fische ist. Die F0-Laichgruppen werden mit Fischen aus einer adulten Laborfischkultur zusammengesetzt. Die Komposition aus 5 Männchen und 5 Weibchen stellt hierbei eine optimierte Einstellung dar, um eine möglichst hohe Eizahl zu erreichen. Typischerweise haben die Fische bereits ein Alter von >6 Monaten, was einen höheren Reifegrad (im Vergleich zu F1) bedeutet. Im Gegensatz dazu stammt die F1 vollständig aus der Testumgebung (=Nachkommen von F0) mit einer kürzeren Entwicklungszeit. Die Erfassung des Fortpflanzungserfolgs beginnt direkt nach Erreichen der Geschlechtsreife der Fische. Obwohl die Fische in der Lage sind, sich fortzupflanzen und die Eier zu befruchten, sind sie "gerade erst" erwachsen und die Reproduktionsorgane noch nicht an ihrer Kapazitätsgrenze.

Derzeit gibt es kein Validitätskriterium für die Reproduktionsleistung von Zebrafischen, das z. B. in der OECD 229 definiert wäre, jedoch heißt es für die Auswahl von Gruppen, die eine angemessene Laichleistung aufweisen, dass mindestes 10 Eier pro Weibchen pro Tag zu beobachten sein sollten. Dieses Kriterium wurde in den ZEOGRT Studien berücksichtigt, um die Reproduktionsleistung sowohl für F0 als auch für F1 zu bewerten.

Die berechneten mittleren Fertilitätsraten waren durch geringe Standardabweichungen gekennzeichnet und waren für F0 und F1 mit 4,2 bzw. 4,7 % sehr ähnlich.

Die Untersuchung des Zeitpunkts der ersten Eiablage muss begonnen werden, sobald die Fische eine ausreichende Reife erreicht haben, die eine Fortpflanzung ermöglicht. In zwei Studien der Phase I Studien wurde festgestellt, dass die Gruppen bereits ab dem ersten Tag, an dem die Laichschalen in den Testgefäßen platziert wurden, ablaichen konnten. Daher war es für diese Studien nicht möglich, das Datum des Laichbeginns genau zu bestimmen. Daher sollte das endgültige ZEOGRT Protokoll einen früheren Beginn der Reproduktionsaufzeichnung in Betracht ziehen.

Das Geschlechterverhältnis stellt einen wichtigen Parameter für die Bewertung endokrin wirkender Chemikalien dar. In den durchgeführten ZEOGRT Studien zeigte das Geschlechterverhältnis die höchste Kontrollvariabilität aller erfassten populationsrelevanten Parameter. In den Studien, die das F1-Akzeptanzkriterium erfüllten, lag das Geschlechterverhältnis für männliche Fische zwischen 43.3 und 60.9 %. Die Gesamtvariabilität kann durch verschiedene Faktoren ausgelöst werden, die z.B. mit den Testbedingungen oder der Genverteilung in den Fischgruppen zusammenhängen. Um diese Einflussfaktoren zu identifizieren, müssen weitere Untersuchungen durchgeführt werden.

Die erfassten Wachstumsparameter (d.h. Gesamtlänge und Nassgewicht) zeigten deutliche geschlechtsspezifische Unterschiede sowohl für adulte Fische von F0 als auch von F1und sollten

daher nach Geschlecht getrennt ausgewertet werden. Eine adäquate Wachstumsleistung kann auch zur Abschätzung einer ausreichenden Reifung herangezogen werden. Die Fische der F1 waren im Allgemeinen kleiner, aber die geschlechtsspezifischen Unterschiede der Wachstumsparameter konnten identifiziert werden.

Die mittlere VTG-Konzentration bei Weibchen war im Vergleich der Kontrollgruppen und auch zwischen den beiden angewandten Generationen sehr ähnlich. Es zeigte sich, dass die mittleren VTG-Konzentrationen in männlichen Fischen im erwarteten niedrigen Bereich lagen, jedoch führten einzelne Ausreißer zu Unsicherheiten in den Datensätzen, was sich auch auf die statistische Auswertung und damit auf die robuste Detektion von Effektkonzentrationen in Bezug auf die VTG-Abnahme auswirkte. Durch die Berechnung der geometrisch-mittleren Konzentrationen von VTG für die einzelnen Replikate soll eine durch die Ausreißer induzierte Verzerrung der Messdaten reduziert werden.

In einem zweiten Schritt der Datenauswertung wurden die ermittelten Kontrollparameter für die Phase II Studien evaluiert. Es ist darauf hinzuweisen, dass die Laboratorien, die an der Validierung beteiligt waren, eigene Zebrafischstämme verwendet haben. Daher konnten unterschiedliche Wachstumsleistungen beobachtet werden.

Grundsätzlich kann postuliert werden, dass alle Studien sehr gute Reproduktionsleistungen der Fische detektieren konnten. Hervorzuheben ist, dass in allen Studien der Zeitpunkt der ersten Eiablage identifiziert werden konnte. Das Akzeptanzkriterium für eine ausreichende Reproduktion wurde sowohl von der F0 als auch von der F1 erfüllt, d.h. mindestens 10 Eier pro Weibchen und Tag wurden gezählt. Wie auch für die Phase-I Studien aufgezeichnet und diskutiert, waren die Eizahlen für F0 in der Regel höher als für die F1. Die Ausnahme war Studie 5, in der die Anzahl der F1 Eier schließlich höher war.

Auch die Befruchtungsraten waren auf einem hohen Niveau, ohne größere Unterschiede für F0 und F1. Wie für den Phase I Datensatz beschrieben, zeichneten sich die Befruchtungsraten durch eine sehr geringe Variabilität unter Kontrollbedingungen aus.

Wie bereits erwähnt, konnten fischstammspezifische Unterschiede in der Wachstumsleistung beobachtet werden. Auch in den Studien der Phase II wurden zudem geschlechtsspezifische Größenunterschiede festgestellt.

Die mittleren Geschlechterverhältnisse der F1 (ausgedrückt in % männlicher Tiere) erfüllten alle die definierten Akzeptanzkriterien. Es wurde jedoch eine hohe Variabilität des Geschlechterverhältnisses in den Kontrollen festgestellt; diese lag zwischen 30.1 % (Studie 6) und 69.3 % (Studie 7, Verdünnungswasserkontrolle) lag. Die Standardabweichungen innerhalb der Kontrollreplikate lagen jedoch in einem akzeptablen Bereich zwischen 12.1 % (Studie 6) und 23.1 % (Studie 7, Lösungsmittelkontrolle).

Die gemessenen Vitellogenin-Konzentrationen waren alle durch eine hohe Variabilität sowohl bei den Weibchen als auch bei den Männchen charakterisiert. Das Auftreten einzelner Ausreißer führte zum Teil zu großen Standardabweichungen innerhalb der gleichen Größenordnung wie der erhaltenen Mittelwerte. Darüber hinaus zeigten die mittleren VTG-Konzentrationen, die in den Studien 5 und 6 bei Männchen gemessen wurden, bereits eine relativ hohe Grundkonzentration an VTG im Blutplasma auf, die höher waren als die im Phase I Datensatz gemessenen Werte. In jedem Fall ergab sich jedoch eine deutliche geschlechtsspezifische Differenz der Mittelwerte für Männchen und Weibchen. Darüber hinaus wurden sowohl für F0 als auch für F1 vergleichbare mittlere VTG-Konzentrationen nachgewiesen.

Um die Datenbasis für die Validierung zu erweitern, insbesondere um eine Bewertung der protokollspezifischen Variablen zu ermöglichen, wurden zusätzliche Kontrolldatensätze aus

beauftragten ZEOGRT-Studien in die Auswertung eingebracht. Insgesamt wurden drei Studien berücksichtigt (Studien 8, 9 und 10). Hervorzuheben ist, dass alle drei Studien mit demselben Zebrafischstamm durchgeführt wurden. Die Daten wurden aus den vorliegenden Studienberichten übernommen.

Die Kontrolldaten aus den Studien 9 und 10 liegen recht nahe an den mittleren Kontrollwerten, die aus der Validierungsphase I herausgearbeitet wurden. Dies bezieht sich z.B. auf die Reproduktionsendpunkte Eizahl und Befruchtungsraten, sowohl für F0 als auch für F1. Auch die Wachstumsparameter in Bezug auf Längen- und Gewichtsmessungen für die verschiedenen Lebensphasen zeigten eine gute Übereinstimmung. Die biologische Leistung in Bezug auf den Schlupferfolg und die lebensphasenspezifischen Überlebensraten entsprach den definierten Akzeptanzkriterien.

Der Vergleich der VTG-Konzentrationen wurde durch Unterschiede in den Datensätzen erschwert. In Studie 8 wurden die VTG-Konzentrationen aus Gewebehomogenaten gemessen, während die VTG-Konzentrationen aus den Studien 9 und 10 aus Blutproben stammten. In Studie 9 bezogen sich die VTG-Ergebnisse jedoch auf das Gesamtprotein und das VTG in Studie 10 nur auf das Blutvolumen. Dennoch ist transparent, dass es geschlechtsspezifische Unterschiede im Mittelwert von mehreren Größenordnungen gibt, die eine Grundvoraussetzung für die VTG-Auswertung erfüllen.

Die Kontrollparameter für Studie 8 waren jedoch durch eine geringere Wachstumsleistung der Tiere gekennzeichnet. Zum Beispiel zeigten sowohl die Länge als auch das Gewicht der F0 keine geschlechtsspezifischen Unterschiede. Verglichen mit den PhaseI Daten schienen vor allem das weibliche Wachstum beeinträchtigt zu sein. Als Nebeneffekt waren auch die Eizahlen im Vergleich zu den anderen Studien niedriger. Diese Beobachtung wurde auch für die F1 Fische gemacht, mit der gleichen reduzierten Wachstumsleistung sowie der im Vergleich verminderten Eizahl. Die Befruchtungsleistung zeigte jedoch keine Beeinträchtigung.

Vergleich von Effektkonzentrationen, Studien mit Tamoxifen-Citrat

Die verfügbaren Daten aus diesem Validierungsprojekt ermöglichten final den Vergleich von zwei Studien, die mit Tamoxifen-Citrat im gleichen Bereich der Testkonzentrationen durchgeführt wurden. Für beide Studien wurde eine stabile Applikation der Testsubstanz analytisch bestätigt. Für die F0 Generation wurde in der Studie 1 (Phase 1) ein sensitiver Effekt auf die Reproduktion beobachtet. In der entsprechenden Studie der Phase II wurde jedoch kein Effekt auf die F0 Reproduktion beobachtet.

Während der F1 Generation zeigten beide Studien entsprechende Auswirkungen auf die biologischen Leistungen in der frühen Lebensphase. Eine Studie zeigte bereits einen reduzierten Schlupferfolg, aber für beide Studien wurde festgestellt, dass die Überlebensrate nach dem Schlüpfen bei gleichem Konzentrationsbereich reduziert war. In Studie 1 (Phase I) zeigte sich auch in der juvenilen Phase ein Effekt auf das Überleben.

Darüber hinaus war die Anzahl der Eier in beiden Studien signifikant reduziert. Eine konzentrationsabhängige Verzögerung des Beginns des ersten Laichens sowie eine verminderte Befruchtungskapazität wurde jedoch nur in einer der beiden Studien gefunden.

Die Wachstumsleistung der adulten Fische wurde in beiden Studien unterschiedlich charakterisiert. Während die übrigen Weibchen in Studie 1 ein verstärktes Wachstum zeigten, waren die Männchen in Studie 5 (Phase II) im Wachstum verzögert. Beide Studien zeigten jedoch eindeutig eine Verschiebung des Geschlechterverhältnisses hin zu einer erhöhten Anzahl männlicher Fische in den exponierten Gruppen. Diese Beobachtungen waren konzentrationsabhängig, und es wurde eine 100%ige Verschiebung zugunsten der Männchen

festgestellt. Im weiteren Verlauf wurde in Studie 1 festgestellt, dass der Schlupferfolg in der F2-Generation reduziert war, während in der entsprechenden Studie 5 kein Effekt festgestellt wurde.

Konsistente Ergebnisse wurden hinsichtlich der Messung von VTG identifiziert. In beiden Studien waren die VTG-Konzentrationen für die F0 Weibchen signifikant reduziert. Für die F1 wurde der Rückgang des weiblichen VTG in der Studie 1 bestätigt.

Die histopathologische Analyse der Fischgonaden war nur für Studie 1 aussagekräftig, die eine konzentrationsabhängige Zunahme der Reifestadien sowohl der männlichen als auch der weiblichen Gonaden feststellte.

Vergleich von Effektkonzentrationen, Studien mit Prochloraz

Im Rahmen des Validierungsprojekts wurden drei Studien mit Prochloraz im gleichen Bereich der Testkonzentrationen durchgeführt. Für alle Studien wurde eine stabile Applikation der Testsubstanz analytisch bestätigt. Für die F0 Generation wurde ein sensitiver Effekt auf die Reproduktion nur in einer Studie aus Phase II beobachtet (Studie 6). In den entsprechenden Studien von Phase I (Studie 2) und Phase II (Studie 7) wurde jedoch kein Effekt auf die F0 Reproduktion beobachtet. Bemerkenswert ist, dass beide Studien, die in Phase II durchgeführt wurden, eine zunehmende Wachstumsleistung für die erwachsenen F0 Fische zeigten.

In allen drei Studien wurde festgestellt, dass die frühen Lebensphasen der F1 Generation betroffen waren. In einer Studie war die Schlupfrate bereits reduziert, aber am auffälligsten war ein verringertes Überleben nach dem Schlüpfen. Obwohl der Effekt in jeder Studie identifiziert wurde, unterschieden sich die entsprechenden Wirkungskonzentrationen. Zwei der Studien zeigten eine zusätzliche Wachstumsverzögerung der Fischlarven in Form einer reduzierten Länge. Die jugendlichen Stadien wurden in keiner der Studien beeinflusst.

In keiner der Studien wurde ein Einfluss auf die F1 Fortpflanzung festgestellt. Darüber hinaus traten mit Ausnahme einer erhöhten Länge der Männchen in Studie 2 keine Wachstumseffekte auf die adulten Fische auf. Der auffälligste Effekt, der in allen Studien konsistent festgestellt wurde, war eine signifikante Verschiebung des Geschlechterverhältnisses hin zu einer erhöhten Anzahl männlicher Fische. Bei der höchsten Konzentration wurden in allen drei Studien 100 % männliche Fische identifiziert.

Die abschließende Evaluierung der F2 Generation ergab in allen verfügbaren Studien keinen signifikanten Effekt auf den Schlupferfolg.

Die Messung von VTG ergab für zwei der Studien eine signifikante Abnahme der VTG-Konzentrationen sowohl für F0 als auch für F1. In der dritten Studie wurde kein Effekt festgestellt. Interessanterweise wurde in dieser Studie eine erhöhte Reife der männlichen Gonaden festgestellt, während in den anderen Studien keine Auswirkungen auf die Fischgonaden festgestellt wurden.

Acknowledgements

The authors would like to thank the contributing laboratories and responsible scientists spending efforts and resources to conduct the studies. These were:

- National Institute for Environmental Studies NIES
  Health and Environmental Risk Division
  Ecotoxicity Research Section
  Dr. Haruna Watanabe
  Dr. Hiroshi YAMAMOTO
  Dr. Takahiro YAMAGISHI
  16-2 Onogawa, Tsukuba, Ibaraki, 305-8506,
  Japan

- Scymaris Ltd.
  Brixham Laboratory
  Becky Cahill, née Burden
  Dr. Richard Maunder
  Dr. Stephen Derrington
  Freshwater Quarry
  Brixham Devon TQ5 8BA UK
  United Kingdom

Moreover, the authors would like to thank the collaborating partner laboratories contributing with lab capacities and their excellent expertise. These were:

Vitellogenin measurement

- University of Southern Denmark
  Faculty of Science
  Department of Biology
  Ecotoxicology
  Prof. Dr. Henrik Holbech
  Campusvej 55
  5230 Odense M
  Denmark

Histopathology

- University of Bern
  Vetsuisse Faculty
  Institute for Fish and Wildlife Health
  Prof. Dr. Helmut Segner
  Prof. Dr. Heike Schmidt-Posthaus
  Länggasstrasse 122
  3012 Bern
  Switzerland

- Fraunhofer Institute for Toxicology and Experimental Medicine ITEM
  Dr. Dirk Schaudien
  Nikolai-Fuchs-Str. 1
  30625 Hannover

1 Introduction

This document was prepared to summarize the validation of the Zebrafish extended one generation reproduction test (ZEOGRT). The test design covers a one-generation test approach including an adult parental reproduction phase (F0), a full filial 1 generation (F1), and the hatching period of a second filial generation (F2).

The test aims to detect potential endocrine acting substances by assessing both impact on population relevant endpoints as well as effects on physiological parameters like biomarker concentrations. Histological and histopathological examination can be added to obtain information on the specific mode of action. The test procedure is flexible to include further assessments on e.g. gene expression or thyroid endpoints, as well as on transgenerational effects by expanding the F2 generation towards a full two-generation test. However, these were not part of the validation.

The project was proposed by Germany and first discussed in October 2015 at the meeting of the OECD Validation Management Group for Ecotoxicity Testing (VMGeco) by the submission of a Standard project submission form (SPSF). The project was included on the test guidelines workplan by the Working Group of National Coordinators for the OECD Test Guidelines Programme (WNT) in April 2016.

This report presents the results from in total four ZEOGRT studies performed at the Fraunhofer Institute for Molecular Biology and Applied Ecology (IME), Germany between 2018 and 2020. This part represents phase I of the validation. In these studies, zebrafish were exposed to an estrogen receptor antagonist, an aromatase inhibitor, a synthetic progestin and a glucocorticoide. This report summarizes the results obtained from the ZEOGRT studies and assesses the intra-laboratory variability. Furthermore, the effect concentrations derived were compared with available data from the literature. Two ZEOGRT studies of that study package had been reported in the context of another project (Project No. (FKZ) 3717 67 405 0 (Teigeler, 2024)). These results will only be shortly summarized.

For the second part of the validation process (phase II), which examines interlaboratory variability, it was possible to identify two laboratories that contributed with own studies. Finally, three additional studies were added to the data pool.

To extend the data set, additional control mean values for the different biological parameters were collected from available industry studies to allow a calculation and evaluation of control variability.

Within an expert meeting held at the German Environment Agency (UBA), Dessau in June 2024, available results were discussed. Moreover, the discussion included modifications of the test protocol and strategies how to implement the new test guideline in the regulatory context.

1.1 Scientific background

The effect of hormone-active substances to humans and the environment has been described in numerous studies over the past three decades. These substances became known to the public in the 1990s under the term "endocrine disruptors" following some ground-breaking publications, highlighting observed impacts on humans and wildlife (e.g. Colborn et al. (Colborn 1993) or Sumpter et al. (Sumpter 1995)).

To assess endocrine-disrupting substances, the OECD has established the OECD Conceptual Framework (CF) for Testing and Assessment of Endocrine Disrupters (ED). The CF lists available standardized methods and methods under development that provide information for ED

assessment. The CF aims to cover the most relevant groups of organisms (mammals, fish, amphibians and invertebrates).

The work presented here aims to establish a new OECD Test Guideline (TG) covering a multigeneration test with the zebrafish (*Danio rerio*) as a part of the OECD CF for ED. This test covers a parental generation of adult spawning fish, a full filial 1 generation and finally a filial 2 generation at an early life stage.

The Medaka extended one generation reproduction test (MEOGRT, TG 240) was adopted in 2014 and revised in 2023 (OECD 2023). The development and validation of a protocol considering zebrafish aims to provide a supplement to the existing test guideline and allows to use species-specific advantages. Moreover, the implementation of a further species extends the possibility to use existing data within a regulatory process.

The basic test design of the ZEOGRT considers a broad range of different life stages which are covered by existing fish TGs, i.e. the early life stages (OECD 2013, TG 210), the sexual development (OECD 2011, TG 234) and reproduction (OECD 2012, TG 229). However, the protocol also considers existing procedures to cover a fish full life cycle, e.g. the Detailed Review Paper on Fish Life-Cycle Tests (OECD 2008) or the zebrafish life cycle test procedure as published by Nagel et al. (Nagel 1998).

For all of these test protocols, zebrafish is a validated species. Moreover, a broad database of studies on population relevant endpoints is available. Both aspects underline the need to establish an EOGRT design also for this species.

1.2 Validation of an OECD test guideline

1.2.1 Course of the validation project

The standardization project on the ZEOGRT was included in the work plan of the OECD Test Guidelines Programme in 2016. A first draft study protocol (standard operation procedure - SOP) was presented to the OECD Validation and Management Group for Ecotoxicity Testing (VMGeco) in October 2016.

1.2.1.1 Validation, phase I

The first study was initiated in 2018 and conducted at Fraunhofer IME, Germany. The substance tested was tamoxifen-citrate representing an estrogen receptor antagonist. In a second study conducted in 2019, prochloraz was tested as candidate for aromatase inhibitors.

As part of another UBA project (Teigeler, 2024), two further ZEOGRT studies with the synthetic progestin dienogest and the glucocorticoid dexamethasone were conducted, and the results are included in the evaluation of the validity of the ZEOGRT protocol in this report. These studies followed the same protocol as applied for the test with tamoxifen-citrate and prochloraz.

The main goals of the first part of the validation (phase I) were:

- proof of applicability of the test protocol for the test species zebrafish
- transfer of known procedures from existing guidelines towards a life cycle/ multi generation protocol
- compare the results from the ZEOGRT studies with existing data from the MEOGRT testing program and the literature.

Finally, the applicability of the protocol could be proved. A summary report of validation phase I was prepared in 2020 and submitted to VMGeco for commenting.

1.2.1.2 Validation, phase II

Within the second part of the project, it was possible to identify external partner laboratories which were able to conduct own studies according to the study ZEOGRT protocol. The search for further participants of an - at least limited - laboratory ring trail was impeded by either the Covid pandemic, but also by the high effort to implement the ZEOGRT procedure, if not available. For contract research labs, financial aspects were the main driver to refuse a participation, although the importance of the validation was recognized. Moreover, experience with the test species zebrafish was partly not available even at routine fish labs. However, by beginning of 2021, three external labs were finally willing to participate and to contribute to the validation project with at least one ZEOGRT study.

Besides the theoretical aspects of the protocol, Fraunhofer IME was able to provide technical knowledge on important procedures including application procedures of the test substances, organizing of working steps, and blood sampling of the exposed fish. Moreover, Fraunhofer IME provided data sheets to allow an effective data recording.

Two of the three labs had been visited by Fraunhofer staff to discuss the procedures and check the infrastructure. Where travelling was not possible, the procedures were recorded with video equipment at the Fraunhofer IME laboratories and provided to the labs. Open questions were discussed by email correspondence or online meetings.

Finally, one of the three labs refused to participate due to capacity reasons. The remaining laboratories contributed to the project with in total three ZEOGRT studies.

1.2.1.3 Use of control data

During the project, it became clear that the final number of contributing studies would be limited. However, to extend the data base, especially to allow an evaluation of the inter-laboratory variability, additional control data sets were identified from contracted ZEOGRT studies. These were studies awarded to Fraunhofer IME, but also studies conducted in other industry labs could be used. In total, data packages of four studies were provided, while three studies were considered for the final data evaluation. In any case written permissions to use the data were granted.

Two of them were conducted at the Fraunhofer IME, a third one was conducted at an industry laboratory. All three studies were conducted with the same strain of zebrafish, originating from the Fraunhofer zebrafish culture.

1.3 Expert meeting

An expert meeting („UBA-Fachgespräch“) was held in Dessau and online in June 2024. International experts from academics, industry and authorities were invited to discuss the available results and open points regarding the test guideline. The list of topics included:

- How to find an appropriate pre-test strategy?
- How to ensure a maximum value of information from a new animal test method?
- How to implement the ZEOGRT in an appropriate test strategy for endocrine disruptors?
- How should the new ZEOGRT test guideline (TG) be included in the existing OECD TG 240?

The discussion gave important contributions to open aspects of the study design and the future use of the guideline. An appropriate concentration setting must ensure a clear identification of effects primary induced by endocrine acting property in contrast to toxic effects. A suitable pre-test strategy should allow a robust estimate of those concentrations. Two options for a pre-test strategy were proposed and discussed. Finally, it was concluded that a combination of a short-term reproduction phase linked with an exposure of early stages can provide the relevant data.

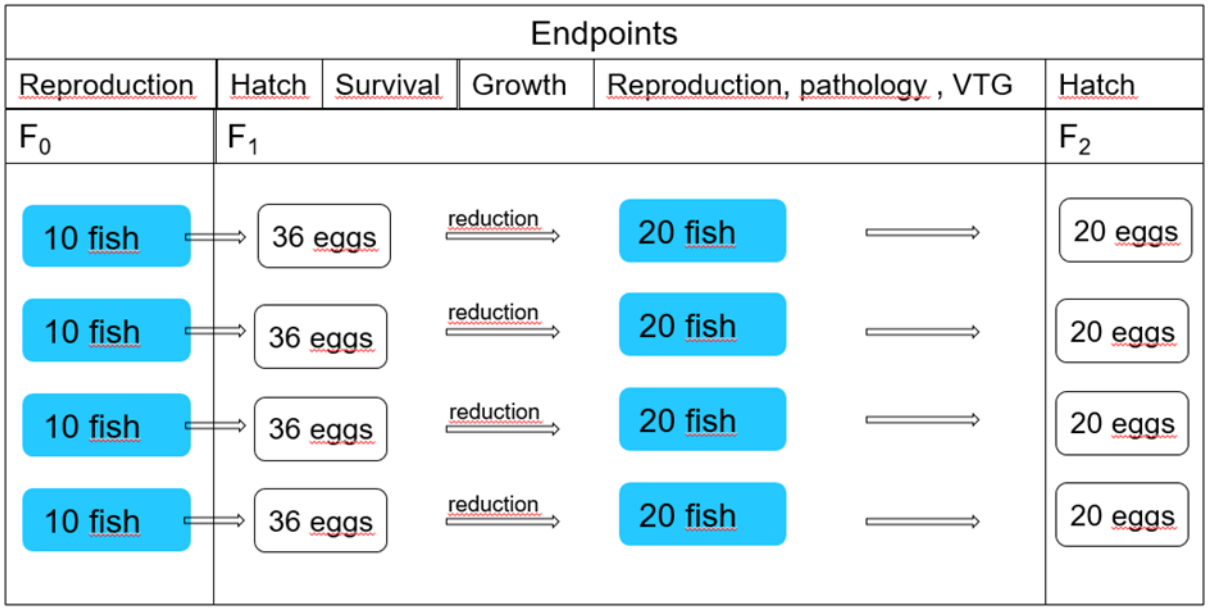
During the meeting, it was discussed if the implementation of new methodologies should be already included in the draft TG text. It was stated that the currently available methods e.g., *omics* are not validated. However, as these are promising tools also to detect the underlying mode of actions, the guideline should reflect and list them as additional data sources. Moreover, also the detection of thyroid related parameters should be considered by the draft TG text.

Finally, there was consensus that the ZEOGRT protocol should be included as an separate part of the OECD TG 240.

1.4 The ZEOGRT protocol

The ZEOGRT protocol was developed following the adopted Medaka EOGRT procedure, i.e. start with a parental adult group in spawning status, followed by a complete filial 1 generation (F1) derived from the parental eggs, and finally a filial 2 generation (F2), limited to the hatching period. The list of endpoints contains egg numbers and fertilization for the parental generation, hatch, survival, juvenile growth, reproduction, sex ratio and life stage-specific growth parameters for the F1 generation and finally hatching success for the F2 generation.

Figure 1: ZEOGRT, fish numbers applied per treatment/concentration and in control



Source: Own illustration, Fraunhofer IME

Figure 1 illustrates the basic setting of the ZEOGRT design, for one treatment or control. Four replicates are used per treatment and concentration respectively.

It is important to mention, that the basic setup of ZEOGRT considered full and true replication throughout the study. There is no pooling of eggs, larvae, juveniles and spawning adults between the test vessels within a treatment group at any time during the study.

Beside the apical endpoints listed above, physiological parameters like vitellogenin concentrations in the blood plasma are measured.

Moreover, a histological examination of fish gonads can be included to determine and assess the determination of the phenotypical sex of the fish. These evaluations can be extended by a detailed histopathological examination following the available OECD guidance document 123 (2010).

Finally, the ZEOGRT is considered to be a suitable tool to identify chemicals with estrogenic and androgenic mode of actions and moreover can be extended by additional focused analysis of tissues (i.e. other organs beside the gonads), additional blood parameters and gene expression analysis aiming to derive mechanistic data to characterize the underlying mode of action. Currently, thyroid specific parameters are of growing interest to allow an identification of thyroid disruption also in long-term fish studies. The endpoints discussed, including e.g. swim bladder inflation, eye histopathology as well as the measurement of thyroid hormones, could be implemented in the ZEOGRT protocol. However, these additional evaluations were not part of the validation process.

1.4.1 Test design

The objective of this method is assessing the effects of continuous exposure to a test chemical on different life stages and life performances of zebrafish (*Danio rerio*) during an extended one generation test. The primary focus is the assessment of the impacts on population relevant endpoints.

This list of relevant life stages includes the spawning period of the parental generation (F0 generation), early life stages, juvenile growth, sexual maturation, and reproduction of the first filial generation (F1 generation), and the early embryonal stage of the second filial generation (F2 generation). Endpoints determined are hatching success, mortalities during different life stages, and juvenile growth (F1 generation, only). The sex ratio of the exposed fish groups is determined either by macroscopic inspection of gonads, and can be confirmed by a histological analysis (F1 generation) of the fish.

Spawning performance (fecundity) and fertilization rate are recorded for adult fish of F0- and F1-generation and the time to first spawning for the F1-generation.

To obtain mechanistic data for further interpretation of the observed effects the study includes the measurement of biomarkers and a histopathological examination of fish gonads.

Blood plasma samples of the adult fish of F0- and F1 generation are taken and measured for vitellogenin (VTG) concentrations. Furthermore, a histopathological examination of the fish gonads of the adult fish of F0- and F1- generation is performed, including maturation stages and pathological lesions.

From the results obtained, the threshold concentrations of the chemical are determined, i.e. the concentration with significant observed sublethal or lethal toxic effects ("LOEC") and the highest concentration tested without any significant effects ("NOEC"). Table 1 gives an overview on the basic test design of the ZEOGRT.

Table 5: Zebrafish extended one generation reproduction test (ZEOGRT), basic test design

Time of exposure	Fish age	Phase	Course	Endpoint
0 d	approx. 6-12 months (<1 year of age)	Reproduction F0 generation	Start with spawning groups 5 male/5 female fish	Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility) (Cumulative egg no.)
21 d	0 d	Early life stage F1 generation	Start with 36 fertilized eggs per vessel (2x18 eggs in stainless steel fry cages)	Time to hatch Hatching success
24 d	3 d		Begin of hatch (hatch completion between 4 to 6 dpf)	
27 d	6 d		Feeding with breeding food ad libitum	
5 w	14 d		Feeding with *Artemia salina* (Lifefood)	
6 w	21 d	F0 generation	Termination	Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) vitellogenin content in males and females
6 w	21 d	Early life stage F1 generation	Photographic determination of survival. Transfer to main aquaria	Post-hatch survival
8 w	35 d		Photographic determination of length and survival Random reduction to 20 individuals	Post-hatch survival Length
12 w	63 d	Juvenile growth F1 generation	Photographic determination of length and survival	Survival Length
13 - 18 w	70 - 105 d	Reproduction F1 generation	Introduction of spawning trays Daily evaluation of egg numbers and fertilization rates	Time to first spawning
15 - 20 w	84 d - 119 d			Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility)
20 w	0d	F2 generation	Start with 20 fertilized eggs per vessel (2x10 eggs in fry cages)	Time to hatch Hatching success
	96 h		hatch	

Time of exposure	Fish age	Phase	Course	Endpoint
20 – 22 w	119 – 133 d	Test termination F1 generation	End of F1 generation	Survival, Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) vitellogenin content in males and females

1.4.2 Test acceptability criteria

The following criteria were defined to assess the test performance and data quality. To ensure consistency with existing fish test guidelines on endocrine acting properties of substances, the list contains accepted values and ranges for the different parameters.

This includes validity criteria as defined in OECD TGs 210, 229, 234 and 240.

Test conditions

- Dissolved oxygen: ≥ 60 %
- Mean water temperature: 26 ± 2 °C

Biological parameters

- Parental generation (F0)
  - Successful reproduction: at least 10 eggs per female and day, 80 % fertilization
  - Survival of adults: ≥ 90 %
- First filial generation (F1)
  - Hatching success: ≥ 80 %
  - Post-hatch survival: ≥ 75 %
  - Survival of juveniles and adults: ≥ 90 %
  - Successful reproduction: at least 10 eggs per female and day, 80 % fertilization
  - Sex ratio: between 30 % to 70 % (males or females)
- Chemical analysis: an analytical measurement of the test concentrations is compulsory.

1.5 ZEOGRT validation, studies and data sources

The following Table summarizes the studies conducted for both phase I and phase II of the validation as well as the available control data sets.

Table 6: ZEOGRT validation: Studies for both phase I and II, and additional data

Validation	Study number	Test substance
Phase I	1	Tamoxifen-Citrate
	2	Prochloraz
	3	Dienogest
	4	Dexamethasone
Phase II	5	Tamoxifen-Citrate
	6	Prochloraz
	7	Prochloraz
Additional data	8	control data only
	9	control data only
	10	control data only

2 Material and methods

2.1 Validation, phase I: Studies performed at Fraunhofer IME

2.1.1 Fish strain

The presented studies of phase I were conducted with the zebrafish strain established at Fraunhofer IME, commonly identified as the “West aquarium strain”.

A barcoding analysis of cultured fish was performed to analyse the genetic characteristics of the strain used and the genetic similarity to other zebrafish strains. The analysis revealed a 100% similarity with other common zebrafish lines like AB or HL strain. The analysed DNA barcode regions included parts of the mitochondrial genome (published sequences under the gene assessing numbers KT_624623 and KT_624627).

The test fish which served as parental generation (F0) were reared from fertilized eggs at the test facility and were maintained in glass aquaria (150-L max) under conditions which ensure a good reproductive status. The age of the parental fish was approx. between 6 and 11 months of age at test start.

2.1.2 Number of animals used

The parental generation (F0) of each study was started with spawning groups of 10 fish (5 males, 5 females). To obtain the F1 generation, 36 embryos from the parental group were kept for hatching. To prepare spawning groups of equal numbers, the F1 groups were reduced to 20 juvenile fish per test vessel at day 35 pf. The surplus fish were anaesthetized and measured for the growth parameter total length. The F2 generation was prepared of 20 fertilized eggs from the F1 spawning groups.

The presented fish numbers referred to one test vessel.

Figure 1 illustrates the outline of the protocol referred to the animal numbers per treatment concentration.

If a setup with 5 test concentrations and a dilution water control is considered, the total fish numbers used were finally:

- 240 fish for parental generation (F0)
- 864 embryos for first filial generation (F1) generation.

The total fish number is thus 1104 animals. The study with the glucocorticoid dexamethasone was conducted with 6 test concentrations. Thus, the total fish number in that study was finally 1288.

As the embryos for second filial generation (F2) generation were terminated before they enter a stage of free feeding, they were not considered as protective life stage according to the European Animal welfare legislation (EEC 2010) and thus not part of the calculation.

The chosen numbers both considered the specification of fish numbers as given for specific life stages in existing fish test guidelines, i.e. OECD 229 (2012) for setting up the adult spawning groups of F0 (i.e. 5 males, 5 females for zebrafish) or OECD 210 (2013) for preparing early life stages.

Existing life cycle test protocols described a preparation method for F1 using 50 to 100 fertilized eggs (OECD 2008). The embryo number chosen for ZEOGRT should be minimized to a necessary

number, but to ensure that an adult group of 20 fish could be achieved for the start of the reproduction period.

Considering the requirements for control hatch (at least 80%) and post-hatch survival (at least 75%), a minimum number of 36 eggs per vessel was found to be sufficient. Moreover, the preparation of F1 groups was splitted over two days to prevent genetic bias.

These assumptions will be assessed under consideration of the available data.

2.1.3 Animal welfare application

All studies performed at the Fraunhofer IME have been registered and approved with the Federal Animal Welfare Authority of North Rhine Westphalia, under the reference numbers 84-02.04.2017.A317 and 81-02.04.2018.A031.

2.1.4 Test substances

The following test substances were used in the studies and applied in the concentrations as shown in Table 7.

Table 7: ZEOGRT validation phase I: Test substances and test concentrations applied

Study number	Test substance	CAS number	Purity	Supplier	Nominal test concentrations	
1	Tamoxifen-Citrate	54965-24-1	≥98 %	Sigma-Aldrich	[µg/L]	0.20, 0.63, 2.0, 6.3, 20
2	Prochloraz	67747-09-5	99.6 %	Kindly provided by BASF	[µg/L]	3.2, 10, 32, 100, 320
3	Dienogest	65928-58-7	99.9 %	Selleckchem, USA	[ng/L]	3.2, 10, 32, 100, 320
4	Dexamethasone	50-02-2	98 %	ABCR GmbH, Germany	[µg/L]	0.32, 1.0, 3.2, 10, 32, 100

2.1.5 Preparation of test solutions

All experiments were conducted under flow through conditions. Pre-dissolved test substance was continuously pumped into the test vessels to ensure stable test concentrations throughout the test period. For application of the substances, appropriate stock solutions were prepared and diluted and stored in glass tanks. The final dilution was achieved by a dosing pump system, mixing dilution water and application solution to the target concentration. The amounts of stock and application solutions were calculated to ensure an exchange of 5 test vessel volumes per day. All substances were stable under the aqueous conditions, thus, the intended basic application regime, e.g. including a 5-fold exchange per day and test vessel, was suitable for all studies conducted.

2.1.6 Chemical analysis of the test substance concentrations

The concentrations of the applied substances were measured in regular intervals throughout every study. Before starting the in-life phases, the methods were validated following the basic requirements of the guideline SANCO/3029/99 rev. 4 (EC 2000). This included the measurement of fortification samples at the LOQ determined for each substance and the 10-fold

LOQ. A further fortification step was set to encompass the calibration range of each method. Table 8 presents the used analytical methods for each substance and the corresponding LOQ values.

The analyses were conducted at the analytical laboratories of Fraunhofer IME, Schmallenberg, Germany.

Table 8: Chemical analysis of the test substances: Analytical method and LOQ applied

Study number	Test substance	Analytical method	Limit of quantification (LOQ)		Mean RSD [%] of validation (fortification experiment)	Lowest test concentration applied	
1	Tamoxifen-citrate	LC-MS/MS	0.05	[µg/L]	9.62	0.20	[µg/L]
2	Prochloraz	LC-MS/MS	1.0	[µg/L]	3.48	3.2	[µg/L]
3	Dienogest	LC-MS/MS	1.5	[ng/L]	5.76	3.2	[ng/L]
4	Dexamethasone	LC-MS/MS	0.10	[µg/L]	4.88	0.32	[µg/L]

2.1.7 Test performance

2.1.8 Good laboratory practise

The test facility at Fraunhofer IME is certified according to Good Laboratory Practice (GLP). The present studies were conducted using available SOPs and a data recording system which follows GLP requirements (OECD 1998).

2.1.8.1 Test vessel

The test vessels were glass aquaria with a total volume 28 L and approximately 25 L of test solution. Each replicate group was kept in an individual test vessel. Each test chamber was labelled with the study number, the test item and test concentrations. Each test vessel was equipped with fry cages for initiation of the filial generations, being glass cylinders with a diameter of 8 cm and a brim height of 10 cm. The bottom of each cage was a Teflon gaze with a mesh width of approximately 350 µm. These fry cages were used to keep the embryos and larvae for both F1 and F2.

2.1.8.2 Test conditions

The light regime was 12 hours light / 12 hours dark. The water temperature was maintained by placing the vessels into a temperature-controlled water bath set to 26.0 °C ± 1.5 °C. The pH and oxygen concentration of the water were measured in each aquarium directly before adding the fish and thereafter at least twice a week. The water temperature was measured on workdays. The oxygen concentration of the test solution should not be lower than 60 %. There was no additional aeration of the test vessels. Uneaten food and faeces were removed from the fry cages and the test aquaria at regular intervals.

2.1.8.3 Flow through conditions

For two replicates each, an individual dosage system was used, i.e., two dosage systems for each treatment concentration. The dilution water was delivered into a mixing chamber placed on a magnetic stirrer via a membrane pump (Prominent, Heidelberg, Germany). A corresponding amount of the application solution was applied to the mixing chamber using a membrane pump with stainless steel head (Prominent, Heidelberg, Germany).

At test start, the flow through system was served by test solutions at least 24 hours before introducing the fish. An appropriate amount of stock solution was prepared, which was renewed daily. The prepared test solution flowed into the test vessels via flexible Teflon tubes, distributed to the two vessels by an electronically regulated distributor driven by compressed air. Controls were served by dilution water only.

Control and all test concentrations were run in 4 replicate aquaria, each. For every test vessel, a water flow rate of approx. 5 L/h was adjusted (max. variability of flow rate of 10 % throughout the test), resulting in a daily turnover of approx. 5 volumes/day. The pumping device as well as the consumption of the stock solution were checked daily.

2.1.8.4 Initiation of the study

At test start, 5 male and 5 female fish were allocated to each replicate. Prior to test start, the fish groups were held under test conditions for at least 14 days to record spawning success during the pre-treatment phase.

Females and males were taken from batches of the same age. If single fish from the main spawning groups had to be replaced, they were taken from the same batch. The spawning groups were composed by randomized distribution of males and females.

When fish in all test vessels achieved daily spawning of at least 10 eggs per female and fertilization rates equal to or above 80 %, the exposure phase was started. One spawning group each was placed in each test vessel. The fish groups were randomly distributed to the test vessels. MS Excel was used for the randomized allocation of the spawning groups to the individual control and treatment vessels.

2.1.8.5 Duration of exposure

From the test start onwards, parental fish from F0 generation were exposed during a spawning period of three weeks. In week 4, fertilized eggs were kept to prepare the F1 generation. The F1 fish were monitored over 5 weeks during their early life stage phase, followed by a juvenile growth phase for further 4 weeks. After reaching sexual maturity, start of reproduction and spawning success was observed and recorded over a period of approx. 5-8 weeks. Finally, an F2 generation was prepared by keeping eggs of F1 parental animals and were exposed until hatch.

For details on the basic test design and time schedule, refer to Table 5.

2.1.8.6 Feeding regime

Adult animals of both F0 and F1 generation were fed once daily ad libitum with commercially available flake food and brine shrimp nauplii (*Artemia salina*).

The early life stages of the F1 generation received a specific feeding regime. When hatch was finished, e.g. from day 5 dpf onwards, larvae were fed twice daily with ground larval diet (TetraMin Baby, Tetra Werke, Melle, Germany) and liquid rearing feed (Nobil fluid, JBL, Neuhofen, Germany). Approximately from day 14 pf onwards, brine shrimp nauplii (Artemia

salina) was added ad libitum to the daily food. From approximately day 21 pf onwards, breeding food was exchanged by ground flake food (ad libitum).

2.1.9 Observations and biological endpoints

Observations on fish of all life stages were made daily. Dead eggs, larvae, fry; dead juvenile and adult fish were recorded and removed immediately. Any abnormal behaviour was recorded.

2.1.9.1 Parental generation (F0)

After start of exposure, spawned eggs were collected daily from each test vessel for at least 20 days. Total egg numbers per replicate as well as the number of fertilizzed eggs were determined. Eggs were collected daily without interruption (i.e. including weekends) as this may cause variability in the data set. At the end of this phase, fertilized eggs were collected from each fish tank to prepare the F1 generation, see chapter 2.1.9.2 for details.

After successful start of the F1 generation, based on fry survival recorded after three weeks pf, the fish of the F0 generation were terminated. All fish were anaesthetized using chloro-butanol. A blood sample was taken from each fish via heart puncture. After successful blood sampling the fish were killed humanly with a dorsal cut.

The phenotypic sex of each fish was determined by macroscopic inspection of fish gonads, i.e. ovary structure for females and testicular tubes for males. This was conducted to confirm the correct settings of the spawning groups. Afterwards, individual lengths and weights were measured to allow a sex-specific evaluation of the growth performance. Finally, the fish were transferred to appropriate fixative to allow a histopathological analysis of fish tissue according to the OECD guidance document 123 (OECD 2010). The phenotypic sex of each fish was confirmed by histological evaluation of fish gonads. Moreover, an analysis of maturation stages was conducted.

2.1.9.2 First filial generation (F1)

At start of the F1 generation, 36 (2 x 18) fertilized eggs, collected on two consecutive days (i.e. 18 eggs per day), were placed randomized in the suitable fry cages fixed under the water surface of each test vessel. The fry cages were glass cylinders with a diameter of at least 8 cm and a brim height of 10 cm, the sieve net at the bottom at a mesh width of approximately 400 µm. Each aquarium was equipped with two fry cages. 144 eggs were used for each test concentration and the control.

Hatch of zebrafish fry started around day 3 pf and was finished after 6 to 7 days pf. Hatching rates was estimated by daily counting of non-hatched eggs between day 2 and at least 90 % hatch.

There was a daily counting of dead fry and fish larvae. Moreover, after 21 and 35 days pf, the number of surviving fish was determined by photographic counting. After 21 days pf, the fish from the two fry chambers were pooled and released into the test vessels. After 35 days pf, length of each individual fish was measured (photographic length determination) and the fish groups were reduced randomly to 20 individuals. After 63 days pf, the length of individual fish was again measured (photographic length determination). From day 56 pf onwards, spawning trays were introduced into the aquaria. Spawned eggs were collected daily, counted and the number of fertilized eggs were determined.

The reproductive endpoints like egg number and fertilization rate were observed for 20 daily counts.

The criteria for starting the quantitative evaluation of the parameters were a daily egg production of minimum 15 eggs and a fertilization rate of ≥80% for controls. Both parameters should be found on three consecutive days.

2.1.9.3 Second filial generation (F2)

For the start of the second filial generation (F2), 20 (2 x 10) fertilized eggs spawned by the adult fish of each replicate were placed randomized in fry cages (two per vessel) and were fixed under the water surface of each test vessel. Hatching success and time to hatch were observed daily and was finished after 96 h. Hatching rates were estimated by daily counting of non-hatched eggs between day 2 and total hatch. After 96 h, all fry were terminated.

2.1.9.4 Test termination

Fish not further used in the study and all fish not being subject to blood collection and tissue sampling at the end of the study were euthanized after over-dosage with chloro-butanol.

At test termination, the fish of the F1 generation were sacrificed and prepared as described below. All fish were anaesthetized using chloro-butanol. A blood sample was taken from each fish via heart puncture. After successful blood sampling, the fish were killed humanly with a dorsal cut.

The phenotypic sex of each fish was determined by macroscopic inspection of fish gonads. Individual lengths and weights were measured.

After determination of length and weight, the fish were transferred to appropriate fixative to allow a histopathological analysis of fish tissue according to the OECD guidance document 123 (OECD 2010). The phenotypic sex of each fish was confirmed by histological evaluation of fish gonads.

2.1.10 Specific methods

2.1.10.1 Egg collection

During the reproduction phase, spawned eggs were collected daily in a glass spawning tray which was placed at the bottom of the test vessels. The tray was covered with a stainless-steel lattice to prevent adult fish from eating the eggs. An artificial plant substrate (modified method according to (Nagel 1986) made of glass pearls was attached to the lattice to stimulate spawning into the tray. The artificial substrate was leached for at least one week in purified tap water to remove residual contaminants.

Spawning started soon after switching on of light. Within two hours after spawning, the eggs were transferred into a sieve, rinsed with clean water to remove debris, put into glass dishes, and counted as total number and number of fertilized eggs on a replicate basis. Fecundity was expressed as the total number of eggs per female and day. The number of fertilized eggs was related to the total number and the respective percentage values of the fertilization rate were calculated. Fertilization was determined by observing cleavage stages (>four cell stage) using a binocular.

2.1.10.2 Photography and image analysis

For fry counts and total length measurements, photographs were made using the digital camera: Canon Cybershot (Canon, Tokio, Japan). Digital image processing was performed by using appropriate photo evaluation software tool ImageJ (National Institutes of Health, Bethesda (Maryland, USA)). Fish were netted and placed in rectangular vessels with a low water level. This was placed in the photo device (light plate with additional illumination from above). A

visible ruler was placed in every vessel. The level of water was adjusted to be the same in each vessel.

From the fish larvae, aged 35 dpf, the total length was measured to the nearest 0.1 cm. After photographing, the fry was carefully re-introduced into the test vessel.

2.1.10.3 Blood collection and preparation

To avoid coagulation of blood and degradation of protein, the samples were collected within phosphate-buffered saline (PBS) buffer containing heparin (1000 units/mL) and the protease inhibitor aprotinin (at least 2TIU/mL). As ingredients for the buffer, heparin as ammonium-salt (Sigma-Aldrich, Darmstadt, Germany) and lyophilized aprotinin (Roth, Karlsruhe, Germany) was used. For blood sampling a syringe (1 mL) with a fixed needle was used. The syringe was prefilled with buffer (approximately 300µL) to completely elute the small blood volumes from each fish. Blood samples ranged from 15-30 µL and were taken by cardiac puncture. At first the fish were anaesthetized with chloro-butanol (20 g/L). Plasma was separated from the blood via centrifugation (30 min; approx. 5000 g; 4°C) and immediately stored at -80°C until further analysis.

2.1.11 Measurement of biomarker vitellogenin

The measurements of zebrafish vitellogenin were performed according to the method published by Holbech et al. (Holbech, Andersen et al. 2001). All measurements were conducted at the laboratory of Henrik Holbech at the University of Southern Denmark (SDU) at Odense, Denmark.

2.1.12 Histopathology

The evaluation of fish gonads of F0 and F1-generation was conducted at the Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM). The tissue slides were finally evaluated, and results were reported by Dr. Dirk Schaudien as the responsible pathologist. The examination was performed following the OECD guidance document 123 (OECD 2010).

2.1.12.1 Tissue fixation procedure for histology

The fish carcasses were fixated following the method described in the OECD guidance document 123 (OECD 2010). After blood collection the fish were opened ventrally by an incision along the midline of abdomen with dissection scissors taking care not to damage the gonads or other organs. Sexing of fish was carried out by macroscopical observation of gonads. Afterwards the fish were fixated to allow a histopathological evaluation.

The whole fish was placed individually in pre-labelled plastic vials which were filled with at least 30 mL of Davidson's fixative (Davidson's fixative: e.g. for 1 L: 200 mL formaldehyde (37%), 100 mL glycerol, 300 mL ethanol, 300 mL distilled water and shortly added before use 100 mL pure (100%) acetic acid). The fixation was done overnight (approx. for 24 h) followed by the transfer on the next day into 10% neutral buffered formalin according to OECD guidance document 123 (OECD 2010).

Of each group, all fish were prepared for histological investigation. The samples were consecutively labelled according to their respective treatment level, replicate and sample number. The samples were sent from the test facility to the histopathological lab at ambient conditions, together with a sample description, by courier as soon as possible after termination of the respective generation.

2.1.12.2 Evaluation of gonad tissue

The occurrence of female, male, undifferentiated, hermaphrodite and ovotestis was recorded based on histological observation for F0 and F1 fish. To assign the fish to the different categories, the following description was considered. During normal sexual development, all zebrafish develop an indifferent gonad with primordial germ cells ('juveniles'). From this stage, all individuals develop so called 'protogynic gonads', with only undeveloped ovaries with oogonia to perinucleolar oocytes (Takahashi 1977, Maack and Segner 2003). Male zebrafish afterwards enter a transition phase, in which the oocytes degenerate. At this stage, no morphologically distinguishable male germ cells are present. Until this stage, the fish are defined as 'undifferentiated', as the sex could not be determined unequivocally. During progression in the transition phase, degenerative oocytes are still present, however also spermatogonia are present. These fish are defined as in 'transition phase' and could be ascribed to the sex category 'male'. In contrast, 'ovotestis' is defined as presence of more than one oocyte in mature testis or spermatogenic cells in mature ovaries. This is a pathological observation in mature gonads. Fish with more mature ovaries are ascribed to the category 'female'. The results were statistically evaluated.

Furthermore, a detailed histopathological examination of gonads was conducted for the males and females of control and all treatment concentrations. During the histological examination the sex of the animals as well as the stage of the development of the gonads were determined according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010).

The results and a report of the histological sex determination for fish treated are presented in the appendix report.

The morphologic criteria for staging ovaries and testes are given in the following table:

Table 9: Criteria for staging gonads according to the OECD guidance document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010)

	Criteria for staging ovaries	Criteria for staging testes
Stage 0	**Undeveloped**: entirely immature phases (oogonia to perinucleolar oocytes); no cortical alveoli	**Undeveloped**: entirely immature phases (spermatogonia to spermatids) with no spermatozoa.
Stage 1	**Early spermatogenic:** vast majority (e.g., > 90%) are pre-vitellogenic follicles, predominantly perinucleolar through cortical alveolar	**Early spermatogenic**: immature phases predominate, but spermatozoa may also be observed; the germinal epithelium is thinner than it is during Stage 2.
Stage 2	**Mid-development**: at least half of observed follicles are early and mid-vitellogenic.	**Mid-spermatogenic**: spermatocytes, spermatids, and spermatozoa are present in roughly equal proportions; the germinal epithelium is thinner than Stage 1, but thicker than Stage 3.
Stage 3	**Late development**: majority of developing follicles are late vitellogenic.	**Late spermatogenic**: all stages may be observed; however, mature sperm predominate; the

	Criteria for staging ovaries	Criteria for staging testes
		germinal epithelium is thinner than it is during Stage 2.
Stage 4	**Late development/hydrated**: majority of follicles are late vitellogenic and mature/spawning follicles; follicles are larger as compared to Stage 3.	**Spent**: loose connective tissue with some remnant sperm.
Stage 5	**Post-ovulatory**: predominately spent follicles, remnants of theca externa and granulosa.	-

Furthermore, the occurrence of the following lesions was investigated as well.

Ovaries:

- Increased oocyte atresia
- Egg debris
- Granulomatous inflammation
- Increased post-ovulatory follicles

Testes:

- Testis-ova
- Increased testicular degeneration
- Interstitial cell hypertrophy/hyperplasia
- Increased proportion of spermatogonia
- Increased interstitial proteinaceous fluid
- Asynchronous germ cell development
- Granulomatous inflammation

These lesions were graded according to the following schema:

Grade 0: Not observable. This grade is used if there are no findings associated with a particular diagnostic criterion.

Grade 1: Minimal. Ranging from inconspicuous to barely noticeable, but so minor, small, or infrequent as to warrant no more than the least assignable grade. For discrete changes, grade 1 is used when there are fewer than 2 occurrences per section. For multifocal or diffusely distributed alterations, this grade is used for processes where ≤ 20 % of the tissue in the section is involved.

Grade 2: Mild. A noticeable feature of the tissue. For discrete changes, grade 2 is used when there are 3 - 5 occurrences per tissue section. For multifocal or diffusely distributed alterations, this grade is used for processes where 20 - 50 % of the tissue in the section are involved.

Grade 3: Moderate. A dominant feature of the tissue. For discrete changes, grade 3 is used when there are 6 - 8 occurrences per tissue section. For multifocal or diffusely distributed alterations, this grade is used for processes where 50 - 80 % of the tissue in the section are involved.

Grade 4: Severe. An overwhelming feature of the tissue. For discrete changes, grade 4 is used when there are more than 9 occurrences per tissue section. For multifocalor diffusely distributed alterations, this grade is used for processes where > 80 % of the tissue in the section are involved

2.1.13 Chemical analysis

2.1.13.1 Equilibration of the flow through system and sampling

Prior to the proposed test start, the flow through system was equilibrated and samples from all test vessels were taken for chemical analysis. This was performed concomitantly to ensure the right test concentrations and the correct adjustment of the dosing pump system.

If it turned that samples could not be measured within 24 h after sampling, they were immediately be kept frozen at ≤ -18 °C.

If the test concentrations could not be confirmed, the dosing system was re-adjusted and after a further equilibration phase with at least one total exchange of test media, samples of the vessels served by the adjusted dosing system were taken, and the procedure was repeated.

After confirmation of the right test concentrations, the experimental phase was started. Samples from all test vessels including controls were taken at test start, one vessel per replicate pair was sampled weekly thereafter. The two vessels of one vessel pair were sampled alternately.

2.1.14 Statistical calculations

For each endpoint, the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values were determined. Alternatively, EC_{10} and EC_{50} concentrations were calculated, if possible and reasonable.

To allow a decision for the appropriate post-hoc test, the data were initially checked for normality and variance homogeneity. NOECs were calculated, using ANOVA, followed by appropriate post hoc tests, e.g. Dunnett's (Dunnett 1955, Dunnett 1964), or Williams test (Williams 1971, Williams 1972) or respective non-parametric approaches (e.g. Jonckheere-Terpsta test (Jonckheere 1954), (Terpstra 1952)).

The computer software ToxRat® Professional 3.3.0 (ToxRat® Solutions GmbH) was used for statistical evaluations.

For NOEC / LOEC-determination, quantal data for fertilization and sex-ratio were arcsine-square-root transformed prior to analysis.

The NOEC / LOEC-determination for the parameters hatch and post-hatch survival were calculated by the step-down Cochran-Armitage test for quantal responses that are consistent with a monotone concentration-response and with no evidence of extra-binomial variance (OECD 2006). When there is evidence of extra-binomial variance, the Rao-Scott modification of the Cochran-Armitage test is recommended (Rao and Scott 1992) (Rao and Scott 1999) or Williams or Dunnett's (after an arcsin-square-root transform) or Jonckheere-Terpstra test applied to replicate proportions.

The statistical evaluation follows the flow chart as presented in Annex 6.1 to this report.

3 Results

3.1 Validation phase I: Studies performed at Fraunhofer IME

3.1.1 Phase I, Study 1: ZEOGRT with Tamoxifen-Citrate

3.1.1.1 Test conditions

The water temperatures did not differ by more than ± 1.5 °C between test vessels at any one time during the exposure period. The mean water temperatures per replicate in controls and treatments were calculated to be between 26.2 and 26.8 °C. The single measurements in all test vessels were between 25.8 and 27.3 °C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C. The mean oxygen concentrations per replicate throughout the test period were determined to be between 86 and 90 %, based on single measurement between 74 and 107 %. The mean pH levels on replicate basis were between 7.60 and 7.94 in controls and treatments. The single pH measurements were between 7.30 and 8.22. There was no impact of the substance application on the pH level.

Finally, stable conditions of exposure could be confirmed.

All mean values and standard deviations are summarized in Table 10 to Table 12. For more details, please refer to the appendix report.

Table 10: Study 1, ZEOGRT with Tamoxifen-Citrate: Water temperature [°C]

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	26.7	0.3	25.9	27.3
	B	26.7	0.4	25.9	27.3
	C	26.7	0.2	26.2	27.2
	D	26.8	0.2	26.3	27.3
0.20	**A**	26.6	0.4	25.8	27.3
	B	26.6	0.4	25.9	27.3
	C	26.7	0.2	26.2	27.3
	D	26.7	0.2	26.1	27.3
0.63	**A**	26.5	0.3	25.9	27.2
	B	26.5	0.3	26.0	27.2
	C	26.7	0.3	26.0	27.3
	D	26.7	0.2	26.0	27.3
2.0	**A**	26.5	0.3	25.9	27.1
	B	26.6	0.3	26.0	27.2
	C	26.6	0.2	26.0	27.1

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
	D	26.6	0.2	25.9	27.2
6.3	**A**	26.6	0.4	25.9	27.3
	B	26.6	0.4	25.8	27.3
	C	26.6	0.2	25.9	27.2
	D	26.7	0.2	26.0	27.1
20	**A**	26.2	0.2	25.8	27.1
	B	26.2	0.2	25.8	27.1
	C	26.6	0.2	26.1	27.2
	D	26.6	0.2	26.2	27.2

SD = Standard deviation

Table 11: Study 1, ZEOGRT with Tamoxifen-Citrate: Oxygen concentration [%]

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	90	4.9	82	105
	B	88	3.8	79	95
	C	88	3.7	79	99
	D	86	3.9	74	96
0.20	**A**	88	4.4	81	100
	B	90	4.5	81	100
	C	87	4.8	77	99
	D	87	5.0	76	104
0.63	**A**	88	4.5	79	99
	B	87	4.8	79	99
	C	88	4.3	77	95
	D	87	4.6	78	99
2.0	**A**	88	4.9	79	102
	B	88	4.0	79	98
	C	87	3.9	77	95
	D	86	4.0	76	92

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
6.3	**A**	90	4.5	80	98
	B	90	5.4	79	107
	C	90	5.5	79	106
	D	89	5.2	77	99
20	**A**	87	6.2	78	100
	B	88	7.3	77	105
	C	89	7.0	77	100
	D	87	5.8	77	95

SD = Standard deviation

Table 12: Study 1, ZEOGRT with Tamoxifen-Citrate: pH value

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.72	0.15	7.42	8.16
	B	7.72	0.15	7.43	8.14
	C	7.67	0.15	7.36	8.06
	D	7.66	0.16	7.30	8.07
0.20	**A**	7.90	0.11	7.60	8.15
	B	7.93	0.13	7.66	8.18
	C	7.94	0.11	7.73	8.20
	D	7.94	0.11	7.74	8.22
0.63	**A**	7.84	0.11	7.64	8.13
	B	7.83	0.11	7.64	8.12
	C	7.87	0.12	7.70	8.16
	D	7.89	0.11	7.70	8.16
2.0	**A**	7.71	0.12	7.50	8.05
	B	7.69	0.13	7.49	8.03
	C	7.72	0.11	7.54	8.04
	D	7.72	0.11	7.53	8.04

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
6.3	**A**	7.82	0.13	7.61	8.09
	B	7.84	0.13	7.66	8.13
	C	7.89	0.14	7.65	8.17
	D	7.91	0.13	7.73	8.16
20.0	**A**	7.63	0.09	7.50	7.87
	B	7.61	0.10	7.49	7.88
	C	7.60	0.09	7.43	7.82
	D	7.60	0.08	7.48	7.77

SD = Standard deviation

3.1.1.2 Chemical analysis

The test substance was applied by using Methanol as a solvent vehicle. To prepare the application solution, methanol was completely evaporated before filling up with water. Decrease of test substance concentrations, e.g. due to degradation or adsorption of the substance could not be observed.

The mean measured concentration were calculated to be 0.20, 0.59. 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L, corresponding to 101, 94.0, 93.7, 103 and 106 % of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations of Tamoxifen-Citrate.

All results of the chemical analysis are shown in Table 13. For more details, please refer to the appendix report.

Table 13: Study 1, ZEOGRT with Tamoxifen-Citrate: Chemical analysis

Nominal concentration Tamoxifen-Citrate	Replicate	Measured concentration Tamoxifen-Citrate							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
[µg/L]		Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	**A**	<LOQ	-	-	-	**<LOQ**	-	-	-
	B	<LOQ	-	-	-				
	C	<LOQ							
	D	<LOQ							
0.20	**A**	0.19	0.02	94.6	11.2	**0.20**		**101**	4.3
	B	0.20	0.03	102	16.8				
	C	0.20	0.03	102	15.8				

Nominal concentration Tamoxifen-Citrate	Replicate	Measured concentration Tamoxifen-Citrate							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
[µg/L]		Mean	SD	Mean	SD	Mean	SD	Mean	SD
	D	0.21	0.04	105	19.5				
0.63	**A**	0.58	0.07	92.8	11.6	**0.59**		**94.0**	5.4
	B	0.64	0.10	102	16.1				
	C	0.56	0.04	89.7	7.0				
	D	0.58	0.07	91.6	11.7				
2.0	**A**	1.8	0.2	92.3	10.1	**1.9**		**93.7**	6.6
	B	2.0	0.3	102	15.0				
	C	1.7	0.2	86.0	8.9				
	D	1.9	0.2	94.3	11.6				
6.3	**A**	6.3	0.7	99.2	10.8	**6.5**		**103**	8.0
	B	5.9	0.7	93.2	11.4				
	C	6.9	0.5	109	8.2				
	D	6.9	0.7	110	11.1				
20	**A**	22	2	108	10.2	**21**		**106**	11.6
	B	24	4	119	20.3				
	C	18	1	91.2	3.8				
	D	22	3	108	15.4				

SD = Standard deviation

3.1.1.3 Biological results, parental generation (F0)

The parental generation was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. At test day 30, one female fish in control (replicate B) had to be sacrificed, since the fish suffered from an infected skin lesion. More details are presented in Table 14.

Reproduction was recorded in terms of egg numbers and fertilization rates in the period from day 1 pf to day 21. For the controls, a mean of 55 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be 47, 58, 48, 42 and 37 at 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean egg numbers and standard deviation are shown in Table 15. The mean fertilization rate for the controls was calculated to be 84.4 %. For the exposed groups, the fertilization rates were determined to be between 78.9 % (at 6.5 µg Tamoxifen-Citrate/L) and 87.3 % (at 0.20 µg Tamoxifen-Citrate/L). The statistical evaluation revealed a significant reduction of egg numbers per female and day compared to control at 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured;

Williams Multiple t-test, $p < 0.05$, one-sided smaller). The fertility rate was not altered by the test item exposure (Dunnett`s Multiple t-test, $\alpha=0.05$, one-sided smaller).

After successful initiation of the first filial generation (see below for details), the adult fish groups of the parental generation were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads and verified by histological analysis. The inspection revealed that most of the groups were set correctly, i.e. 5 males and 5 females were present (see Table 14 for details).

The mean fish total lengths of males were determined to be 3.8 cm in the control and 3.9 cm each in treatment. The mean fish total lengths of females were determined to be 4.0 cm in control and 3.9, 3.9, 3.9, 4.1 and 4.0 cm in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean wet weights of males were determined to be 0.451 g in control and 0.503, 0.495, 0.508, 0.481 and 0.510 g in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean wet weights in females were determined to be 0.688 g in control and 0.642, 0.639, 0.651, 0.720 and 0.660 g in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical evaluation revealed no effect on fish growth as assessed by determination of total length and wet weight for the adult fish at termination (Dunnett`s Multiple t-test, $\alpha=0.05$, two-sided).

All mean values and standard deviation of all biological parameters are summarized in Table 15.

For more details, please refer to the appendix report.

Table 14: Study 1, ZEOGRT with Tamoxifen-Citrate: Overview fish group setting (verified by histopathological analysis) and observed mortality, F0

	Mean measured concentration Tamoxifen-Citrate [µg/L]																							
	Control				**0.20**				**0.59**				**1.9**				**6.5**				**21**			
Replicate	**A**	**B** *	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C** **	**D**	**A**	**B**	**C**	**D**
Parental generation F0																								
Male (m) [n]	5	5	5	4	6	4	3	5	5	3	5	5	5	3	5	4	4	7	5	6	5	5	5	6
Female (f) [n]	5	4	5	6	4	6	7	5	5	7	5	5	5	7	5	6	6	3	5	4	5	5	5	4
Mortality (no. and sex) [n]	-	1 f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

*: At test day 30 (08.03.2018), one female fish in control replicate B had to be sacrificed, since the fish suffered from an infected skin lesion.

**: One male fish was corrected by histopathological examination to female (replicate C at 6.5 µg/L (mean measured)).

Table 15: Study 1, ZEOGRT with Tamoxifen-Citrate: Biological results for parental generation (F0)

Parameters	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.20**	**0.59**	**1.9**	**6.5**	**21**
Mean egg number per day and female [n]	**55**	**47**	**58**	**48**	**42)[a]**	**37)[a]**
SD	9	4	4	4	14	10
Mean fertilization rate [%]	**84.4**	**87.3**	**86.9**	**84.6**	**78.9**	**81.3**
SD	4.5	6.3	6.2	2.9	7.0	7.9
Total length, males [cm]	**3.8**	**3.9**	**3.9**	**3.9**	**3.9**	**3.9**
SD	0.1	0.1	0.0	0.1	0.1	0.1
Total length, females [cm]	**4.0**	**3.9**	**3.9**	**3.9**	**4.1**	**4.0**
SD	0.1	0.1	0.1	0.1	0.1	0.1
Wet weight, males [g]	**0.451**	**0.503**	**0.495**	**0.508**	**0.481**	**0.510**
SD	0.049	0.044	0.039	0.060	0.023	0.034
Wet weight, females [g]	**0.688**	**0.642**	**0.639**	**0.651**	**0.720**	**0.660**
SD	0.061	0.056	0.030	0.079	0.074	0.053
Mean sex ratio, males [%]	**48.9**	**45.0**	**45.0**	**42.5**	**55.0**	**52.5**
SD	6.5	12.9	10.0	9.6	12.9	5.0
Mean sex ratio, females [%]	**51.1**	**55.0**	**55.0**	**57.5**	**45.0**	**47.5**
SD	6.5	12.9	10.0	9.6	12.9	5.0

SD = Standard deviation

)[a] Statistically significant reduction compared to control (Williams Multiple t-test, $p < 0.05$, one-sided smaller)

3.1.1.4 Biological results, first filial generation (F1)

The filial 1 (F1) generation was initiated by keeping fertilized eggs from the parental group and placing them in separate brood chambers placed in each test vessel. The allocation of eggs, in total 36, was divided over two subsequent days, which means that 18 eggs were kept on day 1 and a second set of eggs was kept on day 2. This approach was chosen to prevent that the whole clutch of eggs originated from the same female fish.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80 % in all test vessels (related to the number of eggs introduced). The mean hatching rate was 91.7 % in the controls and 84.0, 84.7, 84.7, 86.1 and 93.8 % in treatments with 0.20, 0.59, 1.9, 6.5 and

21 µg Tamoxifen-Citrate/L (mean measured), respectively. First larvae hatched at day 2 pf and hatch was completed at day 5 pf. No statistically significant effect on hatch was detected.

The number of surviving fish larvae was determined on day 21 and 35 pf by photo evaluation. After each photo recording, the fish were carefully re-introduced to the respective test vessel. The post-hatch survival rate in controls was calculated to be 88.9 % and 86.0 % for day 21 and 35 pf, respectively. The acceptance criteria, i.e. 75 %, was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be 86.4, 80.3, 75.6, 33.7 and 0 % at day 21 pf in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. At day 35 pf, the mean post-hatch survival rates were determined to be 86.4, 77.0, 73.2, 21.9 and 0 % in with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical analyses evaluating both time points revealed a significant reduction of larval survival between controls and treatment, i.e. at day 21 pf all treatments but the lowest one showed a significant higher mortality compared to controls (Step-down Cochran-Armitage Test Procedure; $p<0.05$; one-sided greater), while at day 35 pf only at the two highest treatment levels a statistically significant induced mortality was found (significantly higher mortality; Step-down Rao-Scott-Cochran-Armitage Test; $p<0.05$;one-sided greater).

On day 35 pf, the total length of all remaining fish larvae was measured. The mean total length in controls was calculated to be 1.8 cm. The fish lengths in the exposed groups were determined to be 1.7, 1.7, 1.6 and 1.0 cm in treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured), respectively. Please note, that no length measurement was possible at the highest treatment level since there were no more surviving fish left. The statistical evaluation revealed a significantly reduced total length compared to control in treatments with 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured) (Williams Multiple t-test; $p<0.05$; one-sided smaller).

After the measurement of fish lengths, the fish groups (i.e. treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured)) were randomly reduced to 20 fish each. On day 63 pf, the fish groups were again photographed, revealing a survival rate of 100 % in the controls as well as in all other concentrations tested, with exception of the highest one, i.e. a survival of 75.8 % at 6.5 µg Tamoxifen-Citrate/L (mean measured) was observed. The decrease was found to be statistically significant compared to control (Chi^2 2x2 Table test with Bonferroni Correction; $p<0.05$; one-sided greater). The mean total length at day 63 pf was measured to be 3.0 cm in the controls and 2.9, 2.9, 2.8 and 2.6 cm in treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured), respectively. No statistically significant effect compared to control was found (Williams Multiple t-test; $\alpha=0.05$; two-sided).

At day 63 pf and thereafter, spawning trays were introduced to assess and record the first spawning events. In the controls, the time to first spawning was in the range from day 68 to day 78 pf , while spawning onset was found to be in the range from day 72 to day 83 pf, from day 70 to day 85 pf and from day 70 to day 85 pf in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical analysis revealed no significantly delayed spawning onset compared to control (Dunnett`s Multiple t-test; $\alpha=0.05$; one-sided greater). From day 64 pf onwards, reproduction was recorded in terms of egg numbers and fertilization rates. For the control, a mean of 31 eggs per female and day was calculated. The mean egg numbers per female and day in the exposed groups were determined to be 29, 18 and 26 in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. Since there were no female but only male fish present in treatment with 6.5 µg Tamoxifen-Citrate/L (mean measured), no egg production was possible at all. Notably, at 0.59 µg Tamoxifen-Citrate/L (mean measured) the egg number per female was significantly reduced compared to control (Dunnett`s Multiple t-test; $p<0.05$; one-sided smaller). The mean fertilization rate was calculated

to be 80.2 % in the controls and 87.5, 81.9 and 84.9 % in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. No significant effect compared to control was found (Dunnett`s Multiple t-test; α=0.05; one-sided smaller).

After successful initiation of the second filial generation (F2), the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The obtained results indicated a test item induced shift in sex ratio towards an increased number of males, respective fewer females (Williams Multiple t-test; p<0.05; two-sided). The sex ratio in the controls was 23.8 % males and 73.8 %, which was, however, outside the defined criteria of being between 30 to 70 % for each sex.

The mean fish total lengths in males were determined to be 3.9 cm in the controls and 3.8, 3.9, 3.8 and 3.9 cm in treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured), respectively. No significant effect compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean fish total lengths in females were determined to be 3.8 cm in the controls and 3.9, 4.0 and 4.0 cm in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. A significantly increased length at termination compared to control was found in treatments with 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured; Williams Multiple t-test; p<0.05; two-sided).

The mean fish wet weights of males were determined to be 0.474 g in the controls and 0.469, 0.487, 0.461 and 0.469 g in treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured), respectively. No significant effect compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean wet weights in females were determined to be 0.580 g for the controls and 0.609, 0.648 and 0.704 g in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. A significantly increased weight at termination compared to control was found in treatments with 0.59 and 1.9 µg Tamoxifen-Citrate/L (Williams Multiple t-test; p<0.05; two-sided).

All results, i.e. mean values and standard deviations, are shown in Table 16. For more details, please refer to the appendix report.

Table 16: Study 1, ZEOGRT with Tamoxifen-Citrate: Biological results for first filial generation (F1)

Parameters	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.20*	0.59	1.9	6.5**	21***
Mean hatching rate [%]	**91.7**	**84.0**	**84.7**	**84.7**	**86.1**	**93.8**
SD	6.0	7.3	8.3	2.8	10.4	7.3
Mean post-hatch survival at day 21 pf [%]	**88.9**	**86.4**	**80.3)[a]**	**75.6)[a]**	**33.7)[a]**	**0.0)[a]**
SD	8.5	8.9	12.5	14.7	8.6	0.0

Parameters	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.20*	0.59	1.9	6.5**	21***
Mean post-hatch survival at day 35 pf [%]	**86.0**	**86.4**	**77.0**	**73.2**	**21.9)[b]**	**0.0)[b]**
SD	9.6	8.9	13.7	17.1	15.0	0.0
Mean total length, day 35 pf (all fish pre-reduction) [cm]	**1.8**	**1.7**	**1.7**	**1.6)[c]**	**1.0)[c]**	**-**
SD	0.1	0.0	0.1	0.0	0.1	-
Mean survival day 63 pf (compared to no. post-reduction at day 35 pf) [%]	**100**	**100**	**100**	**100**	**75.8)[d]**	**-**
SD	0	0	0	0	25.4	-
Mean total length, day 63 pf [cm]	**3.0**	**2.9**	**2.9**	**2.8**	**2.6**	**-**
SD	0.1	0.1	0.1	0.1	0.2	-
Time to first spawning [fish age days pf]	A 68	74	85	84	-	-
	B 74	-)*	75	85	-	-
	C 78	72	70	70	-	-
	D 70	83	76	72	-	-
Mean egg number per day and female [n]	**31**	**29**	**18)[e]**	**26**	-	-
SD	10	1	5	8	-	-
Mean fertilization rate [%]	**80.2**	**87.5**	**81.9**	**84.9**	-	-
SD	7.3	1.6	11.3	5.3	-	-
Mean survival, adult stage (day 63 pf until termination) [%]	**100**	**98.3**	**98.8**	**100**	**100**	**-**
SD	0	2.9	2.5	0	0	-
Mean total length, males, adult stage [cm]	**3.9**	**3.8**	**3.9**	**3.8**	**3.9**	**-**
SD	0.1	0.1	0.2	0.1	0.1	-

Parameters	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.20*	0.59	1.9	6.5**	21***
Mean total length, females, adult stage [cm]	**3.8**	**3.9**	**4.0)[f]**	**4.0)[f]**	**-**	**-**
SD	0.1	0.1	0.0	0.0	-	-
Mean wet weight, males, adult stage [g]	**0.474**	**0.469**	**0.487**	**0.461**	**0.469**	**-**
SD	0.035	0.015	0.042	0.015	0.016	-
Mean wet weight, females, adult stage [g]	**0.580**	**0.609**	**0.648)[f]**	**0.704)[f]**	**-**	**-**
SD	0.040	0.045	0.042	0.014		
Mean sex ratio, [% males]	**23.8**	**25.6**	**33.4**	**47.6)[f]**	**100)[f]**	-
SD	10.3	10.9	24.9	8.8	0	-
Mean sex ratio, [% females]	**73.8**	**74.4)[g]**	**66.6)[g]**	**52.4)[g]**	**0)[g]**	**-**
SD	14.4	10.9	24.9	8.8	0	-
Unidentified (no gonads visible) [%]	**2.5**	**0**	**0**	**0**	**0**	**-**
SD	5.0	0	0	0	0	-

SD = Standard deviation

*: The mean treatment value is calculated on basis of three replicates only since replicate B was flooded at test day 45 (23.03.2018) and all larvae but two got lost. This affects all evaluations except hatch.

**: Only male fish present at 6.5 µg test item/L (mean measured).

***: No fish larvae survived at 21 µg test item/L (mean measured) (all larvae dead before day 21 pf).

)[a]: Significantly higher mortality compared to control.; Step-down Cochran-Armitage Test; p<0.05; one-sided greater.

)[b]: Significantly higher mortality compared to control.; Step-down Rao-Scott-Cochran-Armitage Test; p<0-05; one-sided greater.

)[c]: Significantly decreased compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[d]: Significantly higher mortality compared to control.; Chi² 2x2 Table test with Bonferroni Correction; one-sided greater.

)[e]: Significantly reduced egg no./female at 0.59 µg test item/L (mean measured) compared to control.; Dunnett`s Multiple t-test; p<0.05; one-sided smaller.

)[f]: Significantly increased compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[g]: Significantly decreased compared to control.; Williams Multiple t-test; p<0.05; two-sided.

3.1.1.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 88.8 % and 88.3, 68.8 and 37.5 % in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively. For more details, please refer to the appendix report.

A statistically significant difference versus control was determined for treatment with 0.59 and 1.9 µg Tamoxifen/Citrate/L (Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater).

Table 17: Study 1, ZEOGRT with Tamoxifen-Citrate: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.20***	**0.59**	**1.9**	**6.5****	**21*****
Mean hatching rate [%]	**88.8**	**88.3**	**68.8)[a]**	**37.5)[a]**	**-**	**-**
SD	8.5	10.4	15.5	45.0	-	-

SD = Standard deviation

*: The mean treatment value is calculated on basis of three replicates only since replicate B was flooded at test day 45 (23.03.2018) and all larvae but two got lost. This affects all evaluations except hatch.

**: Only male fish present at 6.5 µg Tamoxifen-Citrate/L in F1 generation, thus no reproduction was possible.

***: No fish larvae survived at 21 µg test item/L (all larvae dead before day 21 pf).

)[a]: Significantly higher mortality; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

3.1.1.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 18 (F0 generation) and Table 19 (F1 generation). All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin values for males were determined to be 5.98E+01 ng VTG/mL in controls and 1.45E+02, 1.56E+02, 9.44E+03, 1.47E+03 and 7.35E+02 ng VTG/mL in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively.
The mean VTG values for females were determined to be 1.51E+07 ng VTG/mL in the controls and 1.41E+07, 1.52E+07, 1.52E+07, 7.29E+06 and 7.83E+06 ng VTG/mL in treatments with 0.20, 0.59, 1.9, 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments for male fish (mean measured; Multiple Sequentially-rejective Median test after Bonferroni-Holm; α=0.05; one-sided greater), while female fish showed statistically significant reduced vitellogenin concentrations in treatments with 6.5 and 21 µg Tamoxifen-Citrate/L (mean measured; Step-down Jonckheere-Terpstra Test; p<0.05; one-sided smaller).

Table 18: Study 1, ZEOGRT with Tamoxifen-Citrate: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.20**	**0.59**	**1.9**	**6.5**	**21**
Mean VTG concentration, males [ng VTG/mL]	**5.98E+01**	**1.45E+02**	**1.56E+02**	**9.44E+03**	**1.47E+03**	**7.35E+02**
SD	1.17E+01	1.28E+02	3.46E+01	1.39E+04	1.79E+03	1.93E+02
Mean VTG concentration, females [ng VTG/mL]	**1.51E+07**	**1.41E+07**	**1.52E+07**	**1.52E+07**	**7.29E+06)[a]**	**7.83E+06)[a]**
SD	3.19E+06	8.36E+06	3.26E+06	1.98E+06	8.31E+05	3.79E+06

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

)[a]: Significantly reduced; Step-down Jonckheere-Terpstra Test; $p<0.05$; one-sided smaller.

In first filial generation (F1), the mean vitellogenin (VTG) values for males were determined to be 3.03E+02 ng VTG/L in controls and 1.29E+02, 2.86E+02, 3.83E+02 and 1.89E+02 ng VTG/L in treatments with 0.20, 0.59, 1.9 and 6.5 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean VTG values for females were determined to be 1.46E+07 ng VTG/L in controls and 1.29E+07, 9.36E+06 and 7.51E+06 in treatments with 0.20, 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments for male fish (mean measured; Dunnett`s Multiple t-test; $\alpha=0.05$; one-sided greater), while female fish showed statistically significant reduced vitellogenin values compared to control in treatments with 0.59 and 1.9 µg Tamoxifen-Citrate/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller).

Table 19: Study 1, ZEOGRT with Tamoxifen-Citrate: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.20***	**0.59**	**1.9**	**6.5****	**21*****
Mean VTG concentration, males [ng/mL]	**3.03E+02**	**1.29E+02**	**2.86E+02**	**3.83E+02**	**1.89E+02**	**-**
SD	2.72E+02	3.32E+01	3.08E+02	4.27E+02	1.12E+02	-
Mean VTG concentration, females [ng/mL]	**1.46E+07**	**1.29E+07**	**9.36E+06)[a]**	**7.51E+06)[a]**	**-**	**-**
SD	1.83E+06	3.51E+06	9.39E+05	1.51E+06	-	-

SD = Standard deviation

Remark: The mean VTG concentrations [ng VTG/mL] in controls and treatments were calculated based on the geometric means of each replicate.

*: The mean treatment value is calculated on basis of three replicates only since replicate B was flooded at test day 45 (23.03.2018) and all larvae but two got lost. This affects all evaluations except hatch.

**: Only male fish present at 6.5 µg test item/L.

***: No fish larvae survived at 21 µg test item/L (all larvae dead before day 21 pf).

)[a]: Statistically significant decrease compared to control; Williams Multiple t-test; $p<0.05$; one-sided smaller.

3.1.1.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In the chapters 3.1.1.7.1and 3.1.1.7.2, the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 20 and the results for first filial generation are shown in Table 21.

Finally, the examination of the parental generation (F0) revealed a statistically significant treatment related increased shift to a higher testicular (NOEC: 1.9 µg Tamoxifen-Citrate/L) and ovarial stage (NOEC at 1.9 µg Tamoxifen-Citrate/L) (Step-down Jonckheere-Terpstra test; $p<0.05$; two-sided). However, neither male nor female fish of parental generation showed any treatment-related differences in the occurrence of the investigated lesions. In fact, there were only a few findings at all, subsequently resulting in median values of zero.

In first filial generation (F1) histopathological examination revealed a dose dependent statistically significant shift to a higher testicular (NOEC: 0.20 µg Tamoxifen-Citrate/L) and ovarial stage (NOEC: 0.59 µg Tamoxifen-Citrate/L) (Step-down Jonckheere-Terpstra test; $p<0.05$; two-sided).
Furthermore, a dose-dependent shift to male fish, with only male fish present in treatment with 6.5 µg Tamoxifen-Citrate/L was found.

Histopathological examination of first filial generation F1 revealed an increased occurrence of secondary lesions, i.e. egg debris, granulomatomous inflammation, oocyte atresia and post ovulatory follicles in females in treatments with 1.9 µg Tamoxifen-Citrate/L (mean measured). Furthermore, in male fish of F1 generation an increased interstitial cell hyperthrophy/ hyperplasia and an increased proportion of spermatogonia was seen especially in treatment with 6.5 µg Tamoxifen-Citrate/L (mean measured).

Table 20: Study 1, ZEOGRT with Tamoxifen-Citrate: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Tamoxifen-Citrate [µg/L]					
		Control	**0.20**	**0.59**	**1.9**	**6.5**	**21**
Maturation stages, male [1)]		Stage 0 (0/19) Stage 1 (2/19) Stage 2 (15/19) Stage 3 (2/19) Stage 4 (0/19)	Stage 0 (0/18) Stage 1 (1/18) Stage 2 (12/18) Stage 3 (5/18) Stage 4 (0/18)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (12/18) Stage 3 (6/18) Stage 4 (0/18)	Stage 0 (0/17) Stage 1 (0/17) Stage 2 (8/17) Stage 3 (9/17) Stage 4 (0/17)	Stage 0 (0/22) Stage 1 (0/22) Stage 2 (3/22) Stage 3 (19/22) Stage 4 (0/22)	Stage 0 (1/21) Stage 1 (0/21) Stage 2 (6/21) Stage 3 (13/21) Stage 4 (1/21)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**3**	**2**	**3**	**3**
	B	**2**	**2**	**3**	**2**	**3**	**3**
	C	**2**	**2**	**2**	**2**	**3**	**3**
	D	**2**	**2**	**2**	**3**	**3**	**2**
Result of statistical evaluation (treatment)		-		**)***	**)***	**)[a]**	**)[a]**
Maturation stages, female [2)]		Stage 0 (0/20) Stage 1 (2/20) Stage 2 (16/20) Stage 3 (2/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/22) Stage 1 (0/22) Stage 2 (20/22) Stage 3 (2/22) Stage 4 (0/22) Stage 5 (0/22)	Stage 0 (0/22) Stage 1 (0/22) Stage 2 (18/22) Stage 3 (4/22) Stage 4 (0/22) Stage 5 (0/22)	Stage 0 (0/23) Stage 1 (0/23) Stage 2 (14/23) Stage 3 (9/23) Stage 4 (0/23) Stage 5 (0/23)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (7/18) Stage 3 (11/18) Stage 4 (0/18) Stage 5 (0/18)	Stage 0 (0/19) Stage 1 (1/19) Stage 2 (6/19) Stage 3 (12/19) Stage 4 (0/19) Stage 5 (0/19)
Maturation stage, female, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**3**
	B	**2**	**2**	**2**	**3**	**3**	**3**
	C	**2**	**2**	**2**	**2**	**3**	**3**
	D	**2**	**2**	**2**	**3**	**3**	**2**
Result of statistical evaluation (treatment)		-	-	-	-	**)[a]**	**)[a]**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

)[a]: Statistically significant higher maturation stage compared to control; Step-down Jonckheere-Terpstra test; $p<0.05$; two-sided.

Table 21: Study 1, ZEOGRT with Tamoxifen-Citrate: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Tamoxifen-Citrate [µg/L]					
		Control	**0.20**	**0.59**	**1.9**	**6.5****	**21*****
Maturation stages, male [1)]		Stage 0 (0/19) Stage 1 (8/19) Stage 2 (8/19) Stage 3 (3/19) Stage 4 (0/17)	Stage 0 (0/15) Stage 1 (0/15) Stage 2 (6/15) Stage 3 (9/15) Stage 4 (0/15)	Stage 0 (0/26) Stage 1 (0/26) Stage 2 (2/26) Stage 3 (24/26) Stage 4 (0/26)	Stage 0 (0/34) Stage 1 (0/34) Stage 2 (1/34) Stage 3 (32/34) Stage 4 (1/34)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (0/20) Stage 3 (15/20) Stage 4 (5/20)	-***
Maturation stage, male, median value/ replicate	A	**2**	**2**	**3**	**3**	**3**	-
	B	**2**	**-)***	**3**	**3**	**3**	-
	C	**1**	**3**	**3**	**3**	**4**	-
	D	**1**	**3**	**3**	**3**	**3**	-
Result of statistical evaluation (treatment)		-	-	)[a]	)[a]	)[a]	-
Maturation stages, female [2)]		Stage 0 (0/59) Stage 1 (1/59) Stage 2 (40/59) Stage 3 (18/59) Stage 4 (0/47)	Stage 0 (1/44) Stage 1 (2/44) Stage 2 (26/44) Stage 3 (13/44) Stage 4 (2/44)	Stage 0 (0/53) Stage 1 (0/53) Stage 2 (23/53) Stage 3 (28/53) Stage 4 (2/53)	Stage 0 (0/53) Stage 1 (0/53) Stage 2 (23/53) Stage 3 (28/53) Stage 4 (2/53)	-**	-***
Maturation stage, female, median value/ replicate	A	**2**	**2**	**2**	**3**	-	-
	B	**2**	**-)***	**3**	**3**	-	-
	C	**2**	**3**	**3**	**3**	-	-
	D	**3**	**3**	**3**	**3**	-	-
Result of statistical evaluation (treatment)		-	-	-	)[a]	-	-

[1)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent; [2)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

*: Replicate B was flooded at test day 45 (23.03.2018) and all larvae but two got lost.

**: Only male fish present at 6.5 µg test item/L.

***: No fish larvae survived at 21 µg test item/L (all larvae died before day 21 pf).

)[a]: Statistically significant higher maturation stage compared to control; Step-down Jonckheere-Terpstra test; $p<0.05$; two-sided.

3.1.1.7.1 Histopathology results, parental generation (F0)

Control – group 0

Two female fish (2/20) had the ovary stage 1, sixteen females (16/20) had ovary stage 2 and two females (2/20) had ovary stage 3.
Four female fish showed increased oocyte atresia (two grade 1, two grade 2), five females showed egg debris (three grade 1, two grade 2), four females showed granulomatous inflammation (one grade 1, three grade 2) and three females showed increased post-ovulatory follicles (two grade 1, one grade 2).

Two male fish (2/19) were diagnosed with the testis stage 1, fifteen (15/19) were diagnosed with testis stage 2 and two (2/19) were diagnosed with the testis stage 3.
Additional diagnoses were not observed in the male fish of this group.

0.20 µg Tamoxifen-Citrate/L (mean measured) – group 1

Twenty (20/22) female fish had the ovary stage 2 and two female (2/22) the ovary stage 3.
Three female fish showed increased oocyte atresia (one grade 1, two grade 2), two females showed egg debris (both grade 1), three females showed granulomatous inflammation (one grade 1, two grade 2) and three females showed increased post-ovulatory follicles (all grade 1).

One male (1/18) male fish was diagnosed with the testis stage 1, twelve (12/18) males were diagnosed with the testis stage 2 and five (5/18) males were diagnosed with testis stage 3.
Additional diagnoses were not observed in the male fish of this group.

0.59 µg Tamoxifen-Citrate/L (mean measured) – group 2

Eighteen female fish (18/22) had the ovary stage 2 and four females (4/22) the ovary stage 3.
Two females showed increased oocyte atresia, egg debris and granulomatous inflammation (both grade 1). One female fish showed increased post-ovulatory follicles (all grade 1).

Twelve male fish (12/18) were diagnosed with the testis stage 2 and six (6/18) with testis stage 3. Additional diagnoses were not observed in the male fish of this group.

1.9 µg Tamoxifen-Citrate/L (mean measured) – group 3

Fourteen female fish (14/23) had the ovary stage 2 and nine females (9/23) the ovary stage 3.
Three females showed increased oocyte atresia (two grade 1, one grade 3), two females showed egg debris (one grade 1, one grade 3), three females showed granulomatous inflammation (one grade1, one grade 2, one grade 3) and three females showed increased post-ovulatory follicles (two grade 1, one grade 2).

Eight male fish (8/17) were diagnosed with the testis stage 2 and nine males (9/17) with the testis stage 3.
Additional diagnoses were not observed in the male fish of this group.

6.5 µg Tamoxifen-Citrate/L (mean measured) – group 4

Seven female fish (7/18) had the ovary stage 2, eleven (11/18) the ovary stage 3.
Four females showed increased oocyte atresia (three grade 1, one grade 2), four females showed egg debris (all grade 1), three females showed granulomatous inflammation (all grade1) and one female showed increased post-ovulatory follicles (grade 1).

Three male fish (3/22) were diagnosed with the testis stage 2 and one nineteen males (19/22) with the stage 3.
Additional diagnoses were not observed in the male fish of this group.

21 µg Tamoxifen-Citrate/L (mean measured) – group 5

One female fish (1/19) had the ovary stage 1, six female fish (6/19) had the ovary stage 2 and twelve (12/19) the ovary stage 3.
Two female fish showed increased oocyte atresia and egg debris (all grade 1).

One male fish (1/21) was diagnosed with the testis stage 0, six males (6/21) with the testis stage 2, thirteen (13/21) with the testis stage 3 and one male fish (1/21) with the testis stage 4.
Additional diagnoses were not observed in the male fish of this group.

3.1.1.7.2 Histopathology results, first filial generation (F1)

Control – group 0

One female fish (1/59) had the ovary stage 1, forty females (40/59) the ovary stage 2 and eighteen females (18/59) the ovary stage 3.
Seven females showed increased oocyte atresia (all grade 2), eight females showed egg debris (two grade 1, six grade 2), eight females showed granulomatous inflammation (four grade1, four grade 2) and eight female showed increased post-ovulatory follicles (seven grade 1, one grade 2).

Eight male fish (8/19) were diagnosed with the testis stage 1, eight males (8/19) with the testis stage 2, and three males (3/19) with the testis stage 3.
Additional diagnoses were not observed in male fish of this group.

0.20 µg/ Tamoxifen-Citrate/L (mean measured) – group 1

One female fish (1/44) had the ovary stage 0, two female fish (2/44) the ovary stage 1, twenty-six females (26/44) the ovary stage 2, thirteen females (13/44) the ovary stage 3 and two females (2/44) the ovary stage 4.
Nine females showed increased oocyte atresia (five grade 1, one grade 2, two grade 3, one grade 4), nine females showed egg debris (five grade 1, one grade 2, two grade 3, one grade 4), seven females showed granulomatous inflammation (four grade1, three grade 2) and five female showed increased post-ovulatory follicles (all grade 1).

Six male fish (6/15) were diagnosed with the testis stage 2 and nine males (9/15) with the stage 3. Additional diagnoses were not observed in male fish of this group.

0.59 µg/ Tamoxifen-Citrate/L (mean measured) – group 2

Twenty-three female fish (23/53) had the ovary stage 2, twenty-eight females (28/53) the ovary stage 3 and two females (2/53) the ovary stage 3.
Twelve females showed increased oocyte atresia (six grade 1, two grade 2, four grade 3), twelve females showed egg debris (six grade 1, two grade 2, four grade 3), nine females showed granulomatous inflammation (six grade1, three grade 2) and four female showed increased post-ovulatory follicles (three grade 1, one grade 2).

Two male fish (2/26) were diagnosed with the testis stage 2 and twenty-four male fish (24/26) were diagnosed with the testis stage 3.
One male fish showed increased proportion of spermatogonia (grade 2).

1.9 µg/ Tamoxifen-Citrate/L (mean measured) – group 3

Twenty-three female fish (23/53) had the ovary stage 2, twenty-eight females (28/53) the ovary stage 3 and two females (2/53) the ovary stage 4.
Twenty-one female fish showed increased oocyte atresia (five grade 1, six grade 2, eight grade 3, two grade 4), twenty-one egg debris (four grade 1, nine grade 2, seven grade 3, one grade 4),

nineteen granulomatous inflammation (five grade 1, eleven grade 2, three grade 3) and ten increased post-ovulatory follicles (seven grade 1, two grade 2, one grade 3).

One male fish (1/34) were diagnosed with the testis stage 2 and thirty-two males (32/34) with the testis stage 3 and one male fish (1/34) with testis stage 4.
Three male fish were found to have testis-ova (two grade 3, one grade 4). Furthermore, seven males showed interstitial cell hypertrophy/hyperplasia (three grade 1, four grade 2) and three male fish showed increased proportion of spermatogonia (one grade 1, two grade 2).

6.5 µg/ Tamoxifen-Citrate/L (mean measured) – group 4

There were no female fish present at this treatment level.

Fifteen male fish (15/20) were diagnosed with the testis stage 3 and five males (5/20) with testis stage 4. Three male fish showed increased testicular degeneration (one grade 1, two grade 3). Thirteen male fish (13/20) showed interstitial cell hypertrophy/hyperplasia (eleven grade 2, two grade 3), and five fish were diagnosed with increased proportion of spermatogonia (three grade 2, two grade 3).

3.1.1.8 Summary/Conclusion

The ZEOGRT study aimed to assess the effects of continuous exposure to Tamoxifen-Citrate on different life stages and life performances of zebrafish (*Danio rerio*). The measured water parameters as well as the results of the chemical analysis of Tamoxifen-Citrate concentration in the applied test media suggested stable test conditions throughout the study.

For the **parental generation (F0)**, the evaluation and statistical analysis revealed a significant reduction for the egg numbers per day and female at ≥ 6.5 µg Tamoxifen-Citrate/L (mean measured). Vitellogenin concentration in female blood plasma was found to be significantly reduced at ≥ 6.5 µg Tamoxifen-Citrate/L (mean measured). The histopathological analysis of the fish revealed a significant increase of maturation stages for both males and females in the same concentration range.

The post hatch survival rate of the **first filial generation (F1)** was significantly reduced at ≥ 6.5 µg Tamoxifen-Citrate/L (day 35 pf). All fish larvae died at 21 µg Tamoxifen-Citrate/L (mean measured). The survival of the juvenile life stage was found to be significantly reduced at ≥ 6.5 µg Tamoxifen-Citrate/L (mean measured). No concentration dependent effect was observed for F1 reproduction. The evaluation of sex ratio revealed a significant shift towards an increased number of male fish at ≥ 1.9 µg Tamoxifen-Citrate/L (mean measured). At 6.5 µg Tamoxifen-Citrate/L (mean measured), only male fish were present. The remaining female fish at the lower concentrations expressed an increased growth performance for both total length and weight. As observed for the parental females, the female VTG concentrations were found to be reduced at ≥ 0.59 µg Tamoxifen-Citrate/L (mean measured). The histopathological analysis of the fish revealed a significant increase of maturation stages for both males and females.

The hatching rate of the embryos prepared for the **second filial generation (F2)** was significantly reduced at ≥ 0.59 µg Tamoxifen-Citrate/L (mean measured).

All results are summarized in Table 22.

Table 22: Study 1, ZEOGRT with Tamoxifen-Citrate: Summary of NOEC / LOEC determination during the course of the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Tamoxifen-Citrate [µg/L]	NOEC / LOEC Mean measured concentration Tamoxifen-Citrate [µg/L]
F0-Reproduction	**Fecundity)[a]**	**2.0 / 6.3**	**1.9 / 6.5**
	Fertilization rate	≥20 / >20	≥21 / >21
F0-Growth	Wet weight males	≥20 / >20	≥21 / >21
	Wet weight females	≥20 / >20	≥21 / >21
	Total length males	≥20 / >20	≥21 / >21
	Total length females	≥20 / >20	≥21 / >21
F0-Sex ratio	Sex ratio (% males)	Correct setting of spawning groups was confirmed.	
	Sex ratio (% females)		
F0-Histopathology	**Maturity stage testis)[b]**	**2.0 / 6.3**	**1.9 / 6.5**

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Tamoxifen-Citrate [µg/L]	NOEC / LOEC Mean measured concentration Tamoxifen-Citrate [µg/L]
	Maturity stage ovary)[b]	**2.0 / 6.3**	**1.9 / 6.5**
	Histopathology (males)	No treatment-related differences in the occurrence of the investigated lesions.	
	Histopathology (females)		
F0-Biomarker	Vitellogenin males	≥20 / >20	≥21 / >21
	Vitellogenin females)[c]	**2.0 / 6.3**	**1.9 / 6.5**
F1-ELS	Hatch day 5	≥20 / >20	≥21 / >21
	Survival day 21*)[d]	**0.20 / 0.63**	**0.20 / 0.59**
	Survival day 35*)[e]	**2.0 / 6.3**	**1.9 / 6.5**
	Total length day 35*)[a]	**0.63 / 2.0**	**0.59 / 1.9**
F1-Juveniles	**Survival day 35 – day 63*)[f]**	**2.0 / 6.3**	**1.9 / 6.5**
	Total length day 63*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
F1-Reproduction	Time to first spawning*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
	Fecundity (Egg number per day and female)*)[g]	**decrease only at 0.63**	**decrease only at 0.56**
	Fertilization rate*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
F1-Termination	Survival (test end)	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
	Total length males*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
	Total length females)[h]**	**0.20 / 0.63**	**0.20 / 0.59**
	Wet weight males*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
	Wet weight females)[h]**	**0.20 / 0.63**	**0.20 / 0.59**
	Sex ratio (% males)*)[h]	**0.63 / 2.0**	**0.59 / 1.9**
	Sex ratio (% females))[h]**	**<0.02 / ≤0.02**	**<0.02 / ≤0.02**
F1-Biomarker	**Vitellogenin females*)[k]**	**0.20 / 0.63**	**0.20 / 0.59**

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Tamoxifen-Citrate [µg/L]	NOEC / LOEC Mean measured concentration Tamoxifen-Citrate [µg/L]
	Vitellogenin males*	≥6.3 / >6.3 (as no surviving at highest treatment)	≥6.5 / >6.5 (as no surviving at highest treatment)
F1-Histopathology	**Maturity stage testis*)[b]**	**0.20 / 0.63**	**0.20 / 0.59**
	Maturity stage ovary*)[b]	**0.63 / 2.0**	**0.59 / 1.9**
	Histopathology (females)*	Increased occurence of secondary lesions in particular at highest treatment level.	
	Histopathology (males)*		
F2-Embryo	**Hatch day 4*)[e]**	**0.20 / 0.63**	**0.20 / 0.59**

*: No fish larvae survived at nominal 20 µg test item/L (all larvae dead before day 21 pf).

**: Only male fish present at nominal 6.3 µg test item/L.

)[a]: Statistically significant reduction compared to control; Williams Multiple t-test, p < 0.05, one-sided smaller.

)[b]: Statistically significant higher maturation stage compared to control; Step-down Jonckheere-Terpstra test; p<0.05; two-sided.

)[c]: Significantly reduced compared to control.; Step-down Jonckheere-Terpstra Test; p<0.05; one-sided smaller.

)[d]: Significantly higher mortality compared to control.; Step-down Cochran-Armitage Test; p<0.05; one-sided greater.

)[e]: Significantly higher mortality compared to control.; Step-down Rao-Scott-Cochran-Armitage Test; p<0-05; one-sided greater.

)[f]: Significantly higher mortality compared to control.; Chi² 2x2 Table Test with Bonferroni Correction; one-sided greater.

)[g]: Significantly reduced egg no./female at nominal 0.63 µg t.i./L compared to control.; Dunnett`s Multiple t-test; p<0.05; one-sided smaller.

)[h]: Significantly increased compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[j]: Significantly decreased compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[k]: Significantly decreased vitellogenin value compared to control; Williams Multiple t-test; p<0.05; one-sided smaller.

3.1.2 Phase I, Study 2: ZEOGRT with Prochloraz

3.1.2.1 Test conditions

The water temperatures did not differ by more than ± 1.5 °C between test vessels at any one time during the exposure period. The mean water temperatures per replicate in controls and treatments were calculated to be between 27.0 and 27.3 °C. The single measurements were between 26.4 and 27.8°C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C. The mean dissolved oxygen concentrations were determined to be between 98 and 103 %. The corresponding single values were between 80 and 116 %, thus in line with the acceptance criteria of >60 %. The mean pH values were calculated to be between 7.89 and 8.01. There was no impact of the test substance application on the pH level.

All mean values and standard deviations are summarized in Table 23 to Table 25. For more details, please refer to the appendix report

Table 23: Study 2, ZEOGRT with Prochloraz: Water temperature [°C]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	27.3	0.2	26.7	27.7
	B	27.3	0.2	26.7	27.7
	C	27.0	0.2	26.5	27.4
	D	27.0	0.2	26.5	27.4
3.2	**A**	27.1	0.2	26.7	27.5
	B	27.1	0.2	26.7	27.4
	C	27.1	0.2	26.4	27.5
	D	27.1	0.2	26.4	27.5
10	**A**	27.0	0.2	26.6	27.3
	B	27.0	0.2	26.6	27.3
	C	27.1	0.2	26.5	27.6
	D	27.1	0.2	26.4	27.6
32	**A**	27.2	0.2	26.5	27.5
	B	27.3	0.2	26.6	27.7
	C	27.3	0.2	26.5	27.7
	D	27.3	0.2	26.6	27.7

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	27.3	0.2	26.6	27.8
	B	27.3	0.1	26.7	27.7
	C	27.2	0.2	26.5	27.6
	D	27.2	0.2	26.5	27.6
320	**A**	27.2	0.2	26.5	27.5
	B	27.2	0.2	26.5	27.5
	C	27.1	0.2	26.5	27.5
	D	27.2	0.2	26.4	27.6

SD = Standard deviation

Table 24: Study 2, ZEOGRT with Prochloraz: Oxygen concentration [%]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	101	6.5	85	110
	B	103	5.6	90	110
	C	102	6.2	89	116
	D	101	6.5	83	109
3.2	**A**	102	5.5	89	112
	B	102	5.5	89	110
	C	102	6.2	83	110
	D	102	5.7	89	112
10	**A**	101	5.9	85	108
	B	101	5.9	84	108
	C	101	5.4	86	108
	D	101	5.5	86	107
32	**A**	99	5.7	82	106
	B	98	6.0	81	105
	C	98	5.9	80	105
	D	98	5.6	81	106

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	99	4.6	84	105
	B	100	5.3	84	107
	C	99	4.9	85	106
	D	99	5.2	84	105
320	**A**	99	5.4	81	109
	B	98	5.0	84	106
	C	98	4.9	80	105
	D	98	4.9	81	108

SD = Standard deviation

Table 25: Study 2, ZEOGRT with Prochloraz: pH values

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.89	0.16	7.55	8.21
	B	7.96	0.14	7.72	8.25
	C	7.98	0.14	7.75	8.29
	D	7.95	0.14	7.68	8.20
3.2	**A**	7.99	0.15	7.75	8.26
	B	8.00	0.15	7.76	8.32
	C	7.98	0.15	7.74	8.27
	D	8.01	0.16	7.78	8.35
10	**A**	7.97	0.15	7.79	8.31
	B	7.98	0.14	7.78	8.31
	C	7.97	0.13	7.77	8.25
	D	7.97	0.14	7.79	8.25
32	**A**	7.93	0.16	7.71	8.25
	B	7.92	0.16	7.69	8.24
	C	7.94	0.16	7.70	8.26
	D	7.94	0.15	7.70	8.24

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	7.97	0.17	7.72	8.32
	B	7.99	0.19	7.72	8.42
	C	7.98	0.18	7.73	8.39
	D	7.96	0.18	7.70	8.35
320	**A**	7.98	0.20	7.70	8.39
	B	7.97	0.19	7.69	8.33
	C	7.96	0.18	7.66	8.29
	D	7.98	0.20	7.68	8.40

SD = Standard deviation

3.1.2.2 Chemical analysis

The preparation approach was suitable to apply the test substance Prochloraz in stable concentrations. Decrease of test substance concentration, e.g. due to degradation or adsorption of the substance could not be observed.

The mean measured concentration were calculated to be 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L. These concentrations correspond to 102, 89.6, 105, 108 and 109 % of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations of Prochloraz.

Table 26: Study 2, ZEOGRT with Prochloraz: Chemical analysis

Nominal concentration Prochloraz	Replicate	Measured concentration Prochloraz							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
[µg/L]		Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	**A**	<LOD	-	-	-	**<LOD**	-	-	-
	B	<LOD	-	-	-				
	C	<LOD	-	-	-				
	D	<LOD	-	-	-				
3.2	**A**	3.34	0.95	104.2	29.8	**3.26**	0.16	**102**	5.0
	B	3.04	0.46	94.9	14.4				
	C	3.41	0.28	106.4	8.8				
	D	3.26	0.31	102.0	9.7				

Nominal concentration Prochloraz	Replicate	Measured concentration Prochloraz							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
[µg/L]		Mean	SD	Mean	SD	Mean	SD	Mean	SD
10	**A**	9.15	2.34	91.5	23.4	**8.96**	0.36	**89.6**	3.6
	B	8.53	1.66	85.3	16.6				
	C	9.34	0.64	93.4	6.4				
	D	8.82	0.84	88.2	8.4				
32	**A**	33.5	8.25	104.6	25.8	**33.6**	2.42	**105**	7.5
	B	30.4	5.13	94.9	16.0				
	C	36.2	4.85	113.0	15.1				
	D	34.2	3.43	106.9	10.7				
100	**A**	103.6	7.62	103.6	7.6	**108**	4.91	**108**	4.9
	B	113.8	17.60	113.8	17.6				
	C	103.6	8.20	103.6	8.2				
	D	109.0	8.14	109.0	8.1				
320	**A**	358	31.1	112.0	9.7	**348**	16.4	**109**	5.1
	B	365	30.7	114.0	9.6				
	C	330	21.02	103.1	6.6				
	D	339	29.1	105.8	9.1				

SD = Standard deviation

3.1.2.3 Biological results, parental generation (F0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. In total, 4 fishes died during the study, i.e. 2 female fish and 2 fish without sex determination (due to the degree of decomposition a sex determination was not possible). The two female fish died during recording reproduction and were considered for calculation of mean egg number per day and female (i.e. one female in replicate C in the control at test day 11, another female fish died in replicate B at 348 µg test item/L at test day 16). Since only one fish died in the control, the validity criterion of ≥ 90 % survival rate of adult fish in the controls was met (Table 27).

Reproduction was recorded in terms of egg numbers and fertilization rates in the period from test day 1 to day 22. For the controls, a mean of 54 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be 58, 63, 65, 59 and 42 at 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The mean egg numbers and standard deviation are shown in Table 28. The mean fertilization rate for the controls was calculated to be 84.9 %. For the exposed groups, the fertilization rates were determined to be between 82.9 % (at 3.26 µg Prochloraz/L) and 92.3 % (at 108 µg Prochloraz/L). The statistical evaluation revealed no significant difference compared to control (egg number per day and female: Dunnett`s Multiple t-test; α=0.05; one-sided smaller; fertility: Williams Multiple t-test; α=0.05; one-sided smaller).

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads and verified by histopathological analysis. The inspection revealed that the groups were set correctly, i.e. for most groups, 5 males and 5 females were present (see Table 27 for details).

The mean fish total lengths of males were determined to be 3.7 cm in the controls and 3.7, 3.8, 3.6, 3.7 and 3.7 cm in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The mean fish total lengths of females were determined to be 4.0 cm in controls and 3.8, 4.0, 3.9, 3.9 and 4.0 cm in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The mean wet weights of males were determined to be 0.461 g in controls and 0.438, 0.452, 0.407, 0.452 and 0.447g in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The mean wet weights in females were determined to be 0.700 g in controls and 0.618, 0.712, 0.733, 0.687and 0.760 g in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. Statistical evaluation revealed, that compared to control the test item Prochloraz had no influence on fish growth as assessed by determination of total length and wet weight at termination (Dunnett`s Multiple t-test, α=0.05, two-sided).

All mean values and standard deviation of all biological parameters are summarized in Table 28.

For more details, please refer to the appendix report.

Table 27: Study 2, ZEOGRT with Prochloraz: Overview fish group setting (verified by histopathological analysis) and mortality, F0

	Mean measured concentration Prochloraz [µg/L]																							
	Control				3.26				8.96				33.6				108				348			
Replicate	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Parental generation (F0)																								
Male (m) [n]	5	5	5	5	5	7	4	5	5	5	5	5	5	5	4	5	4	5	4	5	5	5	5	5
Female (f) [n]	5	5	4	5	5	3	6	5	5	5	5	5	5	5	5	5	6	5	5	5	5	4	5	5
Mortality (no. and sex) [n]	-	-	1 f	-	-	-	-	-	-	-	-	-	-	-	1 *	-	-	-	1 *	-	-	1 f	-	-

Remark: The two female fish died during recording reproduction and were considered for calculation of mean egg number per day and female (i.e. one female in replicate C in the control at test day 11, one female in replicate B at 348 µg test item/L (mean measured) at test day 16).

*: Due to a progressed autolysis of the fish, a sex determination was not possible.

Table 28: Study 2, ZEOGRT with Prochloraz: Biological results for parental generation (F0)

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	3.26	8.96	33.6	108	348
Mean egg number per day and female [n]	54	58	63	65	59	42
SD	6	16	19	8	8	5
Mean fertilization rate [%]	84.9	82.9	87.7	86.6	92.3	85.6
SD	3.5	3.4	2.7	3.6	3.2	3.5
Total length, males [cm]	3.7	3.7	3.8	3.6	3.7	3.7
SD	0.0	0.1	0.1	0.1	0.1	0.2
Total length, females [cm]	4.0	3.8	4.0	3.9	3.9	4.0
SD	0.1	0.0	0.1	0.1	0.2	0.2
Wet weight, males [g]	0.461	0.438	0.452	0.407	0.452	0.447
SD	0.013	0.032	0.009	0.037	0.048	0.060

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	**3.26**	**8.96**	**33.6**	**108**	**348**
Wet weight, females [g]	**0.700**	**0.618**	**0.712**	**0.733**	**0.687**	**0.760**
SD	0.064	0.009	0.047	0.074	0.107	0.097
Mean sex ratio, males [%]	**51.4**	**52.5**	**50.0**	**48.6**	**46.1**	**51.4**
SD	2.8	12.6	0.0	2.8	4.8	2.8
Mean sex ratio, females [%]	**48.6**	**47.5**	**50.0**	**51.4**	**53.9**	**48.6**
SD	2.8	12.6	0.0	2.8	4.8	2.8

SD = Standard Deviation

3.1.2.4 Biological results, first filial generation (F1)

The filial 1 (F1) generation was initiated by keeping fertilized eggs from the parental group and placing them in separate brood chambers placed in each test vessel. The allocation of eggs, in total 36, was divided over two subsequent days.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80 % in all test vessels (related to the number of eggs introduced). The mean hatching rate was 100 % in the controls and 98.6, 91.0, 100, 100 and 99.3 % in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. First larvae hatched at day 2 pf and hatch was completed on day 5 pf. A significantly lower number of hatched eggs compared to control was found at 8.96 µg Prochloraz/L, only (Chi^2 2x2 Table Test with Bonferroni Correction; $p \leq 0.05$; one-sided greater). As the hatching success was very high, i.e. 91 %, which is even above the validity criterion for the controls, the finding is considered as less biologically relevant.

The post-hatch survival rate in controls was calculated to be 88.2 % and 84.7 % for day 21 and 35 pf, respectively. The acceptance criteria, i.e. 75 %, was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be 72.5, 78.6, 74.3, 91.0 and 77.5 % at day 21 pf in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. At day 35 pf, the mean post-hatch survival rates were determined to be 57.8, 70.4, 72.9, 85.4 and 76.8 % in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. Statistical analyses evaluating both time points revealed a significant reduction in larval survival in treatments compared to controls, i.e. at day 21 pf all treatments but 108 µg/L (mean measured) showed a significant higher mortality compared to control (Chi^2 2x2 Table Test with Bonferroni Correction; $p<0.05$; one-sided greater), while at day 35 pf only the three lowest treatment levels displayed a higher mortality compared to the control (Chi^2 2x2 Table Test with Bonferroni Correction; $p<0.05$; one-sided greater).

On day 35 pf, the total length of all remaining fish larvae was measured. The mean total length in controls was calculated to be 1.8 cm. The fish lengths in the exposed groups were determined to be 1.9, 1.9, 2.0, 1.9 and 1.6 cm in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The statistical evaluation revealed a significantly reduced length compared to control at the highest treatment level, i.e. 348 µg Prochloraz/L (Williams Multiple t-test; $p<0.05$; one-sided smaller).

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel. On day 63 pf, the fish groups were again photographed, revealing a surviving rate of 98.8 % in the controls and 100, 100, 98.8, 100 and 97.4 % in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. No statistically significant difference compared to control was found in terms of survival at day 63 pf (Chi^2 2x2 Table Test with Bonferroni Correction; a=0.05; one-sided greater). The mean total length at day 63 pf was measured to be 3.0 cm in the controls and 3.1, 3.1, 3.1, 3. and 2.9 cm in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The statistical evaluation revealed no effect on total length at day 63 pf compared to control (Williams Multiple t-test; α=0.05; two-sided).

At day 55 pf spawning trays were introduced in order to assess first spawning events. As most fish groups were already spawning from the first day of tray introduction, no mean values could be obtained.

From day 56 pf onwards, reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 22 eggs per female and day was calculated. The mean egg numbers per female and day in the exposed groups were determined to be 32, 30, 25, and 12 in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively. Since there were no female but only male fish present at the highest treatment level of 348 µg Prochloraz/L, no egg production was possible at all. The mean fertilization rate was calculated to be 86.5 % in the controls and 89.9, 87.1, 85.1 and 86.9 % in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively. Neither for fecundity nor for fertilization, a significant effect compared to control was found. Although, a trend of decreasing egg numbers per day and female is obvious (fecundity: Williams Multiple t-test; α=0.05; one-sided smaller; fertility: Dunnett`s Multipl t-test; α=0.05; one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The mean sex ratio in the controls was determined to be 43.6 % males / 56.4 % females, and thus in line with the validity critarion of 30 to 70 % males or females. In the exposed groups mean sex ratios of 45.4 %males / 54.6 % females, 48.6 % males / 51.4 % females, 42.3 % males / 57.7 % females, 78.8 %males / 21.3 % females and 100 %males / 0 % females were determined in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured concentration), respectively. Statistical evaluation revealed a test item induced shift in sex ratio towards an increased number of males, respective a decreased number of females (Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm; $p<0.05$; two-sided).

The mean fish total lengths in males were determined to be 3.4 cm in the controls and 3.5, 3.5, 3.4, 3.4 and 3.5 cm in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured concentration), respectively. A significantly induced length compared to control was found at 8.96 µg Prochloraz/L only (Dunnett`s Multiple t-test; $p\leq0.05$; two-sided). The mean fish total lengths in females were determined to be 3,4 cm in the controls and 3.6, 3.5, 3.5 and 3.3 cm in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively. No significant effect on length was found compared to control, although a trend to decreasing length with increasing Prochloraz concentration was seen (Williams t-test; α=0.05; two-sided).

The mean fish wet weights of males were determined to be 0.356 g in the controls and 0.383, 0.401, 0.411, 0.402 and 0.410 g in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. A statistically significant increase compared to control was found in treatments with 8.96, 33.6, 108 and 348 µg Prochloraz/L (Williams Multiple t-test;

p<0.05; two-sided). The mean wet weights in females were determined to be 0.447 g for the controls and 0.515, 0.507, 0.486 and 0.412 g in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively. No significant effect compared to control was found.

All results, i.e. mean values and standard deviations, are shown in Table 29.

For more details, please refer to the appendix report.

Table 29: Study 2, ZEOGRT with Prochloraz: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	3.26	8.96	33.6	108	348*
Mean hatching rate [%]	100	98.6	91.0)[a]	100	100	99.3
SD	0	2.8	12.7	0	0	1.4
Mean post-hatch survival at day 21 pf [%]	88.2	72.5)[b]	78.6)[b]	74.3)[b]	91.0	77.5)[b]
SD	4.2	4.1	9.3	4.2	7.3	13.1
Mean post-hatch survival at day 35 pf [%]	84.7	57.8)[b]	70.4)[b]	72.9)[b]	85.4	76.8
SD	4.8	11.2	14.5	3.5	12.3	15.7
Mean total length, day 35 pf (all fish pre-reduction) [cm]	1.8	1.9	1.9	2.0	1.9	1.6)[c]
SD	0.1	0.1	0.1	0.0	0.1	0.1
Mean survival, day 63 pf (compared to no. post-reduction at day 35 pf) [%]	98.8	100	100	98.8	100	97.4
SD	2.5	0	0	2.5	0	5.3
Mean total length, day 63 pf [cm]	3.0	3.1	3.1	3.1	3.0	2.9
SD	0.1	0.1	0.0	0.0	0.1	0.0

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control		**3.26**	**8.96**	**33.6**	**108**	**348***
Time to first spawning [fish age days]	A	≤56	≤56	≤56	≤56	58	-
	B	≤56	57	≤56	≤56	62	-
	C	≤56	57	≤56	≤56	58	-
	D	≤56	≤56	57	≤56	60	-
	As most fish groups were already spawning from the first day of tray introduction, no mean value can be presented. *Only male fish were present in the highest treatment level.						
Mean egg number per day and female [n]	**22**		**32**	**30**	**25**	**12**	**-)***
SD	6		12	4	8	7	-
Mean fertilization rate [%]	**86.5**		**89.9**	**87.1**	**85.1**	**86.9**	**-)***
SD	9.5		1.0	5.1	3.9	5.0	
Mean survival, adult stage (day 63 pf until test end) [%]	**98.8**		**97.1**	**97.5**	**96.3**	**98.8**	**97.5**
SD	2.5		3.4	5.0	4.6	2.5	5.0
Mean total length, males, adult stage [cm]	**3.4**		**3.5**	**3.5)[d]**	**3.4**	**3.4**	**3.5**
SD	0.1		0.1	0.1	0.1	0.0	0.0
Mean total length, females, adult stage [cm]	**3.4**		**3.6**	**3.5**	**3.5**	**3.3**	**-**
SD	0.1		0.1	0.0	0.1	0.1	-
Mean wet weight, males, adult stage [g]	**0.356**		**0.383**	**0.401)[e]**	**0.411)[e]**	**0.402)[e]**	**0.410)[e]**
SD	0.028		0.017	0.014	0.030	0.012	0.016
Mean wet weight, females, adult stage [g]	**0.447**		**0.515**	**0.507**	**0.486**	**0.412**	**-**
SD	0.046		0.056	0.047	0.025	0.017	-

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	3.26	8.96	33.6	108	348*
Mean sex ratio, [% males]	**43.6**	**45.4**	**48.6**	**42.3**	**78.8**	**100)[f]**
SD	17.9	12.4	17.9	15.3	14.4	0
Mean sex ratio, [% females]	**56.4**	**54.6**	**51.4**	**57.7**	**21.3**	**0)[f]**
SD	17.9	12.4	17.9	15.3	14.4	0

SD = Standard deviation

*: Only male fish present at 348 µg Prochloraz/L in F1 generation.

)[a]: Sign. lower no. of hatched eggs compared to control only at 8.96 µg Prochloraz/L (mean measured); Chi² 2x2 Table Test with Bonferroni Correction; p≤0.05; one-sided greater.

)[b]: Significantly higher mortality compared to control.; Chi² 2x2 Table test with Bonferroni Correction; p≤0.05; one-sided greater.

)[c]: Significant reduction compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[d]: Significant increase compared to control. only at 8.96 µg Prochloraz/L; Dunnett`s Multiple t-test; p≤0.05; two-sided.

)[e]: Significant increase compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[f]: Significant increase (males), respective decrease (females) compared to control.; Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm;p<0.05; two-sided.

3.1.2.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 85.0 % and 72.5, 90.0, 86.3 and 85.0 % in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively. For more details, please refer to the appendix report.

Statistical evaluation revealed no substance related effect on hatching success (Chi^2 2x2 Table Test with Bonferroni Correction; one-sided greater α=0.05; one-sided greater).

Table 30: Study 2, ZEOGRT with Prochloraz: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	3.26	8.96	33.6	108	348*
Mean hatching rate [%]	**85.0**	**72.5**	**90.0**	**86.3**	**85.0**	**-***
SD	14.1	15.5	7.1	6.3	15.8	-

SD = Standard deviation

*: Only male fish present at 320 µg test Prochloraz/L, thus no reproduction in F1 was possible.

3.1.2.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 31 (F0 generation) and Table 32 (F1 generation). All single values are given in the appendix report.

In parental generation (F0), the mean vitellogenin values for males were determined to be 1.41E+02 ng VTG/mL in controls and 1.28E+02, 6.98E+01, 7.34E+01, 7.51E+01 and 6.93E+01 ng VTG/mL in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively.

The mean VTG values for females were determined to be 4.07E+07 ng VTG/mL in the controls and 4.53E+07, 3.15E+07, 5.58E+07, 2.23E+07 and 6.08E+06 ng VTG/mL in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively.

Statistical analyses revealed no significant difference between controls and treatments for male fish (Multiple Sequentially-rejective Median Test after Bonferroni-Holm; α=0.05; one-sided greater), while female fish displayed a significantly reduced vitellogenin value compared to control at the highest treatment level of 348 µg Prochloraz/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller).

Table 31: Study 2, ZEOGRT with Prochloraz: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	3.26	8.96	33.6	108	348
Mean VTG concentration, males [ng VTG/mL]	**1.41E+02**	**1.28E+02**	**6.98E+01**	**7.34E+01**	**7.51E+01**	**6.93E+01**
SD	1.04E+02	1.63E+02	9.81E+00	1.62E+01	3.11E+01	3.66E+01
Mean VTG concentration, females [ng VTG/mL]	**4.07E+07**	**4.53E+07**	**3.15E+07**	**5.58E+07**	**2.23E+07**	**6.08E+06)[a]**
SD	9.12E+06	3.36E+07	1.58E+06	2.11E+07	1.54E+07	2.59E+06

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

)[a]: Significantly reduced compared to control.; Williams Multiple t-test; $p<0.05$; one-sided smaller

In first filial generation (F1), the mean vitellogenin (VTG) values for males were determined to be 4.21E+01 ng VTG/mL in controls and 7.21E+01, 9.62E+01, 4.28E+02, 5.18E+01 and 2.50E+01 ng VTG/mL in treatments with 3.26, 8.96, 33.6, 108 and 348 µg Prochloraz/L (mean measured), respectively. The mean VTG values for females were determined to be 1.23E+07 ng VTG/mL in controls and 1.66E+07, 3.29E+07, 1.48E+07 and 5.64E+06 ng VTG/mL in treatments with 3.26, 8.96, 33.6 and 108 µg Prochloraz/L (mean measured), respectively.

Statistical analyses revealed a significantly induced vitellogenin value in males compared to control at only one treatment level, i.e. 33.6 µg Prochloraz/L (mean measured; Multiple Sequentially-rejective U-test after Bonferroni-Holm; p≤0.05; one-sided greater). The statistical evaluation of the female vitellogenin values revealed a reduction compared to control at 108 µg Prochloraz/L (please remark: no females present at 348 µg Prochloraz/L; Multiple Sequentially-rejective U-test After Bonferroni-Holm; p≤0.05; one-sided smaller).

Table 32: Study 2, ZEOGRT with Prochloraz: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng VTG/ml]

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	**3.26**	**8.96**	**33.6**	**108**	**348***
Mean VTG concentration, males [ng/mL]	**4.21E+01**	**7.21E+01**	**9.62E+01**	**4.28E+02)[a]**	**5.18E+01**	**2.50E+01**
SD	4.98E+01	4.23E+01	4.77E+01	3.36E+02	3.21E+01	1.87E+01
Mean VTG concentration, females [ng/mL]	**1.23E+07**	**1.66E+07**	**3.29E+07**	**1.48E+07**	**5.64E+06)[b]**	**-**
SD	2.19E+06	2.59E+06	1.60E+07	3.79E+06	6.35E+05	-

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

*: Only male fish present at 348 µg Prochloraz/L (mean measured) in F1 generation.

)[a]: Significantly induced compared to control.; Multiple Sequentially-rejective U-test After Bonferroni-Holm; p≤0.05; one- sided greater.

)[b]: Significantly reduced compared to control.; Multiple Sequentially-rejective U-test After Bonferroni-Holm; p≤0.05; one-sided smaller.

3.1.2.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In chapters 3.1.2.7.1 and 3.1.2.7.2, the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 33 and the results for first filial generation are shown in Table 34.

Summarizing, in the male fish no treatment-related changes were observed neither for the parental generation (F0) nor for the first filial generation (F1). In fact, in parental generation F0 testicular stage was assessed to be 2 in almost all male fish, while ovarial stage was found to be predominatly 3 in female fish. The same pattern was assessed in first filial generation.

Further, in female fish, no obvious dose-dependent effects could be detected between controls and treatment with 3.26, 8.96 and 33.6 µg Prochloraz/L neither for F0 nor for F1generation. However, an increased atresia and granulomatous inflammation was observed in the female ovaries of the parental generation in treatments with 108 and 348 µg Prochloraz/L. In addition, in the first filial generation F1, the number of female fish was decreased in treatment with 108 µg Prochloraz/L and an ovary stage of mainly 2 was assessed. In treatment with 348 µg Prochloraz/L no females were present at all.

Table 33: Study 2, ZEOGRT with Prochloraz: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Prochloraz [µg/L]					
		Control	**3.26**	**8.96**	**33.6**	**108**	**348**
Maturation stages, male [1)]		Stage 0 (0/20) Stage 1 (0/20) Stage 2 (15/20) Stage 3 (4/20) Stage 4 (1/20)	Stage 0 (0/21) Stage 1 (0/21) Stage 2 (15/21) Stage 3 (6/21) Stage 4 (0/21)	Stage 0 (0/20) Stage 1 (1/20) Stage 2 (18/20) Stage 3 (1/20) Stage 4 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (16/19) Stage 3 (3/19) Stage 4 (0/19)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (13/18) Stage 3 (5/18) Stage 4 (0/18)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (19/20) Stage 3 (1/20) Stage 4 (0/20)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**
	B	**2**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**2**
	D	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 0 (0/19) Stage 1 (0/19) Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19) Stage 5 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (2/19) Stage 3 (16/19) Stage 4 (1/19) Stage 5 (0/19)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (2/20) Stage 3 (17/20) Stage 4 (1/20) Stage 5 (0/20)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (1/20) Stage 3 (19/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/21) Stage 1 (0/21) Stage 2 (2/20) Stage 3 (17/20) Stage 4 (2/20) Stage 5 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (2/19) Stage 3 (17/19) Stage 4 (0/19) Stage 5 (0/19)
Maturation stage, female, median value/ replicate	A	**3**	**3**	**3**	**3**	**3**	**3**
	B	**3**	**3**	**3**	**3**	**3**	**3**
	C	**3**	**3**	**3**	**3**	**3**	**3**
	D	**3**	**3**	**3**	**3**	**3**	**3**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Table 34: Study 2, ZEOGRT with Prochloraz: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Prochloraz [µg/L]					
		Control	**3.26**	**8.96**	**33.6**	**108**	**348**
Maturation stages, male [1)]		Stage 0 (0/34) Stage 1 (0/34) Stage 2 (33/34) Stage 3 (1/34) Stage 4 (0/34)	Stage 0 (0/34) Stage 1 (1/34) Stage 2 (32/34) Stage 3 (1/34) Stage 4 (0/34)	Stage 0 (0/37) Stage 1 (3/37) Stage 2 (32/37) Stage 3 (2/37) Stage 4 (0/37)	Stage 0 (0/33) Stage 1 (1/33) Stage 2 (31/33) Stage 3 (1/33) Stage 4 (0/33)	Stage 0 (0/65) Stage 1 (0/65) Stage 2 (61/65) Stage 3 (4/65) Stage 4 (0/65)	Stage 0 (0/76) Stage 1 (1/76) Stage 2 (65/76) Stage 3 (10/76) Stage 4 (0/76)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**
	B	**2**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**2**
	D	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 0 (0/44) Stage 1 (1/44) Stage 2 (13/44) Stage 3 (30/44) Stage 4 (0/44) Stage 5 (0/44)	Stage 0 (0/39) Stage 1 (0/39) Stage 2 (11/39) Stage 3 (28/39) Stage 4 (0/39) Stage 5 (0/39)	Stage 0 (0/39) Stage 1 (0/39) Stage 2 (6/39) Stage 3 (33/39) Stage 4 (0/39) Stage 5 (0/39)	Stage 0 (0/44) Stage 1 (0/44) Stage 2 (13/44) Stage 3 (29/44) Stage 4 (2/44) Stage 5 (0/44)	Stage 0 (0/14) Stage 1 (0/14) Stage 2 (9/14) Stage 3 (5/14) Stage 4 (0/14) Stage 5 (0/14)	-
Maturation stage, female, median value/ replicate	A	**3**	**3**	**3**	**3**	**2**	**-**
	B	**2**	**3**	**3**	**3**	**3**	**-**
	C	**3**	**3**	**3**	**3**	**3**	**-**
	D	**3**	**3**	**3**	**3**	**2**	**-**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

3.1.2.7.1 Histopathology results, parental generation (F0)

Control – group 0

Three female fish (3/19) had the ovary stage 2, fifteen females (15/19) the ovary stage 3 and one female (1/19) the ovary stage 4. Five female fish showed increased oocyte atresia (all grade 1), eleven females egg debris (seven females grade 1 and four females grade 2), four females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (three females with grade 1 and one female with grade 2).

Fifteen male fish (15/20) were diagnosed with the testis stage 2, four males (4/20) with the stage 3 and one male (1/20) with the stage 4. One male fish showed a granulomatous inflammation in the testes (grade 1). Additional diagnoses were not observed in the male fish of this group.

3.26 µg Prochloraz/L (mean measured concentration) – group 1

Two female fish (2/19) had the ovary stage 2, sixteen females (16/19) the ovary stage 3 and one female (1/19) the ovary stage 4. Seven female fish showed increased oocyte atresia (five females grade 1, one female grade 2 and one female grade 3), eleven females egg debris (seven females grade 1 and four females grade 2), six females a granulomatous inflammation (four females grade 1 and two females grade 2) and three females increased post-ovulatory follicles (two females grade 1 and one female grade 2).

Fifteen male fish (15/21) were diagnosed with the testis stage 2 and six males (6/21) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

8.96 µg Prochloraz/L (mean measured concentration) – group 2

Two female fish (2/20) had the ovary stage 2, seventeen females (17/20) the ovary stage 3 and one female (1/20) the ovary stage 4. Three female fish showed increased oocyte atresia (all grade 1), nine females egg debris (seven females grade 1 and two females grade 2), eleven females a granulomatous inflammation (eight females grade 1 and three females grade 2) and six females increased post-ovulatory follicles (five females grade 1 and one female grade 2).

One male fish (1/20) were diagnosed with the testis stage 1, eighteen males (18/20) with the stage 2 and one male (1/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

33.6 µg Prochloraz/L (mean measured concentration) – group 3

One female fish (1/20) had the ovary stage 2 and nineteen females (19/20) the ovary stage 3. One female fish showed ovarian spermatogenesis of grade 1, ten females increased oocyte atresia (seven females grade 1, two females grade 2 and one female grade 3), thirteen females egg debris (six females grade 1, six females grade 2 and one female grade 3), nine females a granulomatous inflammation (six females grade 1, two females grade 2 and one female grade 3) and three females increased post-ovulatory follicles (two females grade 1 and one female grade 2). One female fish with the granulomatous inflammation in the ovaries had a concurrent grade 3 granulomatous peritonitis.

Sixteen male fish (16/19) were diagnosed with the testis stage 2 and three males (3/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

108 µg Prochloraz/L (mean measured concentration) – group 4

Two female fish (2/21) had the ovary stage 2, seventeen females (17/21) the ovary stage 3 and two females (2/21) the ovary stage 4. Fourteen female fish showed increased oocyte atresia (ten females grade 1 and four females grade 2), twelve females egg debris (nine females grade 1, two females grade 2 and one female grade 3), eight females a granulomatous inflammation (four females grade 1, three females grade 2 and one female grade 3) and one female increased post-ovulatory follicles (grade 2).

Thirteen male fish (13/18) were diagnosed with the testis stage 2 and five males (5/18) with the stage 3.

One male fish showed increased proportion of spermatogonia (grade 1).
Additional diagnoses were not observed in the male fish of this group.

348 µg Prochloraz/L (mean measured concentration) – group 5

Two female fish (2/19) had the ovary stage 2 and seventeen females (17/19) the ovary stage 3. Fifteen female fish showed increased oocyte atresia (eleven females grade 1 and four females grade 2), twelve females egg debris (three females grade 1, seven females grade 2 and two females grade 3) and ten females a granulomatous inflammation (nine females grade 1 and one female grade 2).

Nineteen male fish (19/20) were diagnosed with the testis stage 2 and one male (1/20) with the stage 3. One male fish showed a testis-ova (grade 1).
Additional diagnoses were not observed in the male fish of this group.

3.1.2.7.2 Histopathology results, first filial generation (F1)

Control – group 0

The sex of one fish of the group 0 was not determinable macroscopically. This fish was diagnosed as male based on histology. One female fish (1/44) had the ovary stage 1, thirteen females (13/44) the ovary stage 2 and thirty females (30/44) the ovary stage 3. Three female fish showed increased oocyte atresia (all grade 1), six females egg debris (three females grade 1, two females grade 2 and one female grade 3), seven females a granulomatous inflammation (six females grade 1 and one female grade 2) and eleven females increased post-ovulatory follicles (ten females grade 1 and one female grade 2).

Thirty-three male fish (33/34) were diagnosed with the testis stage 2 and one male (1/34) with the stage 3. Four male fish showed a testis-ova (three males grade 1 and one male grade 2) and one male fish an increased proportion of spermatogonia (grade 1).

3.26 µg Prochloraz/L (mean measured concentration) – group 1

The sex of one fish of the group 1 was not determinable macroscopically. This fish was diagnosed as male based on histology. Eleven female fish (11/39) had the ovary stage 2 and twenty-eight females (28/39) the ovary stage 3. Two female fish showed increased oocyte atresia (one female grade 1 and one female grade 2), ten females egg debris (nine females grade 1 and one female grade 2), six females a grade 1 granulomatous inflammation and four females increased post-ovulatory follicles (three females grade 1 and one female grade 2).

One male fish (1/34) was diagnosed with the testis stage 1, thirty-two males (32/34) with the stage 2 and one male (1/34) with the stage 3. Three male fish showed a testis-ova (one male grade 1 and two males grade 2).
Additional diagnoses were not observed in the male fish of this group.

8.96 µg Prochloraz/L (mean measured concentration) – group 2

The sex of one animal of the group 2 was not determinable macroscopically. This animal was diagnosed as male based on histology. Six female fish (6/39) had the ovary stage 2 and thirty-three females (33/39) the ovary stage 3. One female fish showed increased oocyte atresia (grade 1), thirteen females egg debris (twelve females grade 1 and one female grade 2), eight females a granulomatous inflammation (seven females grade 1 and one female grade 2) and five females increased post-ovulatory follicles (three females grade 1 and two females grade 2).

Two male fish (2/37) were diagnosed with the testis stage 1, thirty-two males (32/37) with the stage 2 and three males (3/37) with the stage 3. Two male fish showed a testis-ova (all grade 1). One male had increased proportion of spermatogonia (grade 1).

33.6 µg Prochloraz/L (mean measured concentration) – group 3

The sex of one animal of the group 3 was determined macroscopically as female but was changed to male based on histology. The sex of another animal of the group 3 was not determinable macroscopically. This animal was diagnosed as male based on histology.

Thirteen female fish (13/44) had the ovary stage 2, twenty-nine females (29/44) the ovary stage 3 and two females (2/44) the ovary stage 4. Five female fish showed increased oocyte atresia (all grade 1), eighteen females egg debris (ten females grade 1, seven females grade 2 and one female grade 3), fifteen females a granulomatous inflammation (thirteen females grade 1 and two females grade 2) and five females increased post-ovulatory follicles (three females grade 1, one female grade 2 and one female grade 3).

One male fish (1/33) was diagnosed with the testis stage 1, thirty-one males (31/33) with the testis stage 2 and one male (1/33) with the stage 3. Three male fish showed a testis-ova (grade 1) and one male increased proportion of spermatogonia (grade 1). In addition, in one male fish a complete (grade 4) unilateral atrophy/hypoplasia of the testis was observed.

108 µg Prochloraz/L (mean measured concentration) – group 4

Nine female fish (9/14) had the ovary stage 2 and five females (5/14) the ovary stage 3. One female fish showed increased oocyte atresia (grade 1), one female egg debris (grade 1) and one female increased post-ovulatory follicles (grade 1).

Sixty-one male fish (61/65) were diagnosed with the testis stage 2 and four males (4/65) with the stage 3. Fifteen male fish showed a testis-ova (thirteen males grade 1 and two males grade 2). Additional diagnoses were not observed in the male fish of this group.

348 µg Prochloraz/L (mean measured concentration) – group 5

The sex of one animal of the group 5 was determined macroscopically as female but was changed to male based on histology. The sex of four fish of the group 5 was not determinable macroscopically. These animals were diagnosed as males based on histology.

There were no female fish in this group.

One male fish (1/76) was diagnosed with the testis stage 1, sixty-five males (65/76) with the testis stage 2 and ten males (10/76) with the stage 3. Eight male fish showed a testis-ova (grade 1) and one male increased proportion of spermatogonia (grade 1). In three male fish focal histiocytic cells (grade 1) were seen.
Additional diagnoses were not observed in the male fish of this group.

3.1.2.8 Summary/Conclusion

The ZEOGRT study aimed to assess the effects of continuous exposure to Prochloraz on different life stages and life performances of zebrafish (*Danio rerio*). The measured water parameters as well as the results of the chemical analysis of Prochloraz concentration in the applied test media suggested stable test conditions throughout the study.

For the **parental generation (F0)**, the evaluation and statistical analysis revealed no significant effect of Prochloraz on growth in terms of lengths and weights. The inspection of the fish gonads confirmed the correct setting of the spawning groups for most of the fish groups applied. Furthermore, Prochloraz did not alter reproduction in terms of fecundity and fertility. The vitellogenin concentration in female fish was significantly reduced compared to control at the highest treatment level of 348 µg Prochloraz/L (NOEC: 108 µg Prochloraz/L (mean measured)). The histopathological analysis showed no effect on the maturation stages due to Prochloraz exposure. However, in female fish an increased atresia and granulomatous inflammation was observed in treatments with 108 and 348 µg Prochloraz/L.

In **first filial generation (F1)**, the early larval survival was affected by Prochloraz treatment. More precisely, the fish numbers were significantly reduced compared to control at 3.26, 8.96 and 33.6 µg Prochloraz/L, resulting in a NOEC of < 3.26 µg Prochloraz/L (mean measured concentration). Notably, almost no further mortality was observed thereafter, i.e. during the phase of juvenile growth and until termination. Total length at day 35 pf was significantly reduced compared to control at the highest treatment level (NOEC: 108 µg Prochloraz/L (mean measured)). Reproduction was not affected by Prochloraz in the F1 generation. However, weight of adult males was significantly increased compared to control in treatments with 8.96, 33.6, 108 and 348 µg Prochloraz/L (NOEC: 3.26 µg Prochloraz/L (mean measured)). The sex ratio was altered by Prochloraz exposure, resulting in a male biased sex ratio with no females present at the highest treatment level (NOEC: 108 µg Prochloraz/L (mean measured)). Furthermore, vitellogenin concentrations in female fish were significantly reduced compared to control at 108 µg Prochloraz/L (at 348 µg Prochloraz/L, no females were present; NOEC: 33.6 µg Prochloraz/L (mean measured)).

Hatching success of the **second filial generation (F2)** was not affected by Prochloraz.

Based on the endpoint early larval survival of the first filial generation (F1), which was the most sensitive endpoint throughout the study, the overall NOEC was determined to be < 3.26 µg Prochloraz/L (mean measured).

All results of the ZEOGRT are summarized in Table 35.

Table 35: Study 2, ZEOGRT with Prochloraz: Summary of NOEC / LOEC determination during the course of the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
F0-Reproduction	Fecundity (Egg number per day and female)	≥320 / >320	≥348 / >348
	Fertilization rate	≥320 / >320	≥348 / >348
F0-Growth	Wet weight males	≥320 / >320	≥348 / >348
	Wet weight females	≥320 / >320	≥348 / >348
	Total length males	≥320 / >320	≥348 / >348
	Total length females	≥320 / >320	≥348 / >348
F0-Sex ratio	Sex ratio (% males)	Right setting was confirmed	
	Sex ratio (% females)		
F0-Histopathology	Maturity stage ovary	≥320 / >320	≥348 / >348
	Maturity stage testis	≥320 / >320	≥348 / >348
	Histopathology (females)	**Increased atresia and granulomatous inflammation in the ovaries were observed in female fish in treatments with 100 and 320 µg Prochloraz/L**	
	Histopathology (males)	No treatment-related differences in the occurrence of the investigated lesions.	
F0-Biomarker	Vitellogenin males	≥320 / >320	≥348 / >348
	Vitellogenin females)[a]	**100 / 320**	**108 / 348**
F1-ELS	Hatch day 5	≥320 / >320	≥348 / >348
	Survival day 21)[b]	**<3.2 / ≤3.2**	**<3.26 / ≤3.26**
	Survival day 35)[b]	**<3.2 / ≤3.2**	**<3.26 / ≤3.26 ****
	Total length day 35)[a]	**100 / 320**	**108 / 348**
F1-Juveniles	Survival day 35 – day 63	≥320 / >320	≥348 / >348
	Total length day 63	≥320 / >320	≥348 / >348
F1-Reproduction	Time to first spawning	Most fish groups were already spawning from the first day of tray introduction.	
	Fecundity (Egg number per day and female)*	≥100 / >100	≥108 / >108
	Fertilization rate*	≥100 / >100	≥108 / >108
F1-Termination	Survival (test end)	≥320 / >320	≥348 / >348

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
	Total length males)[c]	≥320 / >320	≥348 / >348
	Total length females*	≥100 / >100	≥108 / >108
	Wet weight males)[d]	**3.2 / 10**	**3.26 / 8.96**
	Wet weight females*	≥100 / >100	≥108 / >108
	Sex ratio (% males))[e]	**100 / 320**	**108 / 348**
	Sex ratio (% females))[e]	**100 / 320**	**108 / 348**
F1-Biomarker	Vitellogenin males)f	≥320 / >320	≥348 / >348
	Vitellogenin females)[g]	**32 / 100**	**33.6 / 108**
F1-Histopathology	Maturity stage ovary *	≥100 / >100	≥108 / >108
	Maturity stage testis	≥320 / >320	≥348 / >348
	Histopathology (females)	Decreased no. of female fish in treatments with 100 µg Prochloraz/L. At 320 µg Prochloraz/L no females were present at all.	
	Histopathology (males)		
F2-Embryo	Hatch day 4 *	≥100 / >100	≥108 / >108

*: Only male fish present at nominal 320 µg Prochloraz/L in F1 generation.

**: The NOEC was detected to be ≤ 3.26 µg/L, however, no significant difference was found at 108 and 384 µg/L.

)a: Williams test, p<0.05, one-sided smaller.

)b: Significantly higher mortality compared to control.; Chi² 2x2 Table test with Bonferroni Correction; p≤0.05; one-sided greater.

)c: Significantly increased compared to control. only at nominal 10 µg Prochloraz/L; Dunnett`s Multiple t-test; p≤0.05; two-sided.

)d: Significantly increased compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)e: Significantly increased (males), respective decreased (females) compared to control.; Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm;p<0.05; two-sided.

)f: Significantly induced compared to control. only at nominal 32 µg Prochloraz/L; Multiple Sequentially-rejective U-test After Bonferroni-Holm; p≤0.05; one-sided greater.

)g: Significantly reduced compared to control.; Multiple Sequentially-rejective U-test After Bonferroni-Holm; p≤0.05; one-sided smaller.

3.1.3 Phase I, Study 3: ZEOGRT with Dienogest

3.1.3.1 Test conditions

The mean water temperatures per replicate in control and treatments were calculated to be between 26.6 and 27.0 °C. The single measurements in all test vessels were between 26.0 and 28.0 °C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C. The mean dissolved oxygen concentrations were determined to be between 95 and 98 %. The corresponding single values were between 83 and 107% and thus in line with the acceptance criteria of >60 %. The mean pH levels were calculated to be in the range of 8.01 and 8.27 in controls and treatments. The single pH measurements were between 7.70 and 8.48. There was no impact of the substance application on the pH level.

All mean values and standard deviations are summarized in Table 36 to Table 38. For more details, please refer to the appendix report.

Table 36: Study 3, ZEOGRT with Dienogest: Test conditions, water temperature [°C]

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
control	**A**	26.8	0.2	26.4	27.5
	B	26.8	0.2	26.4	27.6
	C	26.7	0.4	26.1	28.0
	D	26.7	0.4	26.1	28.0
3.20	**A**	26.7	0.2	26.4	27.6
	B	26.7	0.2	26.4	27.5
	C	26.7	0.3	26.3	27.9
	D	26.7	0.3	26.3	27.9
10.0	**A**	26.7	0.2	26.2	27.4
	B	26.7	0.2	26.3	27.6
	C	26.7	0.3	26.3	28.0
	D	26.8	0.3	26.3	28.0
32.0	**A**	26.8	0.2	26.3	27.6
	B	27.0	0.2	26.5	27.7
	C	26.8	0.3	26.3	28.0
	D	26.9	0.3	26.3	28.0
100	**A**	27.0	0.3	26.5	27.7
	B	26.9	0.3	26.3	27.8
	C	26.6	0.3	26.1	27.8

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
	D	26.6	0.3	26.0	27.8
320	**A**	26.9	0.3	26.2	27.8
	B	26.9	0.3	26.2	27.9
	C	26.8	0.3	26.2	27.8
	D	26.7	0.3	26.2	27.9

SD = Standard deviation

Table 37: Study 3, ZEOGRT with Dienogest: Test conditions, oxygen concentration [%]

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	96	5	83	103
	B	98	5	84	107
	C	97	6	83	107
	D	96	6	83	106
3.20	**A**	97	5	83	107
	B	97	5	85	107
	C	97	5	83	107
	D	97	5	83	106
10.0	**A**	96	5	83	105
	B	96	5	83	105
	C	96	5	83	106
	D	96	5	85	105
32.0	**A**	96	5	86	105
	B	96	5	84	105
	C	95	5	83	105
	D	95	4	83	105

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	95	5	90	105
	B	96	4	86	103
	C	96	4	88	103
	D	96	4	87	104
320	**A**	95	4	84	104
	B	95	4	85	103
	C	95	4	85	102
	D	95	3	87	102

SD = Standard deviation

Table 38: Study 3, ZEOGRT with Dienogest: Test conditions, pH value

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	8.01	0.17	7.70	8.32
	B	8.06	0.17	7.80	8.35
	C	8.04	0.15	7.78	8.33
	D	8.03	0.17	7.72	8.31
3.20	**A**	8.09	0.15	7.80	8.33
	B	8.10	0.16	7.82	8.32
	C	8.08	0.16	7.78	8.33
	D	8.10	0.15	7.80	8.35
10.0	**A**	8.07	0.17	7.80	8.36
	B	8.08	0.17	7.80	8.35
	C	8.09	0.17	7.80	8.34
	D	8.09	0.16	7.80	8.35
32.0	**A**	8.11	0.19	7.79	8.35
	B	8.11	0.19	7.78	8.40
	C	8.09	0.19	7.75	8.40
	D	8.10	0.18	7.78	8.40

Nominal concentration Dienogest [ng/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	8.27	0.11	8.07	8.48
	B	8.12	0.19	7.75	8.40
	C	8.15	0.17	7.75	8.42
	D	8.15	0.17	7.75	8.40
320	**A**	8.13	0.18	7.77	8.42
	B	8.12	0.18	7.75	8.41
	C	8.12	0.18	7.77	8.39
	D	8.14	0.18	7.75	8.42

SD = Standard deviation

3.1.3.2 Chemical analysis

The mean measured concentration were calculated to be 3.51, 10.3, 31.7, 105, and 335 ng Dienogest/L. These correspond to 110, 103, 99.1, 105 and 105 % of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations of Dienogest.

All results of the chemical analysis are shown in Table 39. For more details, please refer to the appendix report.

Table 39: Study 3, ZEOGRT with Dienogest: Chemical analysis

Nominal concentration Dienogest [ng/L]	Replicate	Measured concentration Dienogest							
		[ng/L]/vessel		[%]/vessel		[ng/L]/treatment		[%]/treatment	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
control	**A**	<LOQ	-	-	-	**-**	-	**-**	-
	B	<LOQ	-	-	-				
	C	<LOQ	-	-	-				
	D	<LOQ	-	-	-				
3.2	**A**	3.66	2.10	114	65.7	**3.51**	0.30	**110**	8.64
	B	3.22	0.69	101	21.5				
	C	3.87	2.81	121	87.9				
	D	3.30	0.77	103	24.0				
10	**A**	11.0	3.91	110	39.1	**10.3**	0.51	**103**	4.99
	B	10.0	1.38	100	13.8				

Nominal concentration Dienogest [ng/L]	Replicate	Measured concentration Dienogest							
		[ng/L]/vessel		[%]/vessel		[ng/L]/treatment		[%]/treatment	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
	C	10.3	3.45	103	34.5				
	D	9.85	1.28	98.5	12.8				
32	**A**	31.1	11.2	97.2	35.0	**31.7**	2.53	**99.1**	7.96
	B	28.7	3.68	89.7	11.5				
	C	34.8	12.4	109	38.7				
	D	32.3	4.40	101	13.8				
100	**A**	95.5	23.5	95.5	23.5	**105**	7.01	**105**	6.71
	B	102	12.8	102	12.8				
	C	110	19.3	110	19.3				
	D	110	8.60	110	8.60				
320	**A**	318	65.3	99.3	20.4	**335**	13.3	**105**	3.95
	B	343	30.7	107	9.59				
	C	333	67.7	104	21.2				
	D	347	56.2	109	17.6				

SD = Standard deviation

LOQ = 1.5 ng/L

3.1.3.3 Biological results, parental generation (F0)

Reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 64 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be between 64 (at 3.51 ng/L) and 84 (at 31.7 ng/L). The mean egg numbers and standard deviations are shown in Table 40. The mean fertilization rate for controls was calculated to be 90.7%. For the exposed groups, the fertilization rates were determined to be between 90.1% (at 10.3 ng/L) and 93.7% (at 105 ng/L). A concentration related impact on the reproductive capability of the fish could not be observed.

After successful initiation of the first filial generation, the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection and histopathological examination of the fish gonads. The inspection revealed that most of the groups were set correctly, i.e. 5 males and 5 females were present. However, 4 male and 6 female fish were found in replicates B and D at 3.51 ng Dienogest/L and in replicate C at 10.3 ng Dienogest/L, while 6 male and 4 female fish were found in replicate A at 3.51 ng Dienogest/L, in replicate D at 105 ng Dienogest/L and in replicate B at 335 ng Dienogest/L. Due to mortality or technical failure, the fish numbers were reduced down to nine fish only in two tanks, namely in replicate C of the control (four male and five female fish were counted) and in replicate D at 31.7 ng Dienogest/L (five male and four female fish were counted). At 10.3 ng Dienogest/L, a single fish was

identified as hermaphrodite. This individual fish was excluded from further calculation of the mean values.

The mean fish total length in controls was determined to be 3.9 cm for both, males and females. The mean fish total lengths of males in the treatments were determined to be between 3.8 cm (at 3.51, 10.3 and 31.7 ng Dienogest/L) and 3.9 cm (at 105 and 335 ng Dienogest/L). The mean fish total lengths of females in the treatments were determined to be between 3.9 cm (at 3.51, 10.3, 105 and 335 ng Dienogest/L) and 4.0 cm (at 31.7 ng Dienogest/L). The mean wet weight in controls was calculated to be 0.503 g for males, and 0.711 g for females. The mean wet weights of males in the treatments were determined to be between 0.479 g (at 3.51 ng Dienogest/L) and 0.518 g (at 105 ng Dienogest/L). The mean wet weights of females in the treatments were determined to be between 0.657 g (at 105 ng Dienogest/L) and 0.713 g (at 31.7 ng Dienogest/L).

Statistical evaluation of endpoint data could not reveal any statistically significant difference between control and treatment with Dienogest.

All mean values and standard deviation of all biological parameters are summarized in Table 40. Details can be found in the appendix report.

Table 40: Study 3, ZEOGRT with Dienogest: Biological results for parental generation (F0)

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean egg number per day and female[n]	**64**	**64**	**73**	**84**	**64**	**77**
SD	11	24	12	13	11	16
Mean fertilization rate [%]	**90.7**	**91.5**	**90.1**	**91.5**	**93.7**	**90.2**
SD	3.4	2.2	0.9	3.3	2.0	3.8
Mean total length, males [cm]	**3.9**	**3.8**	**3.8**	**3.8**	**3.9**	**3.9**
SD	0.1	0	0.1	0	0.1	0
Mean total length, females [cm]	**3.9**	**3.9**	**3.9**	**4.0**	**3.9**	**3.9**
SD	0.2	0.1	0.1	0.1	0	0.1
Mean wet weight, males [g]	**0.503**	**0.479**	**0.491**	**0.497**	**0.518**	**0.498**
SD	0.019	0.018	0.036	0.025	0.063	0.012

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean wet weight, females [g]	**0.711**	**0.680**	**0.683**	**0.713**	**0.657**	**0.665**
SD	0.055	0.118	0.046	0.064	0.040	0.054
Mean sex ratio, [% males]	**48.6**	**47.5**	**47.5**	**51.4**	**52.5**	**52.5**
SD	2.8	9.6	5.0	2.8	5.0	5.0
Mean sex ratio, [% females]	**51.4**	**52.5**	**50.0**	**48.6**	**47.5**	**47.5**
SD	2.8	9.6	8.2	2.8	5.0	5.0
Mean hermaphrodite ratio [%]	**0**	**0**	**2.50**	**0**	**0**	**0**
SD	0	0	5.0	0	0	0

SD = Standard deviation

3.1.3.4 Biological results, first filial generation (F1)

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80% in all test vessels (related to the number of eggs introduced). The mean hatching rate in controls and treatments was calculated to be 100 %.

The number of surviving fish larvae were determined on day 21 and 35 pf. The post-hatch survival rate in controls was calculated to be 93.1 % and 92.4 % for day 21 and 35 pf, respectively. The acceptance criteria, i.e. 75 %, was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be 80.6, 70.8, 72.9, 38.2 and 47.9 % at day 21 pf in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. At day 35 pf the mean post-hatch survival rates were determined to be 79.2, 70.8, 71.5, 37.5 and 47.2 % in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Statistical analyses evaluating both time points revealed a significant reduction in larval survival between controls and treatment with 105 and 335 ng Dienogest/L (Williams test, $p \leq 0.050$, one-sided smaller).

On day 35 pf, the total length of all remaining fish larvae was measured. The mean total length in controls was calculated to be 1.9 cm. The fish lengths in the exposed groups were determined to be 1.8, 1.7, 1.8, 1.9 and 1.9 cm in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The statistical evaluation revealed a significant difference at 10.3 ng Dienogest/L compared to the control, however, due to a missing concentration related response, this finding was considered to have no biological relevance.

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish size. On day 63 pf, the fish groups were again photographed, revealing a surviving rate of 98.8% in the controls and 100% in all Dienogest concentrations tested. The mean total length at day 63 pf was measured to be 3.1 cm in the controls and 3.1, 3.1, 3.1, 3.3 and 3.2 cm in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Neither survival nor total length at day 63 pf showed any statistical differences between control and treatment with Dienogest.

From day 64 pf onwards, reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 31 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration) were determined to be 30, 26, 37, 50 and 43, respectively. The mean fertilization rate was calculated to be 86.4% in the controls and 88.8, 88.5, 84.1, 80.5 and 72.1% in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. Compared to control, the treatment with Dienogest did not show any statistically significant effect on the mean egg number per day and female. However, a trend towards an increased egg number could be observed at the top concentrations. The fertilization rate was significantly reduced in treatment with 335 ng Dienogest/L (Williams test, $p \leq 0.050$, one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The obtained results indicated no test item induced shift of the sex ratio. In controls, the ratio was at 60.9% males/39.1 % females. Under treatment conditions, the percentage of males ranged between 42.9% (at 105 ng Dienogest/L) and 56.8% (at 3.51 ng Dienogest /L). Correspondingly, the number of females remained at similar levels in controls and treatments.

The mean fish total length in males was determined to be 3.7 cm in controls and 3.8, 3.8, 3.8, 3.9 and 3.8 in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean fish total length in females was determined to be 3.6 cm in controls and 3.7, 3.7, 3.7, 4.0 and 3.8 cm in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

The mean fish wet weight of males was determined to be 0.436 g in controls and 0.461, 0.449, 0.445, 0.516 and 0.495 g in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean wet weight in females was determined to be 0.512 g for the controls and 0.534, 0.565, 0.549, 0.700 and 0.648 g in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Neither the total fish length nor the wet weight showed any statistical difference between control and treatment with Dienogest. Notably, a trend for an increase of wet weights was observed for the female fish at the two top concentrations.

All results, i.e. mean values and standard deviations, are shown in Table 41. For more details, please refer to the appendix report.

Table 41: Study 3, ZEOGRT with Dienogest: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean hatching rate	**100**	**100**	**100**	**100**	**100**	**100**
SD	0	0	0	0	0	0
Mean post-hatch survival at day 21 pf [%]	**93.1**	**80.6**	**70.8**	**72.9**	**38.2)[a]**	**47.9)[a]**
SD	3.6	2.3	12.9	13.5	37.4	25.8
Mean post-hatch survival at day 35 pf [%]	**92.4**	**79.2**	**70.8**	**71.5**	**37.5)[a]**	**47.2)[a]**
SD	4.7	2.8	12.9	14.9	36.2	24.5
Mean total length, day 35 pf (all fish, before reduction) [cm]	**1.9**	**1.8**	**1.7)[b]**	**1.8**	**1.9**	**1.9**
SD	0.0	0.0	0.0	0.0	0.2	0.2
Mean survival day 63 pf (compared to no. after reduction at day 35 pf) [%]	**98.8**	**100**	**100**	**100**	**100**	**100**
SD	2.5	0	0	0	0	0
Mean total length, day 63 pf [cm]	**3.1**	**3.1**	**3.1**	**3.1**	**3.3**	**3.2**
SD	0.1	0.0	0.0	0.0	0.3	0.2
Time to first spawning [fish age days]	A 65	≤64	≤64	≤64	-	≤64
	B ≤64	≤64	≤64	≤64	≤64	≤64
	C ≤64	≤64	≤64	≤64	≤64	≤64
	D ≤64	65	≤64	≤64	≤64	≤64
	As most fish groups were already spawning from the first day of tray introduction, no mean value can be presented.					
Mean egg number per day and female [n]	**31**	**30**	**26**	**37**	**50**	**43**
SD	9	8	7	11	18	15
Mean fertilization rate [%]	**86.4**	**88.8**	**88.5**	**84.1**	**80.5**	**72.1)a**
SD	3.6	3.3	2.5	2.2	13.3	11.8

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean survival, adult stage (day 63 pf until test end) [%]	**100**	**98.8**	**100**	**95.0**	**98.3**	**97.9**
SD	0	2.5	0	10.0	2.9	4.2
Mean total length, males, adult stage [cm]	**3.7**	**3.8**	**3.8**	**3.8**	**3.9**	**3.8**
SD	0.1	0.1	0	0.1	30.4	0.1
Mean total length, females, adult stage [cm]	**3.6**	**3.7**	**3.7**	**3.7**	**4.0**	**3.8**
SD	0.1	0	0.1	0	0.4	0.2
Mean wet weight, males, adult stage [g]	**0.436**	**0.461**	**0.449**	**0.445**	**0.516**	**0.495**
SD	0.027	0.044	0.010	0.044	0.161	0.056
Mean wet weight, females, adult stage [g]	**0.512**	**0.534**	**0.565**	**0.549**	**0.700**	**0.648**
SD	0.063	0.015	0.027	0.060	0.284	0.087
Mean sex ratio, [% males]	**60.9**	**56.8**	**43.8**	**51.4**	**42.9**	**53.3**
SD	10.7	9.5	12.5	15.5	13.9	13.8
Mean sex ratio, [% females]	**39.1**	**43.2**	**56.3**	**48.6**	**57.1**	**46.7**
SD	10.7	9.5	12.5	15.5	13.9	13.8

)[a]: Statistically significant reduction compared to control, $p<0.05$, Williams test, one-sided smaller.

)[b]: Statistically significant reduction compared to control, $p<0.05$, Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, one-sided-smaller, heterogenous variances. Due to a missing concentration related response, this finding was considered to have no biological relevance.

3.1.3.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 83.8% and 75.0, 53.8, 46.3, 56.7 and 40.0% in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively (Table 42). For more details, please refer to the appendix report.

A statistically significant difference versus control was determined for all treatment levels ≥10.3 ng Dienogest/L (Williams test, one-sided smaller, $p<0.05$).

Table 42: Study 3, ZEOGRT with Dienogest: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	3.51	10.3	31.7	105	335
Mean hatching rate [%]	83.8	75.0	53.8)[a]	46.3)[a]	56.7)[a]	40.0)[a]
SD	11.1	12.2	26.9	14.4	10.4	29.7

SD = Standard deviation

)[a]: Statistically significant reduced compared to control, $p<0.05$, Williams test, one-sided smaller.

3.1.3.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 43 and Table 44. All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin values in controls were determined to be 62.01 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 451.93, 207.48, 346.49, 138.67 and 339.60 ng Dienogest/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean VTG values in controls were determined to be 1.64E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 1.43E+07, 1.94E+07, 2.70E+07, 2.19E+07 and 1.34E+07 ng Dienogest/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments, neither for males nor for females of parental generation.

Table 43: Study 3, ZEOGRT with Dienogest: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng/mL]

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean VTG concentration, males [ng VTG/mL]	**62.01**	**451.93**	**207.48**	**346.49**	**138.67**	**339.60**
SD	21.52	439.43	131.87	423.59	56.20	330.27
Mean VTG concentration, females [ng VTG/mL]	**1.64E+07**	**1.94E+07**	**2.70E+07**	**2.19E+07**	**1.43E+07**	**1.34E+07**
SD	2.58E+06	7.01E+06	9.40E+06	6.11E+06	3.28E+06	4.76E+06

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

In the first filial generation (F1), the mean vitellogenin (VTG) values in controls were determined to be 177.70 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 55.16, 84.10, 228.48, 13638.42 and 295.18 ng VTG/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively. The mean VTG values in controls were determined to be 1.81E+07 ng VTG/L for females. The mean VTG values of females in treatments 1.36E+07, 1.73E+07, 1.62E+07, 3.62E+07 and 2.93E+07 ng VTG/L in treatments with 3.51, 10.3, 31.7, 105 and 335 ng Dienogest/L (mean measured concentration), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments, neither for males nor for females of first filial generation.

Table 44: Study 3, ZEOGRT with Dienogest: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng/mL]

Parameter	Mean measured concentration Dienogest [ng/L]					
	Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Mean VTG concentration, males [ng VTG/mL]	**177.70**	**55.16**	**84.10**	**228.48**	**13638.42**	**295.18**
SD	75.77	19.26	45.42	316.75	22986.12	301.40
Mean VTG concentration, females [ng VTG/mL]	**1.81E+07**	**1.36E+07**	**1.73E+07**	**1.62E+07**	**3.62E+07**	**2.93E+07**
SD	1.11E+07	4.19E+06	5.70E+06	3.72E+06	2.31E+07	8.57E+06

SD = Standard deviation

3.1.3.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In chapters 3.1.3.7.1 and 3.1.3.7.2 the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 45 and the results for first filial generation are shown in Table 46.

Summarizing the results, neither parental generation (F0) nor first filial generation (F1) showed any treatment-related differences in male or female gonad staging.

Table 45: Study 3, ZEOGRT with Dienogest: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Dienogest [ng/L]					
		Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Maturation stages, male [1)]		Stage 1 (2/19) Stage 2 (16/19) Stage 3 (1/19)	Stage 2 (16/19) Stage 3 (3/19)	Stage 1 (2/19Stage 1 (2/19) Stage 2 (15/19) Stage 3 (2/19))	Stage 2 (14/20) Stage 3 (6/20)	Stage 2 (18/21) Stage 3 (3/21)	Stage 1 (1/21) Stage 2 (19/21) Stage 3 (1/21)
Maturation stage, male, median value/ replicate	A	2	2	2	2	2	2
	B	2	2	2	2	2	2
	C	2	2	2	2	2	2
	D	2	2	2	2	2	2
Maturation stages, female [2)]		Stage 2 (5/20) Stage 3 (13/20) Stage 4 (2/20)	Stage 1 (1/21) Stage 2 (9/21) Stage 3 (11/21)	Stage 2 (6/20) Stage 3 (14/20)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 1 (1/19) Stage 2 (5/19) Stage 3 (13/19)
Maturation stage, female, median value/ replicate	A	3	3	2	3	3	3
	B	3	2	2	3	3	3
	C	3	3	3	3	3	3
	D	3	3	3	3	3	2

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Table 46: Study 3, ZEOGRT with Dienogest: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Dienogest [ng/L]					
		Control	**3.51**	**10.3**	**31.7**	**105**	**335**
Maturation stages, male [1)]		Stage 1 (2/19) Stage 2 (16/19) Stage 3 (1/19)	Stage 2 (16/19) Stage 3 (3/19)	Stage 1 (2/19Stage 1 (2/19) Stage 2 (15/19) Stage 3 (2/19))	Stage 2 (14/20) Stage 3 (6/20)	Stage 2 (18/21) Stage 3 (3/21)	Stage 1 (1/21) Stage 2 (19/21) Stage 3 (1/21)
Maturation stage, male, median value/ replicate	A	2	2	2	2	2	2
	B	2	2	2	2	2	2
	C	2	2	2	2	2	2
	D	2	2	2	2	2	2
Maturation stages, female [2)]		Stage 2 (5/20) Stage 3 (13/20) Stage 4 (2/20)	Stage 1 (1/21) Stage 2 (9/21) Stage 3 (11/21)	Stage 2 (6/20) Stage 3 (14/20)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 2 (3/19) Stage 3 (15/19) Stage 4 (1/19)	Stage 1 (1/19) Stage 2 (5/19) Stage 3 (13/19)
Maturation stage, female, median value/ replicate	A	3	3	2	3	3	3
	B	3	2	2	3	3	3
	C	3	3	3	3	3	3
	D	3	3	3	3	3	2

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Furthermore, neither parental generation (F0) nor first filial generation (F1) showed any treatment-related differences in the occurrence of the investigated lesions. In fact, there were only a few findings at all, subsequently resulting in median values of zero. For more details, please refer to the chapters 3.1.3.7.1 (F0) and 3.1.3.7.2 (F1).

3.1.3.7.1 Histopathology results, parental generation (F0)

Control – group 0

The sex of one animal of the group 0 was determined macroscopically as male but was changed to female based on histology.

Five female animals (5/20) had the ovary stage 2, thirteen animals (13/20) the ovary stage 3 and two animals (2/20) the ovary stage 4.

Five female fish showed increased oocyte atresia (four females grade 1 and one female grade2), two females egg debris (grade 1), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and five females increased post-ovulatory follicles (three females grade 1 and two females grade 2).

Two male fish (2/19) were diagnosed with the testis stage 1, sixteen males (16/19) with the stage 2 and one male (1/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

3.51 ng Dienogest/L (mean measured) – group 1

The sex of one fish of the group 1 was determined macroscopically as male but was changed to female based on histology.

One female animal (1/21) had the ovary stage 1, nine females (9/21) the ovary stage 2 and eleven females (11/21) the ovary stage 3.

Four females showed increased oocyte atresia (three females grade 1 and one female grade 2), eight females egg debris (two females grade 1 and six females grade 2), one female a grade 1 granulomatous inflammation and five females increased post-ovulatory follicles (four females grade 1 and one female grade 2).

Sixteen male fish (16/19) were diagnosed with the testis stage 2 and three males (3/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

10.3 ng Dienogest/L (mean measured) – group 2

One fish was diagnosed as a hermaphrodite with two ovaries of stage 1 and one testis of stage 1.

The sex of two animals of the group 1 was determined macroscopically as male but was changed to female based on histology.

Of these 20 female animals, six (6/20) had the ovary stage 2 and fourteen (14/20) females the ovary stage 3.

Two female fish showed increased oocyte atresia (both with grade 1), five animals egg debris (four females grade 1 and one female grade 2), five females a granulomatous inflammation (two females grade 1 and three females grade 2) and five females increased post-ovulatory follicles (four females grade 1 and one female grade 2).

Two male fish (2/19) were diagnosed with the testis stage 1, fifteen males (15/19) with the stage 2 and two males (2/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

31.7 ng Dienogest/L (mean measured) – group 3

Three female animals (3/19) had the ovary stage 2, fifteen females (15/19) the ovary stage 3 and one female (1/19) the ovary stage 4.

Three female fish showed increased oocyte atresia (two females grade 1 and one female grade 2), four females egg debris (three females grade 1 and one female grade 2), four females a granulomatous inflammation (two females with grade 1, one female grade 2 and one female grade 3) and three females increased post-ovulatory follicles (two females grade 1 and one female grade 2).

Fourteen male fish (14/20) were diagnosed with the testis stage 2 and six males (6/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

105 ng Dienogest/L (mean measured) – group 4

Three female animals (3/19) had the ovary stage 2, fifteen animals (15/19) the ovary stage 3 and one female (1/19) the ovary stage 4.

Two female fish showed increased oocyte atresia (one female grade 1 and one female grade 2), two females egg debris (one female grade 1 and one female grade 2), one female a granulomatous inflammation of grade 1 and two females increased post-ovulatory follicles (one female grade 1 and one female grade 2).

Eighteen male fish (18/21) were diagnosed with the testis stage 2 and three males (3/21) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

335 ng Dienogest/L (mean measured) – group 5

One female fish (1/19) had the ovary stage 1, five females (5/19) the ovary stage 2 and thirteen females (13/19) the ovary stage 3.

One female fish showed egg debris (grade 2), four females a granulomatous inflammation (three females grade 1 and one female grade 4) and seven females increased post-ovulatory follicles (three females grade 1, three females grade 2 and one female grade 3).

One male fish (1/21) was diagnosed with the testis stage 1, nineteen male fish (19/21) were diagnosed with the testis stage 2 and one male (1/21) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

3.1.3.7.2 Histopathology results, first filial generation (F1)

Control - group 0

The sex of one fish of the group 0 was determined macroscopically as male but was changed to female based on histology. Another animal was determined macroscopically as female but was changed to male based on histology.

Thirteen female fish (13/31) had the ovary stage 2, seventeen females (17/31) the ovary stage 3 and one female (1/31) the ovary stage 4.

One female fish showed increased oocyte atresia (grade 1), five females egg debris (four females grade 1 and one female grade 2), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (all grade 1).

Two male fish (2/48) were diagnosed with the testis stage 1, thirty-nine males (39/48) with the stage 2 and seven males (7/48) with the stage 3.

Six male fish showed a testis-ova (five males grade 1 and one male grade 2). Additional diagnoses were not observed in the male fish of this group.

3.51 ng Dienogest/L (mean measured) – group 1

Nine female fish (9/34) had the ovary stage 2, twenty-four females (24/34) the ovary stage 3 and one female (1/34) the ovary stage 4.

Five female fish showed egg debris (three females grade 1 and two females grade 2), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (all grade 1).

Thirty-five male fish (35/45) were diagnosed with the testis stage 2 and ten males (10/45) with the stage 3.

Three male fish showed a testis-ova (all grade 1). Additional diagnoses were not observed in the male fish of this group.

10.3 ng Dienogest/L (mean measured) – group 2

The sex of one fish of the group 2 was determined macroscopically as male but was changed to female based on histology.

One female fish (1/45) had the ovary stage 1, fifteen females (15/45) the ovary stage 2, twenty-eight females (28/45) the ovary stage 3 and one female (1/45) the ovary stage 4.

Two female fish showed egg debris (both with grade 1), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (one female grade 1 and three females grade 2).

Two male fish (2/35) were diagnosed with the testis stage 1, twenty-eight males (28/35) with the stage 2 and five males (5/35) with the stage 3.

Four male fish showed a testis-ova (all grade 1). One male had a grade 1 granulomatous inflammation.

31.7 ng Dienogest/L (mean measured) – group 3

Six female animals (6/37) had the ovary stage 2 and thirty-one females (31/37) the ovary stage 3.

Three female fish showed egg debris (all grade 1), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (two females grade 1 and two females grade 2).

One male fish (1/38) were diagnosed with the testis stage 1, thirty-one males (31/38) with the testis stage 2 and 6 males (6/38) with the stage 3.

One male fish showed a testis-ova (grade 1). Additional diagnoses were not observed in the male fish of this group.

105 ng Dienogest/L (mean measured) – group 4

The sex of one animal of the group 4 was determined macroscopically as female but was changed to male based on histology. The sex of another animal of the group 4 was not determinable macroscopically. This fish was diagnosed as female based on histology

Six female fish (6/26) had the ovary stage 2 and twenty females (20/26) the ovary stage 3.

Three female fish showed egg debris (all grade 2), two females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (six females grade 1 and one female grade 2).

Thirteen male fish (13/17) were diagnosed with the testis stage 2 and four males (4/17) with the stage 3.

Two male fish showed a testis-ova (one male grade 1 and the other grade 2). One male fish had a grade 2 asynchronous germ cell development and another male a grade 2 granulomatous inflammation.

335 ng Dienogest/L (mean measured) – group 5

Seven female fish (7/26) had the ovary stage 2, eighteen females (18/26) the ovary stage 3 and one female (1/26) the ovary stage 4.

Three female fish showed increased oocyte atresia (all grade 1), seven females egg debris (four females grade 1, two females grade 2 and one female grade 4), three females a granulomatous inflammation (one female grade 1, one female grade 2 and one female grade 3) and seven females increased post-ovulatory follicles (four females grade 1 and three females grade 2).

Three male fish (3/32) were diagnosed with the testis stage 1, twenty-four males (24/32) with the testis stage 2 and five males (5/32) with the stage 3.

One male fish showed a testis-ova (grade 4) as well as a granulomatous inflammation (grade 1). The contralateral gonad of this animal showed a unilateral atrophy/hypoplasia (grade 4). Additional diagnoses were not observed in the male fish of this group.

3.1.3.8 Summary/Conclusion

The ZEOGRT study aimed to assess the effects of continuous exposure to Dienogest on different life stages and life performances of zebrafish (*Danio rerio*). The measured water parameters as well as the results of the chemical analysis of Dienogest concentration in the applied test media suggested stable test conditions throughout the study.

For the **parental generation (F0)**, the evaluation and statistical analysis revealed no significant effect of Dienogest exposure on any population-relevant endpoint determined. The measurement of growth in terms of lengths and weights did not show an effect. The inspection of the fish gonads confirmed the correct setting of the spawning groups for most of the fish groups applied. Furthermore, Dienogest did not alter the vitellogenin concentration in blood plasma of both males and females. The histopathological analysis of maturation stages and further lesions did not reveal an effect linked to test substance exposure.

In contrast to that, reproduction in terms of fertility and early larval survival was affected by Dienogest in **first filial generation (F1)**. More precisely, fertility was significantly reduced compared to control at the highest treatment level of 335 ng Dienogest/L (NOEC: 105 ng Dienogest/L (mean measured concentration)). A significant reduction of post-hatch survival at day 21 pf and 35 pf was observed starting at a concentration of 105 ng Dienogest /L (NOEC: 31.7 ng Dienogest/L (mean measured concentration). Notably, mortality of larvae occurred mainly before day 21 pf, during the phase of feed transition from yolk sac feeding to external feeding. Thereafter, i.e. during the phase of juvenile growth, no further mortality was observed until test end.

Hatching success of the **second filial generation (F2)** was significantly reduced compared to control in all treatment levels except the lowest concentration at 3.51 ng Dienogest/L (mean measured concentration). It can be assumed that a low quality of F1 eggs was the reason for a reduced hatching success of the F2 generation larvae. However, a reduced VTG concentration in the blood plasma of F1 females was not measured, suggesting that the yolk amount in the eggs was not impacted.

Based on the endpoint hatching success of the **second filial generation (F2)**, which was the most sensitive endpoint throughout the study, the NOEC was determined to be 3.51 ng Dienogest/L. The observed effect on the early life stage survival of F1 underlines the overall observation that especially embryonic and larval stages were directly impacted by this substance.

All results of the ZEOGRT are summarized in Table 47.

Table 47: Study 3, ZEOGRT with Dienogest: Summary of NOEC / LOEC determination during the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dienogest [ng/L]	NOEC / LOEC Mean measured concentration Dienogest [ng/L]
F0-Reproduction	Fecundity (Egg number per day and female)	≥ 320 / >320	≥ 335 / >335
	Fertilization rate	≥ 320 / >320	≥ 335 / >335
F0-Growth	Wet weight males	≥ 320 / >320	≥ 335 / >335
	Wet weight females	≥ 320 / >320	≥ 335 / >335
	Total length males	≥ 320 / >320	≥ 335 / >335
	Total length females	≥ 320 / >320	≥ 335 / >335
F0-Sex ratio	Sex ratio (% males)	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% females)	≥ 320 / >320	≥ 335 / >335
F0-Histopathology	Maturity stage ovary	≥ 320 / >320	≥ 335 / >335
	Maturity stage testis	≥ 320 / >320	≥ 335 / >335
	Histopathology (females)	≥ 320 / >320	≥ 335 / >335
	Histopathology (males)	≥ 320 / >320	≥ 335 / >335
F0-Biomarker	Vitellogenin females	≥ 320 / >320	≥ 335 / >335
	Vitellogenin males	≥ 320 / >320	≥ 335 / >335
F1-ELS	Hatch day 5	≥ 320 / >320	≥ 335 / >335
	Survival day 21)[a]	**32.0 / 100**	**31.7 / 105**
	Survival day 35)[a]	**32.0 / 100**	**31.7 / 105**
	Total length day 35 **)[1]**	≥ 320 / >320	≥ 335 / >335
F1-Juveniles	Survival day 35 – day 63	≥ 320 / >320	≥ 335 / >335
	Total length day 63	≥ 320 / >320	≥ 335 / >335

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dienogest [ng/L]	NOEC / LOEC Mean measured concentration Dienogest [ng/L]
F1-Reproduction	Time to first spawning	≥ 320 / >320	≥ 335 / >335
	Fecundity (Egg number per day and female)	≥ 320 / >320	≥ 335 / >335
	Fertilization rate)[a]	**100 / 320**	**105 / 335**
F1-Termination	Survival (testend)	≥ 320 / >320	≥ 335 / >335
	Total length males	≥ 320 / >320	≥ 335 / >335
	Total length females	≥ 320 / >320	≥ 335 / >335
	Wet weight males	≥ 320 / >320	≥ 335 / >335
	Wet weight females	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% males)	≥ 320 / >320	≥ 335 / >335
	Sex ratio (% females)	≥ 320 / >320	≥ 335 / >335
F1-Biomarker	Vitellogenin females	≥ 320 / >320	≥ 335 / >335
	Vitellogenin males	≥ 320 / >320	≥ 335 / >335
F1-Histopathology	Maturity stage ovary	≥ 320 / >320	≥ 335 / >335
	Maturity stage testis	≥ 320 / >320	≥ 335 / >335
	Histopathology (females)	≥ 320 / >320	≥ 335 / >335
	Histopathology (males)	≥ 320 / >320	≥ 335 / >335
F2-Embryo	**Hatch day 4)[a]**	**3.20 / 10.0**	**3.51 / 10.3**

)[a]: Williams test, $p<0.05$, one-sided smaller

)[1]: The statistical evaluation revealed a significant difference of fish lengths at 10.3 ng Dienogest/L compared to the control, however, due to a missing concentration related response, this finding was considered to have no biological relevance.

3.1.4 Phase I, Study 4: ZEOGRT with Dexamethasone

3.1.4.1 Test conditions

The mean water temperatures per replicate in control and treatments were calculated to be between 26.1 and 26.7 °C. The single measurements in all test vessels were between 25.6 and 27.2 °C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C. The mean dissolved oxygen concentrations were determined to be between 100 and 104 %. The corresponding single values were between 88 and 115 % and thus in line with the acceptance criteria of >60 %. The mean pH levels were calculated to be in the range of 7.93 and 8.08 in controls and treatments. The single pH measurements were between 7.77 and 8.28. There was no impact of the substance application on the pH level.

All mean values and standard deviations are summarized in Table 48 to Table 50. For more details, please refer to the appendix report.

Table 48: Study 4, ZEOGRT with Dexamethasone: Test conditions, water temperature [°C]

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	26.4	0.19	25.9	26.8
	B	26.4	0.20	25.8	26.8
	C	26.4	0.27	25.9	27.1
	D	26.5	0.25	26.0	27.1
0.32	**A**	26.2	0.21	25.6	26.7
	B	26.2	0.23	25.7	26.7
	C	26.4	0.28	25.9	27.1
	D	26.4	0.26	25.9	27.1
1.0	**A**	26.1	0.18	25.7	26.6
	B	26.1	0.19	25.7	26.5
	C	26.3	0.25	25.9	27.0
	D	26.3	0.28	25.8	27.1
3.2	**A**	26.4	0.23	25.8	26.9
	B	26.5	0.23	25.8	27.0
	C	26.4	0.26	25.9	27.1
	D	26.4	0.24	25.9	27.2

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
10	**A**	26.2	0.18	25.8	26.7
	B	26.2	0.20	25.8	26.7
	C	26.4	0.20	25.9	27.0
	D	26.4	0.20	25.9	27.0
32	**A**	26.3	0.20	25.9	26.8
	B	26.3	0.23	25.9	26.8
	C	26.4	0.19	26.0	27.0
	D	26.5	0.19	26.1	27.1
100	**A**	26.5	0.19	26.2	26.9
	B	26.5	0.19	26.1	26.9
	C	26.7	0.16	26.4	27.2
	D	26.7	0.16	26.4	27.2

SD = Standard deviation

Table 49: Study 4, ZEOGRT with Dexamethasone: Test conditions, oxygen concentration [%]

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	103	6	88	112
	B	104	6	91	115
	C	104	6	92	113
	D	104	5	93	113
0.32	**A**	104	5	95	114
	B	104	5	96	114
	C	104	5	96	114
	D	104	6	93	115
1.0	**A**	103	5	95	114
	B	103	5	94	114
	C	103	5	94	114
	D	103	5	95	113

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
3.2	**A**	103	5	94	113
	B	102	5	95	112
	C	102	5	95	112
	D	102	5	94	111
10	**A**	102	5	95	111
	B	103	5	95	112
	C	102	5	95	112
	D	101	5	92	110
32	**A**	102	5	94	111
	B	102	5	94	111
	C	102	5	95	111
	D	102	5	94	111
100	**A**	102	6	95	112
	B	101	5	94	112
	C	101	5	94	111
	D	100	5	93	111

SD = Standard deviation

Table 50: Study 4, ZEOGRT with Dexamethasone: Test conditions, pH value

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.93	0.10	7.73	8.11
	B	7.97	0.12	7.77	8.30
	C	7.97	0.10	7.79	8.20
	D	8.00	0.09	7.85	8.18
0.32	**A**	8.02	0.10	7.86	8.22
	B	8.02	0.10	7.84	8.22
	C	8.04	0.11	7.84	8.22
	D	8.02	0.10	7.86	8.22

Nominal concentration Dexamethasone [µg/L]	Replicate	Mean	SD	Min value	Max value
1.0	**A**	8.03	0.09	7.87	8.21
	B	8.02	0.08	7.88	8.19
	C	8.03	0.08	7.89	8.19
	D	8.03	0.08	7.89	8.20
3.2	**A**	8.04	0.09	7.87	8.19
	B	8.03	0.09	7.87	8.20
	C	8.05	0.08	7.90	8.23
	D	8.06	0.09	7.90	8.22
10	**A**	8.04	0.09	7.90	8.21
	B	8.04	0.09	7.90	8.27
	C	8.05	0.09	7.90	8.28
	D	8.04	0.09	7.88	8.22
32	**A**	8.06	0.09	7.92	8.21
	B	8.08	0.10	7.93	8.25
	C	8.07	0.09	7.93	8.24
	D	8.08	0.09	7.94	8.25
100	**A**	8.08	0.09	7.93	8.26
	B	8.06	0.09	7.85	8.22
	C	8.08	0.10	7.83	8.24
	D	8.06	0.10	7.72	8.23

SD = Standard deviation

3.1.4.2 Chemical analysis

It was possible to apply the test chemical Dexamethasone without the use of a solvent vehicle. Decrease of test substance concentration, e.g. due to degradation or adsorption of the substance could not be observed.

The mean measured concentration were calculated to be 0.33, 0.91, 3.2, 10.5, 34.7 and 100.1 µg Dexamethasone/L. These correspond to 102.6, 91.4, 99.7, 105.4, 108.5 and 100.1% of the nominal concentrations. The evaluation of the effect concentrations was based on mean measured concentrations.

All results of the chemical analysis are shown in Table 51. For more details, please refer to the appendix report.

Table 51: Study 4, ZEOGRT with Dexamethasone: Chemical analysis

Nominal concentration Dexamethasone [µg/L]	Replicate	Measured concentration Dexamethasone							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	**A**	<LOQ	-	-	-	**-**	-	**-**	-
	B	<LOQ	-	-	-				
	C	<LOQ	-	-	-				
	D	<LOQ	-	-	-				
0.32	**A**	0.32	0.04	99.5	13.0	**0.33**	0.01	**103**	2.1
	B	0.33	0.05	103	14.2				
	C	0.33	0.03	104	9.7				
	D	0.33	0.06	104	17.6				
1.0	**A**	0.87	0.14	87.4	14.3	**0.91**	0.04	**91.4**	4.1
	B	0.89	0.13	89.1	12.8				
	C	0.94	0.07	94.2	7.3				
	D	0.95	0.10	95.0	10.4				
3.2	**A**	3.2	0.3	99.6	9.0	**3.2**	0.05	**99.7**	1.7
	B	3.2	0.31	99.3	9.8				
	C	3.1	0.2	97.9	6.7				
	D	3.3	0.38	102	11.8				
10	**A**	10.1	1.0	101	10.1	**10.5**	0.51	**105**	4.8
	B	10.1	0.78	101	7.8				

Nominal concentration Dexamethasone [µg/L]	Replicate	Measured concentration Dexamethasone							
		[µg/L]/vessel		[%]/vessel		[µg/L]/treatment		[%]/treatment	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
	C	11.1	1.2	111	11.6				
	D	10.9	1.12	109	11.2				
32	**A**	35.6	2.7	111	8.5	**34.7**	1.56	**109**	4.5
	B	36.5	2.78	114	8.7				
	C	33.2	2.8	104	8.8				
	D	33.7	3.02	105	9.4				
100	**A**	101.2	9.7	101.2	9.7	**100**	2.7	**100**	2.7
	B	103.3	11.6	103.3	11.6				
	C	97.5	7.9	97.5	7.9				
	D	98.3	7.8	98.3	7.8				

LOQ=0.10 µg/L

3.1.4.3 Biological results, parental generation (F0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. Reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 75 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments was determined to be between 53 (at 0.91 µg/L) and 73 (at 10.5 µg/L). The mean egg numbers and standard deviation are shown inTable 52. The mean fertilization rate for controls was calculated to be 91.1%. For the exposed groups, the fertilization rates were determined to be between 82.3% (at 10.5 µg/L) and 90.4% (at 100 µg/L). The statistical evaluation revealed a significant reduction of egg numbers at 0.33 and 0.91 µg Dexamethasone/L, and for fertilization rate at 10.5 and 34.7 µg Dexamethasone/L. However, a concentration related impact on the reproductive capability of the fish could not be observed. Thus, the biological relevance of these observations was considered as minor. See Table 52 for details.

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads. The inspection revealed that most of the groups were set correctly, i.e. 5 males and females were present. For two test vessels, i.e. vessels 3-2 and 3-4 (at 3.20 µg/L), 6 female and 4 male fish were found. Due to mortality or technical failure, the fish numbers were reduced in two tanks, i.e. in vessel 5-1 (at 32.0 µg/L) five male and four female fish were found, and in vessel 6-2 (at 100 µg/L) only four male and five female fish were detected.

The mean fish total lengths of males were determined to be 3.8 cm in controls and 3.8, 3.7, 3.8, 3.8, 3.6 and 3.6 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean fish total lengths of females were determined to be 3.9 cm in controls

and 3.9, 3.8, 3.8, 3.9, 3.8 and 3.8 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

The mean wet weights of males were determined to be 0.482 g in controls and 0.482, 0.465, 0.482, 0.504, 0.430 and 0.406 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean wet weights in females were determined to be 0.704 g in controls and 0.697, 0.689, 0.671, 0.652, 0.638 and 0.559 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Evaluation of data revealed a statistically significant reduction in female fish total length and wet weight compared to control in treatment with 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller). Male fish in treatments with 34.7 and 100 µg Dexamethasone/L showed a significant reduction in wet weight, too (Jonckheere-Terpstra, $p<0.05$, one-sided smaller).
All mean values and standard deviation of all biological parameters are summarized in Table 52.

For more details, please refer to the appendix report.

Table 52: Study 4, ZEOGRT with Dexamethasone: Biological results for parental generation (F0)

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean egg number per day and female [n]	**75**	**57)[a]**	**53)[a]**	**62**	**73**	**68**	**67**
SD	9	6	11	7	8	12	10
Mean fertilization rate [%]	**91.1**	**87.2**	**86.3**	**87.8**	**82.3)[a]**	**85.3)[a]**	**90.4**
SD	2.5	2.9	3.6	1.7	5.8	2.2	0.6
Total length, males [cm]	**3.8**	**3.8**	**3.7**	**3.8**	**3.8**	**3.6**	**3.6**
SD	0.1	0.1	0.1	0.1	0.1	0.0	0.1
Total length, females [cm]	**3.9**	**3.9**	**3.8**	**3.8**	**3.9**	**3.8**	**3.8)[b]**
SD	0.1	0.2	0.1	0.1	0.1	0.1	0.1
Wet weight, males [g]	**0.482**	**0.482**	**0.465**	**0.486**	**0.504**	**0.430)[c]**	**0.406)[c]**
SD	0.036	0.029	0.014	0.024	0.104	0.009	0.039
Wet weight, females [g]	**0.704**	**0.697**	**0.689**	**0.675**	**0.652**	**0.638**	**0.559)[b]**
SD	0.054	0.113	0.052	0.046	0.044	0.060	0.037

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean sex ratio, males [%]	**50.0**	**50.0**	**50.0**	**47.5**	**50.0**	**51.4**	**48.6**
SD	0.0	0.0	0.0	5.0	0.0	2.8	2.8
Mean sex ratio, females [%]	**50.0**	**50.0**	**50.0**	**52.5**	**50.0**	**48.6**	**51.4**
SD	0.0	0.0	0.0	5.0	0.0	2.8	2.8

)[a]: Statistically significant reduction compared to control, p<0.05, Dunnett`s Multiple t-test, one-sided smaller.

)[b]: Statistically significant reduction compared to control, p<0.05, Williams test, one-sided smaller.

)[c]: Statistically significant reduction compared to control, p<0.05, Jonckheere-Terpstra test, one-sided smaller.

3.1.4.4 Biological results, first filial generation (F1)

Hatch was sufficient to obtain enough larvae to proceed and was observed to be > 80% in all test vessels (related to the number of eggs introduced). The mean hatching success in controls was calculated to be 98.6%. In the treatments, the hatching rates were between 89.6% (at 10.5 µg Dexamethasone/L) and 100% (at 100 µg Dexamethasone/L). A concentration related effect on hatching was not evident. All mean hatching rates and standard deviations are shown in Table 53.

The number of surviving fish larvae were determined on day 21 and 35 pf. The post-hatch survival rate in controls was calculated to be 97.2% and 94.4% for day 21 and 35, respectively. The acceptance criteria, i.e. at least 75%, was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be between 90.5% (at 10.5 µg/L) and 100% (at 34.7 µg/L). No significant effect on larval survival was observed. Due to a handling mistake in the second control replicate B, the fish larvae were unintendedly released to the main vessels and finally got lost. Thus, the control was continued with only three tank replicates. A re-set of this replicate was considered as not appropriate, as there would have been a time difference of 4 weeks compared to the other groups.

On day 35 pf, the total lengths of all remaining fish larvae were measured. The mean length in controls was calculated to be 1.8 cm. The fish lengths in the exposed groups were determined to be 1.8, 1.8, 1.7, 1.8, 1.7 and 1.6 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. A decrease of fish lengths was observed and was statistically significant at 100 µg Dexamethasone/L (Jonckheere-Terpstra test, p<0.05, one-sided smaller).

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish size. On day 63 pf, the fish groups were again photographed, and the number of surviving fishes was determined, and the fish total length was measured. The mean control length was determined to be 3.0 cm. In the treatment groups, the mean fish total lengths were calculated to be between 2.7 cm (at 100 µg/L) and 3.1 cm (at 0.91 µg/L). Again, as observed for the larval stages, a decrease of fish growth was also evident for the juvenile stages. The decrease was statistically significant at 34.7 and 100 µg/L (Williams test, p<0.05, one-sided smaller). All fish groups were reduced to an equal number of 20 fish per tank (if possible), an effect induced by fish density can be excluded. Thus, it can be

postulated that the growth effect was induced by the test chemical itself. There was no effect on survival of the juvenile stages.

From day 57 pf onwards, reproduction was recorded in terms of egg number and fertilization rates. Evaluation of data did not show any significant differences between treatments and controls. For the controls, a mean value of 21 eggs per female and day was calculated, while mean values in treatments were determined to be between 17 (at 34.7 µg/L) and 23 (at 0.91, 3.20 and 10.5 µg/L) eggs per female and day. The mean fertilization rate for the controls was determined to be 89.9%. In treatments with Dexamethasone the fertilization rates were between 90.0% (at 100 µg/L) and 93.5% (at 0.33 µg/L).

After successful initiation of the second filial generation, the adult fish groups of F1 were sacrificed. Measurement of the male fish total length revealed a mean value of 3.8 cm for the controls and 3.9, 3.8, 3.9, 3.8, 3.6 and 3.5 cm when treated with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. Compared to control, a statistically significant reduction of total fish length was evident in treatments with 34.7 and 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller). In female fish the mean total length was determined to be 3.7 cm in the controls and 3.8, 3.8, 3.8, 3.8, 3.7 and 3.6 cm in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. Female fish did not show any statistical difference compared to control in terms of fish total length.

The mean fish wet weight in males was determined to be 0.425 g for controls and 0.454, 0.432, 0.453, 0.449, 0.361 and 0.296 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. The mean wet weight in females was determined to be 0.528 g for the controls and 0.573, 0.568, 0.534, 0.531, 0.459 and 0.394 g in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (nominal concentration), respectively. Male as well as female fish showed a significant reduction in wet weight when treated with 34.7 and 100 µg Dexamethasone/L (Williams test, $p<0.05$, one-sided smaller.

The sex ratio was first calculated based on the macroscopic inspection of fish gonads and was afterwards verified by histopathological evaluation. The obtained results indicated no test item induced shift of the sex ratio. In controls, the ratio was at 43.3% males/56.7% females. Under treatment conditions, the percentage of males ranged between 29.1% (at 34.7 µg Dexamethasone/L) and 50.0% (at 10.5 µg Dexamethasone/L). Correspondingly, the number of females remained at similar levels in controls and treatments.
All results, i.e. mean values and standard deviations, are shown in Table 53.

For more details, please refer to the appendix report.

Table 53: Study 4, ZEOGRT with Dexamethasone: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean hatching rate [%]	**98.6**	**93.1**	**95.1**	**99.3**	**89.6**	**92.4**	**100**
SD	2.8	8.6	4.7	1.4	12.5	6.5	0
Mean post-hatch survival at day 21 pf [%]	**97.2***	**100**	**100**	**99.3**	**99.3**	**100**	**98.6**
SD	4.8	0	0	1.4	1.4	0	2.8
Mean post-hatch survival at day 35 pf [%]	**94.4***	**95.3**	**98.5**	**97.2**	**90.5**	**100**	**98.6**
SD	9.6	5.7	1.8	3.9	11.3	0	2.8
Mean total length, day 35 pf (all fish pre-reduction) [cm]	**1.8***	**1.8**	**1.8**	**1.7**	**1.8**	**1.7**	**1.6)[a]**
SD	0.0	0.1	0.0	0.0	0.1	0.1	0.1
Mean survival day 63 pf (compared to no. post-reduction at day 35 pf) [%]	**100***	**100**	**100**	**98.8**	**100**	**100**	**100**
SD	0*	0	0	2.5	0	0	0
Mean total length, day 63 pf [cm]	**3.0***	**3.0**	**3.1**	**3.0**	**3.0**	**2.9)[b]**	**2.7)[b]**
SD	0.0	0.1	0.0	0.1	0.1	0.1	0.1
Time to first spawning [fish age days pf]	A 61	58	66	65	57	61	62
	B -*	60	68	57	58	58	69
	C 58	59	61	58	57	58	61
	D 59	57	68	71	57	60	57

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean egg number per day and female [n]	**21***	**18**	**23**	**23**	**23**	**17**	**20**
SD	3	7	3	4	1	5	4
Mean fertilization rate [%]	**89.9***	**93.5**	**90.9**	**90.2**	**92.1**	**92.9**	**90.0**
SD	2.5	3.1	3.1	5.6	0.8	2.0	4.4
Mean survival, adult stage (day 63 pf until termination) [%]	**100***	**100**	**98.8**	**98.8**	**97.5**	**98.8**	**96.3**
SD	0	0	2.5	2.5	2.9	2.5	4.8
Mean total length, males, adult stage [cm]	**3.8***	**3.9**	**3.8**	**3.9**	**3.8**	**3.6)[b]**	**3.5)[b]**
SD	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Mean total length, females, adult stage [cm]	**3.7***	**3.8**	**3.8**	**3.8**	**3.8**	**3.7**	**3.6**
SD	0.1	0.1	0.1	0.2	0.0	0.2	0.1
Mean wet weight, males, adult stage [g]	**0.425***	**0.454**	**0.432**	**0.453**	**0.445**	**0.359)[b]**	**0.296)[b]**
SD	0.017	0.040	0.030	0.031	0.061	0.009	0.002
Mean wet weight, females, adult stage [g]	**0.528***	**0.573**	**0.568**	**0.534**	**0.531**	**0.459)[b]**	**0.394)[b]**
SD	0.027	0.050	0.036	0.059	0.005	0.049	0.041

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Mean sex ratio, [% males]	**43.3***	**35.0**	**29.3**	**38.6**	**50.0**	**29.1**	**30.3**
SD	7.6	8.2	14.7	23.0	8.4	15.5	18.8
Mean sex ratio, [% females]	**56.7***	**65.0**	**70.7**	**61.4**	**50.0**	**70.9**	**69.7**
SD	7.6	8.2	14.7	23.0	8.4	15.5	18.8
Unidentified (no gonads visible) [%]	**-**	**-**	**-**	**-**	**1.3**	**1.3**	**-**
SD	-	-	-	-	2.5-	2.5	-

SD = Standard deviation

*: Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value derives from three replicates only.

)[a]: Statistically significant reduced compared to control, p<0.05, Jonckheere-Terpstra test; one-sided smaller.

)[b]: Statistically significant reduced compared to control, p<0.05, Williams test, one-sided smaller.

3.1.4.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 100% and 100, 100, 97.5, 90.0, 90.0 and 97.5% in treatments with 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L (mean measured concentration), respectively. For more details, please refer to Table 54 and the appendix report.

Evaluation of hatching data revealed a significant reduction at 10.5 µg Dexamethasone /L and at 34.7 µg Dexamethasone/L (Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, one-sided-smaller, heterogenous variances). However, as the hatching rates achieved 90% and a concentration response is missing, a biological relevance of this observation is not likely.

Table 54: Study 4, ZEOGRT with Dexamethasone: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean hatching rate [%]	**100***	**100**	**100**	**97.5**	**90.0)[a]**	**90.0)[a]**	**97.5**
SD	0	0	0	2.9	7.1	5.8	2.9

SD = Standard deviation

*: Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value drives from three replicates only.

)[a]: Statistically significant reduction compared to control, Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, p<0.05, one-sided-smaller, heterogeneous variances.

3.1.4.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 55 and Table 56. All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin (VTG) value in controls was determined to be 75.36 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 205.72, 394.89, 110.01, 144.64, 2631.22 and 469.51 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

The mean VTG value in controls was determined to be 4.89E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 3.65E+07, 4.07E+07, 4.52E+07, 5.19E+07, 6.28E+07 and 5.35E+07 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments neither for males nor for females of parental generation (F0), respectively.

Table 55: Study 4, ZEOGRT with Dexamethasone: Biomarker results for parental generation (F0), vitellogenin concentration in blood plasma [ng/mL]

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean VTG concentration, males [ng VTG/mL]	**75.36**	**205.72**	**394.89**	**110.01**	**144.64**	**2631.22**	**469.51**
SD	18.48	135.73	315.32	115.58	115.83	4211.09	231.23
Mean VTG concentration, females [ng VTG/mL]	**4.89E+07**	**3.65E+07**	**4.07E+07**	**4.52E+07**	**5.19E+07**	**6.28E+07**	**5.35E+07**
SD	2.77E+07	9.16E+06	1.35E+07	1.51E+07	1.45E+07	3.01E+07	1.81E+07

SD = Standard deviation
Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate. The data presented is characterized by high variations (± SD). This was mostly due to single measurements which can be interpreted as biological outliers. However, no explicit mathematical outlier test was conducted to exclude those values.

In first filial generation (F1) the mean vitellogenin (VTG) value in controls was determined to be 28.03 ng VTG/L for males. The mean VTG values of males in treatments were determined to be 41.43, 455.70, 131.22, 48.97, 48.77 and 39.44 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively. The mean VTG value in controls was determined to be 2.76E+07 ng VTG/L for females. The mean VTG values of females in treatments were determined to be 1.11E+07, 7.87E+06, 1.33E+07, 1.88E+07, 7.01E+06 and 2.37E+06 ng VTG/L at 0.33, 0.91, 3.20, 10.5, 34.7 and 100 µg Dexamethasone/L, respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments neither for males nor for females of first filial generation (F1), respectively.

Table 56: Study 4, ZEOGRT with Dexamethasone: Biomarker results for parental generation (F1), vitellogenin concentration in blood plasma [ng/mL]

Parameter	Mean measured concentration Dexamethasone [µg/L]						
	Control	0.33	0.91	3.20	10.5	34.7	100
Mean VTG concentration, males [ng VTG/mL]	**28.03***	**41.43**	**455.70**	**131.22**	**48.97**	**48.77**	**39.44**
SD	7.12	18.96	534.11	107.34	41.11	59.27	17.56
Mean VTG concentration, females [ng VTG/mL]	**2.76E+07***	**1.11E+07**	**7.87E+06**	**1.33E+07**	**1.88E+07**	**7.01E+06**	**2.37E+06**
SD	1.78E+07	9.25E+06	5.60E+06	4.36E+06	2.92E+06	3.15E+06	1.88E+06

SD = Standard deviation
Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate. The data presented is characterized by high variations (± SD). This was mostly due to single measurements which can be interpreted as biological outliers. However, no explicit mathematical outlier test was conducted to exclude those values.
*: Larvae of replicate B got lost due to a handling mistake, thus, from day 21 pf on, the mean control value drives from three replicates only.

3.1.4.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In chapters 3.1.4.7.1 and 3.1.4.7.2, the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 57 and the results for first filial generation are shown in Table 58.

Summarizing the results, neither parental generation (F0) nor first filial generation (F1) showed any treatment-related differences in male or female gonad staging.

Table 57: Study 4, ZEOGRT with Dexamethasone: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Dexamethasone [µg/L]						
		Control	0.33	0.91	3.20	10.5	34.7	100
Parental generation (F0)								
Maturation stages, male [1)]		Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (16/20) Stage 3 (4/20)	Stage 2 (15/19) Stage 3 (4/19)	Stage 2 (18/20) Stage 3 (2/20)	Stage 2 (17/20) Stage 3 (3/20)	Stage 2 (14/19) Stage 3 (5/19)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**	**2**
	B	**2**	**2**	**2**	**3**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**2**	**3**
	D	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 2 (6/20) Stage 3 (13/20) Stage 4 (1/20)	Stage 2 (5/20) Stage 3 (15/20)	Stage 2 (4/20) Stage 3 (16/20)	Stage 2 (3/21) Stage 3 (17/21) Stage 4 (1/21)	Stage 2 (2/20) Stage 3 (17/20) Stage 4 (1/20)	Stage 1 (1/19) Stage 2 (4/19) Stage 3 (14/19)	Stage 2 (8/20) Stage 3 (12/20)
Maturation stage, female, median value/ replicate	A	**3**	**3**	**3**	**3**	**3**	**3**	**3**
	B	**3**	**3**	**3**	**3**	**3**	**3**	**3**
	C	**3**	**2**	**3**	**3**	**3**	**3**	**2**
	D	**3**	**3**	**3**	**3**	**3**	**2**	**2**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Table 58: Study 4, ZEOGRT with Dexamethasone: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Dexamethasone [µg/L]						
		Control	**0.33**	**0.91**	**3.20**	**10.5**	**34.7**	**100**
Maturation stages, male [1)]		Stage 2 (26/26)	Stage 1 (1/28) Stage 2 (25/28) Stage 3 (2/28)	Stage 2 (22/23) Stage 3 (1/23)	Stage 1 (1/30) Stage 2 (28/30) Stage 3 (1/30)	Stage 1 (2/38) Stage 2 (35/38) Stage 3 (1/38)	Stage 1 (2/22) Stage 2 (20/22)	Stage 1 (1/23) Stage 2 (22/23)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**	**2**
	B	-	**2**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**2**	**2**
	D	**2**	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 2 (25/34) Stage 3 (9/34)	Stage 2 (38/52) Stage 3 (14/52)	Stage 1 (2/56) Stage 2 (41/56) Stage 3 (13/56)	Stage 2 (34/48) Stage 3 (14/48)	Stage 2 (26/39) Stage 3 (13/39)	Stage 2 (35/46) Stage 3 (21/46)	Stage 2 (31/54) Stage 3 (23/54)
Maturation stage, female, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**	**2**
	B	-	**3**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**3**	**2**
	D	**2**	**2**	**2**	**2**	**2**	**2**	**2**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Furthermore, neither parental generation (F0) nor first filial generation (F1) showed any treatment-related differences in the occurrence of the investigated lesions.

Notably, increased testicular degeneration, interstitial cell hypertrophy/ hyperplasia, interstitial proteinaceous fluid and asynchronous germ cell development were not found to be present in any male fish of parental generation (F0). Testis-ova was diagnosed in one male fish (1/1-8), another single animal (6/4-5) displayed granulomatous inflammation. Only the occurrence of increased proportion of spermatogonia was identified more often.

A very similar pattern was observed for first filial generation (F1). Increased testicular degeneration, interstitial cell hypertrophy/ hyperplasia, interstitial proteinaceous fluid and asynchronous germ cell development were not found to be present in any male fish of first filial generation (F1). Increased proportion of spermatogonia was diagnosed in one male fish of the control (0/4-13), another single animal (2/4-10) displayed granulomatous inflammation. Only the occurrence of testis-ova was found slightly more often.

3.1.4.7.1 Histopathology results, parental generation (F0)

Control – group 0

Six female fish (6/20) had the ovary stage 2, thirteen females (13/20) the ovary stage 3 and one female (1/20) the ovary stage 4.

Fourteen female fish showed increased oocyte atresia (eight females with grade 1 and six females with grade 2), ten females egg debris (seven females grade 1 and three females grade 2), nine females a granulomatous inflammation (eight females grade 1 and one female grade 2) and thirteen females increased post-ovulatory follicles (five females with grade 1, six females with grade 2 and two females with grade 3).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

0.33 µg Dexamethasone/L (mean measured concentration) – group 1

Five female fish (5/20) had the ovary stage 2 and fifteen females (15/20) the ovary stage 3.

Fifteen female fish showed increased oocyte atresia (five females grade 1, seven females grade 2, two females grade 3 and one female grade 4), thirteen females egg debris (five females grade 1, five females grade 2 and three females grade 3), fifteen females a granulomatous inflammation (three females grade 1, seven females grade 2 and five females grade 3) and seven females increased post-ovulatory follicles (four females grade 1, two females grade 2 and one female grade 3).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

One male fish showed a testis-ova (grade 1) and one male fish showed increased proportion of spermatogonia (grade 2). Additional diagnoses were not observed in the male fish of this group.

0.91 µg Dexamethasone/L (mean measured concentration) – group 2

Four female fish (4/20) had the ovary stage 2 and sixteen females (16/20) the ovary stage 3.

Twelve female fish showed increased oocyte atresia (seven females grade 1, two females grade 2, two females grade 3 and one female grade 4), thirteen females egg debris (four females grade 1, seven females grade 2, one female grade 3 and one female grade 4), twelve females a granulomatous inflammation (nine females grade 1, two females grade 2 and one female grade 3) and seven females increased postovulatory follicles (five females grade 1 and two females grade 2).

Sixteen male fish (16/20) were diagnosed with the testis stage 2 and four males (4/20) with the stage 3.

One male fish showed increased proportion of spermatogonia (grade 1). Additional diagnoses were not observed in the male fish of this group.

3.19 µg Dexamethasone/L (mean measured concentration) – group 3

Three female fish (3/21) had the ovary stage 2, seventeen females (17/21) the ovary stage 3 and one female (1/21) the ovary stage 4.

Eleven female fish showed increased oocyte atresia (seven females grade 1, three females grade 2 and one female grade 3), thirteen females egg debris (eight females grade 1, four females grade 2 and one female grade 3), nine females a granulomatous inflammation (four females grade 1,

four females grade 2 and one female grade 4) and eight females increased post-ovulatory follicles (two females grade 1 and six females grade 2).

Fifteen male fish (15/19) were diagnosed with the testis stage 2 and four males (4/19) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

10.5 µg Dexamethasone/L (mean measured concentration) – group 4

Two female fish (2/20) had the ovary stage 2, seventeen females (17/20) the ovary stage 3 and one female (1/20) the ovary stage 4.

Six female fish showed increased oocyte atresia (four females grade 1, one female grade 2 and one female grade 3), six females egg debris (three females grade 1, two females grade 2 and one female grade 3), fourteen females a granulomatous inflammation (eight females grade 1, three females grade 2 and three females grade 3) and seven females increased post-ovulatory follicles (three females grade 1 and four females grade 2).

Eighteen male fish (18/20) were diagnosed with the testis stage 2 and two males (2/20) with the stage 3.

One male fish showed increased proportion of spermatogonia (grade 1). Additional diagnoses were not observed in the male fish of this group.

34.7 µg Dexamethasone/L (mean measured concentration) – group 5

One female fish (1/19) had the ovary stage 1, four female fish (4/19) had the ovary stage 2 and fourteen females (14/19) the ovary stage 3.

Four female fish showed increased oocyte atresia (all grade 1), eleven females egg debris (five females grade 1, five females grade 2 and one female grade 3), eight females a granulomatous inflammation (four females grade 1 and four females grade 2) and six females increased post-ovulatory follicles (three females grade 1 and three females grade 2).

Seventeen male fish (17/20) were diagnosed with the testis stage 2 and three males (3/20) with the stage 3.

Additional diagnoses were not observed in the male fish of this group.

100 µg Dexamethasone/L (mean measured concentration) – group 6

Eight female fish (8/20) had the ovary stage 2 and twelve females (12/20) the ovary stage 3. Seven female fish showed increased oocyte atresia (four females grade 1 and three females grade 2), five females egg debris (two females grade 1 and three females grade 2), three females a granulomatous inflammation (all grade 1) and four females increased post-ovulatory follicles (two females grade 1 and two females grade 2).

Fourteen male fish (14/19) were diagnosed with the testis stage 2 and five males (5/19) with the stage 3.

Three male fish showed increased proportion of spermatogonia (grade 1) and one male a granulomatous inflammation (grade 1).

3.1.4.7.2 Histopathology results, first filial generation (F1)

Control – group 0

Twenty-five female fish (25/34) had the ovary stage 2 and nine females (9/34) the ovary stage 3.

Three female fish showed increased oocyte atresia (all grade 1), seven females egg debris (six females grade 1 and one female grade 2), three females a granulomatous inflammation (two females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (all grade 1).

All twenty-six male fish (26/26) were diagnosed with the testis stage 2.

One male fish showed a testis-ova (grade 1) and one male fish an increased proportion of spermatogonia (grade 1).

0.33 µg Dexamethasone/L (mean measured concentration) – group 1

Thirty-eight female fish (38/52) had the ovary stage 2 and fourteen females (14/52) the ovary stage 3.

Eight female fish showed increased oocyte atresia (seven females grade 1 and one female grade 2), eight females egg debris (all grade 1), nine females a grade 1 granulomatous inflammation and eight females increased post-ovulatory follicles (all grade 1).

One male fish (1/28) was diagnosed with the testis stage 1, twenty-five males (25/28) with the stage 2 and two males (2/28) with the stage 3.

Three male fish showed a testis-ova (all grade 1). Additional diagnoses were not observed in the male fish of this group.

0.91 µg Dexamethasone/L (mean measured concentration) – group 2

Two female fish (2/56) had the ovary stage 1, forty-one females (41/56) the ovary stage 2 and thirteen females (13/56) the ovary stage 3.

Three female fish showed increased oocyte atresia (two females grade 1 and one female grade 2), six females egg debris (four females grade 1 and two females grade 2), eight females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (four females grade 1 and three females grade 2).

Twenty-two male fish (22/23) were diagnosed with the testis stage 2 and one male (1/23) with the stage 3.

One male fish showed a testis-ova (grade 1). One male had a granulomatous inflammation (grade 1).

3.19 µg Dexamethasone/L (mean measured concentration) – group 3

Thirty-four female fish (34/48) had the ovary stage 2 and fourteen females (14/48) the ovary stage 3.

Two female fish showed increased oocyte atresia (all grade 1), seven females egg debris (six females grade 1 and one female grade 2), seven females a granulomatous inflammation (five females grade 1, one female grade 2 and one female grade 3) and six females increased post-ovulatory follicles (four females grade 1 and two females grade 3).

One male fish (1/30) was diagnosed with the testis stage 1, twenty-eight males (28/30) with the testis stage 2 and one male (1/30) with the stage 3.

One male fish showed a testis-ova (grade 4). Additional diagnoses were not observed in the male fish of this group.

10.5 µg Dexamethasone/L (mean measured concentration) – group 4

Twenty-six female fish (26/39) had the ovary stage 2 and thirteen females (13/39) the ovary stage 3.

Four female fish showed increased oocyte atresia (all grade 1), three females egg debris (two females grade 1 and one female grade 2), seven females a granulomatous inflammation (four females grade 1 and three female grade 2) and one female increased post-ovulatory follicles (grade 2).

One male fish of this group had also an ovary stage 1 ovary separated from the testes. Therefore, this animal was diagnosed as hermaphrodite. Two male fish (2/38) was diagnosed with the testis stage 1, thirty-five males (35/38) with the testis stage 2 and one male (1/38) with the stage 3.

The fish diagnosed as hermaphrodite showed a testis-ova (grade 1). Additional diagnoses were not observed in the male fish of this group.

34.7 µg Dexamethasone/L (mean measured concentration) – group 5

Thirty-five female fish (35/56) had the ovary stage 2 and twenty-one females (21/56) the ovary stage 3.

Seven female fish showed increased oocyte atresia (all grade 1), Twelve females egg debris (nine females grade 1, two females grade 2 and one female grade 3), twelve females a granulomatous inflammation (all grade 1) and seven females increased post-ovulatory follicles (six females grade 1 and one females grade 2).

One male fish of this group had also an ovary stage 1 ovary separated from the testes. Therefore, this animal was diagnosed as hermaphrodite. Two male fish (2/22) was diagnosed with the testis stage 1 and twenty-one (21/22) with the testis stage 2.

Additional diagnoses were not observed in the male fish of this group.

100 µg Dexamethasone/L (mean measured concentration) – group 6

Thirty-one female fish (31/54) had the ovary stage 2 and twenty-three females (23/54) the ovary stage 3.

Three female fish showed increased oocyte atresia (all grade 1), eleven females egg debris (six females grade 1, three females grade 2 and two females grade 3), eleven females a granulomatous inflammation (ten females grade 1 and one female grade 2) and three females increased post-ovulatory follicles (two females grade 1 and one females grade 2).

One male fish (1/23) was diagnosed with the testis stage 1 and twenty-two (22/23) with the testis stage 2.

Additional diagnoses were not observed in the male fish of this group.

3.1.4.8 Summary and Conclusion

The presented ZEOGRT study aimed to assess the effects of continuous exposure to Dexamethasone on different life stages and life performances of zebrafish (*Danio rerio*). The measured water parameters and the results of the regular chemical analysis confirmed stable test conditions throughout the study.

In the **parental generation (F0)**, no effects on reproduction in terms of fecundity and fertility were detected. The determination of the phenotypic sex confirmed the correct setting of spawning groups for most of the test vessels. Effects related to test substance exposure were mainly found for the growth parameters. Male wet weight was significantly reduced compared to control starting at a concentration of 34.7 µg Dexamethasone/L (mean measured concentration). Thus, the NOEC was set at 10.5 µg Dexamethasone/L. Female wet weight as well as total length were also reduced in the presence of Dexamethasone, but only at the highest treatment level of 100 µg Dexamethasone/L (mean measured concentration). Accordingly, the NOEC was set as 34.7 µg Dexamethasone/L (mean measured concentration). Vitellogenin concentration in male and female blood plasma and the histopathological pattern of the examined gonads of both males and females were not affected by the test substance.

Also, in the **first filial generation (F1)**, retarded growth of the exposed fish was observed and thus consistent with the findings linked to F0. Notably, the reduction of growth in terms of total length was evident during development from larvae to adult male fish. More precisely, at day 35 pf, the total length was significantly reduced compared to control only at the highest treatment level, whereas measurement at day 63 pf as well as male total length at test end revealed a significant reduction starting even at 34.7 µg Dexamethasone/L (mean measured concentration). However, female total length at test end were not affected by Dexamethasone exposure, but male and female wet weights at test end were significantly reduced starting at 34.7 µg Dexamethasone/L (mean measured concentration).

There were no effects on the reproduction capability of the F1. Although the growth performance was impacted, there was no delay observed regarding the date of spawning start, i.e. day of first spawning. The reproduction parameters fecundity and fertility were not negatively impacted due to test substance exposure. Other apical endpoints like survival or sex ratio as well as vitellogenin concentration in blood plasma and the histopathological pattern of the examined gonads were not affected by the test item.

Hatching success of the **second filial generation (F2)** was found to be significantly reduced at 10.5 and 34.7 µg Dexamethasone/L (mean measured concentration). However, as the hatching rates achieved 90% and a concentration-response is missing, a biological relevance of this observation can be excluded.

Based on the endpoint growth in terms of wet weight and total length the NOEC was determined to be 10.5 µg Dexamethasone/L. The LOEC was set at 34.7 µg Dexamethasone/L (mean measured concentration).

All results of the ZEOGRT with Dexamethasone are shown in Table 59.

Table 59: Study 4, ZEOGRT with Dexamethasone: Summary of NOEC / LOEC determination during the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dexamethasone [µg/L]	NOEC / LOEC Mean measured concentration Dexamethasone [µg/L]
F_0-Growth	**Wet weight males)[a]**	**10.0 / 32.0**	**10.5 / 34.7**
	Wet weight females)[b]	**32.0 / 100**	**34.7 / 100**
	Total length males	≥ 100 / >100	≥ 100 / >100
	Total length females)[b]	**32.0 / 100**	**34.7 / 100**
	Sex ratio (% males)	≥ 100 / >100	≥ 100 / >100
	Sex ratio (% females)	≥ 100 / >100	≥ 100 / >100
F_0-Reproduction [1])	Fecundity (Egg number per day and female)	≥ 100 / >100	≥ 100 / >100
	Fertilization rate	≥ 100 / >100	≥ 100 / >100
F_0-Histopathology	Maturity stage ovary	≥ 100 / >100	≥ 100 / >100
	Maturity stage testis	≥ 100 / >100	≥ 100 / >100
	Histopathology (females)	≥ 100 / >100	≥ 100 / >100
	Histopathology (males)	≥ 100 / >100	≥ 100 / >100
F_0-Biomarker	Vitellogenin females	≥ 100 / >100	≥ 100 / >100
	Vitellogenin males	≥ 100 / >100	≥ 100 / >100
F_1-ELS	Hatch day 5	≥ 100 / >100	≥ 100 / >100
	Survival day 21	≥ 100 / >100	≥ 100 / >100
	Survival day 35	≥ 100 / >100	≥ 100 / >100
	Total length day 35)[a]	**32.0 / 100**	**34.7 / 100**
F_1-Juveniles	Survival day 35 – day 63	≥ 100 / >100	≥ 100 / >100
	Total length day 63)[b]	**10.0 / 32.0**	**10.5 / 34.7**
F_1-Reproduction	Time to first spawning	≥ 100 / >100	≥ 100 / >100
	Fecundity (Egg number per day and female)	≥ 100 / >100	≥ 100 / >100
	Fertilization rate	≥ 100 / >100	≥ 100 / >100

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Dexamethasone [µg/L]	NOEC / LOEC Mean measured concentration Dexamethasone [µg/L]
F_1-Termination	Survival (test end)	≥ 100 / >100	≥ 100 / >100
	Total length males)[b]	**32.0 / 100**	**34.7 / 100**
	Total length females	≥ 100 / >100	≥ 100 / >100
	Wet weight males)[b]	**32.0 / 100**	**34.7 / 100**
	Wet weight females)[b]	**32.0 / 100**	**34.7 / 100**
	Sex ratio (% females)	≥ 100 / >100	≥ 100 / >100
	Sex ratio (% males)	≥ 100 / >100	≥ 100 / >100
F_1-Biomarker	Vitellogenin females	≥ 100 / >100	≥ 100 / >100
	Vitellogenin males	≥ 100 / >100	≥ 100 / >100
F_1-Histopathology	Maturity stage ovary	≥ 100 / >100	≥ 100 / >100
	Maturity stage testis	≥ 100 / >100	≥ 100 / >100
	Histopathology (females)	≥ 100 / >100	≥ 100 / >100
	Histopathology (males)	≥ 100 / >100	≥ 100 / >100
F_2-Embryo	Hatch day 4 **)[c]**	≥ 100 / >100 [2)]	≥ 100 / >100 [2)]

)[a]: Step down Jonckheere-Terpstra test, p<0.05, one-sided smaller.

)[b]: Williams test, p<0.05, one-sided smaller.

)[c]: Multiple sequentially-rejective Welsh-t-test after Bonferroni-Holm, p<0.05, one-sided-smaller.

[1]) The statistical evaluation revealed a significant reduction of egg numbers at 0.33 and 0.91 µg Dexamethasone/L, and for fertilization rate at 10.5 and 34.7 µg Dexamethasone/L. However, a concentration related impact on the reproductive capability of the fish could not be observed. Thus, the biological relevance of these observations was considered as minor.

[2]) Hatching success was found to be significantly reduced at 10.5 and 34.7 µg Dexamethasone/L (mean measured concentration). However, the hatching rate at the top concentration was not significantly different from control. Thus, a biological relevance of this observation can be excluded.

3.2 Validation, Phase II: Studies at external laboratories

3.2.1 Phase II, Study 5: ZEOGRT with Tamoxifen-Citrate

3.2.1.1 Test conditions

The mean water temperatures per replicate in control and treatments were calculated to be between 26.1 and 26.4 °C. The single measurements in all test vessels were between 25.0 and 27.1 °C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C. The mean oxygen concentrations per replicate throughout the test period were determined to be between 89 and 98 %, based on single measurement between 72 and 110 %. The mean pH levels on replicate basis were between 7.51 and 7.63 in controls and treatments. The single pH measurements were between 7.40 and 7.90. There was no impact of the substance application on the pH level.

Summing up, stable conditions of exposure could be confirmed.

All mean values and standard deviations are summarized in Table 60 to Table 62. For more details, please refer to the appendix report.

Table 60: Study 5, ZEOGRT with Tamoxifen-Citrate: Test conditions, water temperature [°C]

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	26.3	0.3	25.4	26.7
	B	26.2	0.3	25.4	27.0
	C	26.1	0.3	25.3	26.8
	D	26.2	0.3	25.4	26.8
0.29	**A**	26.2	0.4	25.0	27.1
	B	26.3	0.3	25.2	27.1
	C	26.3	0.3	25.4	27.0
	D	26.3	0.3	25.4	26.9
0.92	**A**	26.2	0.3	25.4	27.1
	B	26.1	0.3	25.1	27.0
	C	26.3	0.3	25.4	26.9
	D	26.2	0.3	25.4	26.7
2.92	**A**	26.2	0.3	25.3	26.7
	B	26.2	0.3	25.2	26.8
	C	26.2	0.3	25.3	26.9
	D	26.2	0.3	25.4	26.8
8.30	**A**	26.2	0.3	25.3	27.0
	B	26.3	0.3	25.2	27.0

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
	C	26.3	0.3	25.2	26.9
	D	26.3	0.3	25.4	26.8
26.3	**A**	26.4	0.2	26.0	26.8
	B	26.3	0.2	26.0	26.8
	C	26.3	0.2	26.0	26.6
	D	26.3	0.2	25.9	26.6

SD = Standard deviation

Table 61: Study 5, ZEOGRT with Tamoxifen-Citrate: Oxygen concentration [%]

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	94	7	82	104
	B	96	8	81	104
	C	93	9	78	105
	D	96	8	86	110
0.29	**A**	91	8	81	104
	B	96	8	86	107
	C	92	8	86	110
	D	95	8	84	108
0.92	**A**	91	9	72	104
	B	95	8	86	109
	C	93	8	84	106
	D	96	8	86	108
2.92	**A**	90	8	81	103
	B	95	8	83	107
	C	93	7	87	106
	D	95	8	87	107

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
8.30	**A**	96	8	81	105
	B	96	9	82	106
	C	97	7	88	106
	D	98	6	90	106
26.3	**A**	89	4	86	96
	B	94	7	89	106
	C	92	7	85	104
	D	95	6	90	106

SD = Standard deviation

Table 62: Study 5, ZEOGRT with Tamoxifen-Citrate: Test conditions, pH value

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.62	0.11	7.45	7.86
	B	7.63	0.12	7.45	7.86
	C	7.59	0.11	7.40	7.79
	D	7.60	0.08	7.47	7.69
0.29	**A**	7.60	0.12	7.45	7.86
	B	7.62	0.09	7.48	7.81
	C	7.61	0.08	7.47	7.69
	D	7.63	0.09	7.47	7.79
0.92	**A**	7.60	0.12	7.46	7.87
	B	7.61	0.10	7.48	7.85
	C	7.59	0.10	7.44	7.78
	D	7.62	0.10	7.48	7.83
2.92	**A**	7.59	0.13	7.46	7.87
	B	7.60	0.11	7.47	7.87
	C	7.59	0.11	7.45	7.81
	D	7.61	0.11	7.47	7.86

Nominal concentration Tamoxifen-Citrate [µg/L]	Replicate	Mean	SD	Min value	Max value
8.30	**A**	7.59	0.12	7.45	7.86
	B	7.60	0.12	7.46	7.88
	C	7.60	0.12	7.46	7.84
	D	7.61	0.11	7.47	7.87
26.3	**A**	7.51	0.08	7.45	7.64
	B	7.57	0.18	7.46	7.90
	C	7.54	0.15	7.45	7.85
	D	7.57	0.17	7.46	7.89

SD = Standard deviation

3.2.1.2 Chemical analysis

The mean measured concentration were calculated to be 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L. These corresponded to 76, 75, 75, 77 and 86 % of the nominal concentrations, respectively.

Primary stock solution was prepared three times a week as follows: 40 to 55 mg of Tamoxifen-Citrate and 4/5.5 L of MilliQ water were transferred in amber-glass bottle, sonicated for 1h, and stirred for 24h. After stirring for 24h, the primary stock solution was acidified to pH 3.0 with 1 M HCl. Application solution of 5/6.5L (Mon, Wed, Fri) was prepared by dilution of primary stock solution with MilliQ water. The solution was acidified to pH 3 again with 1 M HCl. The flow rate was determined approximately for two vessels supplied by a dosing pump.

The diluted test solution was first stored in a distribution reservoir (just above the tank) which provided the test solution to two vessels through the drop port on the bottom of the distribution reservoir. The flow rate of pump was slightly larger than the volume for two vessels and the excess solution was overflowed from the distribution reservoir.

All results of the chemical analysis are shown in Table 63. For more details, please refer to the appendix report.

Table 63: Study 5, ZEOGRT with Tamoxifen-Citrate: Chemical analysis

Nominal concentration Tamoxifen-Citrate	Mean measured concentration Tamoxifen-Citrate			
[µg/L]	[µg/L]	SD	[%] of nominal	[%] RSD
control	-	-	-	-
0.29	0.220	0.015	76	6.8
0.92	0.692	0.052	75	7.5
2.92	2.19	0.26	75	11.7
8.30	6.38	0.59	77	9.3
26.3	22.5	3.4	86	15.2

SD = Standard deviation; RSD = Relative standard deviation

3.2.1.3 Biological results, parental generation (F0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. One male fish was corrected by histopathological examination to female (replicate D at 2.19 µg Tamoxifen-Citrate/L (mean measured)). This was considered for calculation of mean egg number per day and female. 14 fish died during the study, i.e. 9 female and 5 male fish. Two female fish died during recording reproduction and were considered for calculation of mean egg number per day and female, i.e. one female in replicate C at 0.220 µg Tamoxifen-Citrate /L (mean measured), another female fish died in replicate B at 22.5 µg test item/L (mean measured). More details are presented in Table 64.

Reproduction was recorded in terms of egg numbers and fertilization rates in the period from day 1 pf to day 21 pf. For the controls, a mean of 33 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be 19, 22, 29, 33 and 21 at 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean egg numbers and standard deviations are shown in Table 65. The mean fertilization rate for the controls was calculated to be 94.7 %. For the exposed groups, the fertilization rates were determined to be between 92.9 % (at 0.692 µg Tamoxifen-Citrate/L (mean measured)) and 95.5 % (at 2.19 µg Tamoxifen-Citrate/L (mean measured)). The statistical evaluation revealed a significant reduction of egg numbers per female and day compared to control only at 0.220 µg Tamoxifen-Citrate/L (mean measured; Dunnett`s Multiple t-test, $p<0.05$, one-sided smaller). The fertility rate was not altered by test substance exposure (Dunnett`s Multiple t-test, $\alpha = 0.05$, one-sided smaller).

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads and verified by histopathological analysis. The inspection revealed that the groups were mostly set correctly, i.e. approx. 5 males and females were present. Only one fish (replicate D at 2.19 µg Tamoxifen-Citrate/L (mean measured)) was corrected by histopathological examination to female (see below for details).

The mean fish total lengths of males were determined to be 4.8 cm in the controls and 4.8, 4.7, 4.7, 4.7 and 4.7 cm in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean fish total lengths of females were determined to be

5.0 cm in controls and 5.0, 5.1, 5.1, 5.1, and 5.2 cm in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean wet weights of males were determined to be 0.894 g in controls and 0.891, 0.871, 0.851, 0.855 and 0.878 g in treatments 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean wet weights in females were determined to be 1.191 g in controls and 1.229, 1.271, 1.256, 1.308 and 1.540 g in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical evaluation revealed, that compared to control the test chemical had no influence on male fish growth as assessed by determination of total length and wet weight at termination (Dunnett`s Multiple t-test, α=0.05, two-sided). In contrast to this, female length was increased at the highest treatment level compared to control (Williams Multiple t-test, $p<0.05$, two-sided). The wet weight was increased by Tamoxifen-Citrate as well, however, not statistically significant, but showing a clear trend (Multiple Sequentially-rejective Welsh t-test after Bonferroni-Holm; α=0.05; two-sided).

All mean values and standard deviations of all biological parameters are summarized in Table 65.

For more details, please refer to the appendix report.

Table 64: Study 5, ZEOGRT with Tamoxifen-Citrate: Overview fish group setting (verified by histopathological analysis), F0

	Nominal concentration Tamoxifen-Citrate [µg/L]																							
	Control				**0.29**				**0.92**				**2.92**				**8.30**				**26.3**			
	Mean measured concentration Tamoxifen-Citrate [µg/L]																							
	Control				**0.220**				**0.692**				**2.19**				**6.38**				**22.5**			
Replicate	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D** *	**A**	**B**	**C**	**D**	**A**	**B**	**C**	**D**
Parental generation F0																								
Male (m) [n]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**4**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Female (f) [n]	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**6**	**5**	**5**	**5**	**5**	**5**	**5**	**5**	**5**
Mortality (no. and sex) [n]	-	**1 f**	-	-	**1 f**	-	**1 f**	-	-	**1 m**	-	-	**2 m**	-	**1 f**	-	-	-	**2 f**	-	**1 f**	**2 f**	**2 m**	-

*: One male fish was corrected by histopathological examination to female (replicate D at 2.92 µg test item/L).

Table 65: Study 5, ZEOGRT with Tamoxifen-Citrate: Biological results for parental generation (F0)

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.220**	**0.692**	**2.19**	**6.38**	**22.5**
Mean egg number per day and female [n]	**33**	**19)[a]**	**22**	**29**	**33**	**21**
SD	13	8	6	9	8	2
Mean fertilization rate [%]	**94.7**	**94.0**	**92.9**	**95.5**	**94.3**	**93.5**
SD	0.5	3.1	3.0	1.2	0.7	2.6
Total length, males [cm]	**4.8**	**4.8**	**4.7**	**4.7**	**4.7**	**4.7**
SD	0.1	0.1	0.1	0.1	0.0	0.1
Total length, females [cm]	**5.0**	**5.0**	**5.1**	**5.1**	**5.1**	**5.2)[b]**
SD	0.1	0.1	0.1	0.1	0.1	0.1
Wet weight, males [g]	**0.894**	**0.891**	**0.871**	**0.851**	**0.855**	**0.878**
SD	0.066	0.054	0.043	0.072	0.031	0.081
Wet weight, females [g]	**1.191**	**1.229**	**1.271**	**1.256**	**1.308**	**1.540**
SD	0.045	0.046	0.146	0.030	0.038	0.238
Mean sex ratio, males [%]	**51.4**	**52.8**	**48.6**	**45.8**	**53.1**	**51.4**
SD	2.8	3.2	2.8	8.5	6.3	10.6
Mean sex ratio, females [%]	**48.6**	**47.2**	**51.4**	**54.2**	**46.9**	**48.6**
SD	2.8	3.2	2.8	8.5	6.3	10.6
Mean survival all at termination [%]	**97.5**	**95.0**	**97.5**	**92.5**	**95.0**	**87.5**
SD	5.0	5.8	5.0	9.6	10.0	9.6

SD = Standard deviation

)[a]: Significantly reduced compared to control. only at 0.220 µg/L (mean measured; Dunnett`s Multiple t-test; p<0.05; one-sided smaller).

)[b]: Significantly increased compared to control. only at 22.5 µg/L (mean measured; Williams Multiple t-test; p<0.05, two-sided).

3.2.1.4 Biological results, first filial generation (F1)

The filial 1 (F1) generation was initiated by keeping fertilized eggs from the parental group and placing them in separate brood chambers placed in each test vessel. The allocation of eggs, in total 36, was splitted over two subsequent days, which means that 18 eggs were kept on day 1 and a second set of eggs was kept on day 2.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be >80 % in all test vessels (related to the number of eggs introduced). The mean hatching rate was 100 % in the controls and 99.3, 100, 99.3, 100 and 95.8 % in treatments 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical analysis revealed a significantly reduced hatching rate compared to control at the highest treatment level of 22.5 µg Tamoxifen-Citrate/L (mean measured) (Chi^2 2x2 Table Test with Bonferroni Correction; $p<0.05$; one-sided greater).

The number of surviving fish larvae were determined on day 21 and 35 pf by photo evaluation. The post-hatch survival rate in controls was calculated to be 90.3 % and 88.9 % for day 21 and 35 pf, respectively. The acceptance criterion, i.e. 75 %, was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be 88.2, 83.3, 94.4, 34.7 and 0.7 % at day 21 pf in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. At day 35 pf the mean post-hatch survival rates were determined to be 86.8, 82.6, 92.3, 16.7 and 0 % in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical analyses evaluating both time points (i.e. day 21 and day 35 pf) revealed a significant reduction in larval survival between controls and treatments with 6.38 and 22.5 µg Tamoxifen-Citrate/L both (mean measured) (Step-down Rao-Scott-Cochran-Armitage Test Procedure; $p<0.05$; one-sided greater).

On day 35 pf, the total length of all remaining fish larvae was measured computer-aided, and the mean total length was found to be 1.4 cm in the control as well as in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. Subsequently, there was no statistically significant difference between control and treatments (Dunnett`s Multiple t-test; $\alpha=0.05$; one-sided smaller). No surviving fish were left at the highest treatment level.

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel. On day 65 pf, the fish groups were again photographed, revealing a survival rate compared to day 35 pf upon reduction of 100 % in the controls a well as in all remaining treatments (i.e. 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured). The mean total length at day 63 pf was measured to be 3.7 cm in the controls and 3.7, 3.6, 3.6 and 3.6 cm treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. No statistically significant effect compared to control was found (Dunnett`s Multiple t-test; $\alpha=0.05$; two-sided).

At day 56 pf and afterwards, spawning trays were introduced to assess and record the first spawning events. In the controls, the time to first spawning was in the range from day 70 to day 73 pf, while spawning onset was found to be in the range from day 70 to day 80 pf, from day 74 to day 80 pf and from day 79 to day 87 pf in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. Statistical analysis revealed a significantly delayed spawning onset compared to control in treatments with 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured) (Williams Multiple t-test; $p<0.05$; one-sided greater). From day 108 pf onwards, reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 56 eggs per female and day was calculated. The mean egg numbers per female and day in the exposed groups were determined to be 62, 53, 55 and 0 in

treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean fertilization rate was calculated to be 91.4 % in the control and 93.9, 91.7, 86.3 and 66.7 % in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. Please note, that at 6.38 µg Tamoxifen-Citrate/L fecundity and fertility data were only possible to assess in replicate B due to the presence of one single female fish. In replicates A, C and D, only male fish were present. Statistical evaluation revealed a significant reduction in terms of fecundity and fertility compared to control at 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively (Williams Multiple t-test; $p<0.05$; one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis.

The mean sex ratio for males [%] were determined to be 62.8 % in the controls and 72.6 %, 54.6 %, 89.7 % and 90.0 % in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively.

The mean sex ratio for females [%] were determined to be 33.3 % in the controls and 26.0 %, 45.4 %, 10.3 % and 5.0 % in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively.

The obtained results indicated a test item induced shift in sex ratio towards a significantly increased number of males at 6.38 µg Tamoxifen-Citrate/L (mean measured; Step-down Jonckheere-Terpstra test; $p<0.05$; two-sided). The decreased number of females was not found to be statistically significant, although the trend is clear (mean measured; Williams Multiple t-test; $\alpha=0.05$; two-sided).

The mean fish total lengths of males were determined to be 4.7 cm in controls and 4.6, 4.6, 4.6 and 4.4 in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean fish total lengths of females were determined to be 4.8 cm in controls and 4.9, 4.7, 4.9 and 4.5 cm in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. Please note, that only one single female was present at 6.38 µg Tamoxifen-Citrate/L (mean measured).

The mean fish wet weights of males were determined to be 0.886 g in the controls and 0.806, 0.795, 0.822 and 0.700 g in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. The mean wet weights of females were determined to be 1.086 g for the controls and 1.110, 1.060, 1.201 and 0.900 g in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively. Please note, that only one single female was present at 6.38 µg Tamoxifen-Citrate/L (mean measured).
Statistical analysis revealed, that neither length nor weight of female fish were significantly altered by the test item (Dunnett`s Multiple t-test; $\alpha=0.05$; two-sided). In contrast to this, male length and weight were significantly reduced compared to control at all treatment levels (mean measured; Williams Multiple t-test; $p<0.05$; two-sided).

All results, i.e. mean values and standard deviations, are shown in Table 66. For more details, please refer to the appendix report.

Table 66: Study 5, ZEOGRT with Tamoxifen-Citrate: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.220	0.692	2.19	6.38*	22.5**
Mean hatching rate [%]	**100**	**99.3**	**100**	**99.3**	**100**	**95.8)[a]**
SD	0.0	1.4	0.0	1.4	0.0	8.3
Mean post-hatch survival at day 21 pf [%]	**90.3**	**88.2**	**83.3**	**94.4**	**34.7)[b]**	**0.7)[b]**
SD	9.8	8.9	13.0	6.5	15.1	1.4
Mean post-hatch survival at day 35 pf [%]	**88.9**	**86.8**	**82.6**	**92.3**	**16.7)[b]**	**0.0)[b]**
SD	8.3	8.2	12.9	4.3	8.2	0.0
Mean total length, day 35 pf [cm]	**1.4**	**1.4**	**1.4**	**1.4**	**1.4**	**-**
SD	0.0	0.1	0.1	0.1	0.2	-
Mean survival day 65 pf [%]	**100**	**100**	**100**	**100**	**100**	-
SD	0.0	0.0	0.0	0.0	0.0	-
Mean total length, day 63 pf [cm]	**3.7**	**3.7**	**3.6**	**3.6**	**3.6**	**-**
SD	0.1	0.1	0.1	0.2	0.1	-
Time to first spawning [fish age days] (± SD)	A 73	74	74	87	-	-
	B 73	80	80	81	87	-
	C 73	70	77	81	-	-
	D 70	70	80	79	-	-
Significantly delayed compared to control.	-	**-**	**)[c]**	**)[c]**	**)[c]**	**-**
Mean egg number per day and female [n]	**56**	**62**	**53**	**55**	**0)[d]**	**-**
SD	17	15	4	24	-	-
Mean fertilization rate [%]	**91.4**	**93.9**	**91.7**	**86.3**	**66.7)[d]**	**-**
SD	4.1	0.8	1.9	7.3	-	-

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.220**	**0.692**	**2.19**	**6.38***	**22.5****
Mean survival, adult stage [%]	**95.0**	**91.3**	**95.0**	**97.5**	**90.8**	-
SD	4.1	6.3	7.1	2.9	10.7	-
Mean total length, males, adult stage [cm]	**4.7**	**4.6)[e]**	**4.6)[e]**	**4.6)[e]**	**4.4)[e]**	**-**
SD	0.2	0.1	0.1	0.1	0.1	-
Mean total length, females, adult stage [cm]	**4.8**	**4.9**	**4.7**	**4.9**	**4.5**	**-**
SD	0.1	0.3	0.3	0.1	-	-
Mean wet weight, males, adult stage [g]	**0.886**	**0.806)[e]**	**0.795)[e]**	**0.822)[e]**	**0.700)[e]**	**-**
SD	0.090	0.062	0.020	0.040	0.029	
Mean wet weight, females, adult stage [g]	**1.086**	**1.110**	**1.060**	**1.201**	**0.900**	**-**
SD	0.107	0.199	0.180	0.089	-	-
Mean sex ratio, males [%]	**62.8**	**72.6**	**54.6**	**89.7**	**90.0)[f]**	**-**
SD	15.0	7.8	12.9	5.9	11.5	-
Mean sex ratio, females [%]	**33.3**	**26.0**	**45.4**	**10.3**	**5.0**	**-**
SD	13.4	10.4	12.9	5.9	10.0	-

SD = Standard deviation

Remark: For single fish, gonads were not detected after processing for histological slides.

*: One single female fish was present at 6.38 µg test item/L (mean measured). Thus, the mean value was based on replicate C only.

**: No fish larvae survived at 22.5 µg test item/L (mean measured) (all larvae dead before day 35 pf).

)[a]: Significantly higher mortality compared to ctr.; Chi² 2x2 Table Test with Bonferroni Correction; p<0.05; one-sided greater.

)[b]: Significantly higer mortality compared to ctr.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[c]: First spawning was delayed compared to ctr.; Williams Multiple t-test; p<0.05; one-sided greater.

)[d]: Reduced compared to ctr.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[e]: Significantly reduced compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[f]: Significantly increased compared to control.; Step-down Jonckheere-Terpstra test; p<0.05; two-sided.

3.2.1.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 100 % and 100, 99.3, 100 and 100 % in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured concentration), respectively. Please note, that the mean value given for the treatment with 6.38 µg Tamoxifen-Citrate (mean measured) was based on one single replicate (i.e. replicate C).

For more details, please refer to the appendix report.

Since the hatching success was assessed to be 100 % at almost all treatment levels, statistical assessment was unnecessary to perform.

Table 67: Study 5, ZEOGRT with Tamoxifen-Citrate: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.220	0.692	2.19	6.38)[b]	22.5)[a]
Mean hatching rate [%]	100	100	99.3	100	100	-
SD	0.0	0.0	1.4	0.0	-	-

SD = Standard deviation

)[a]: No fish larvae survived at 22.5 µg test item/L (mean measured) (all larvae dead before day 35 pf).

)[b]: One single female fish was present at 6.38 µg test item/L (mean measured). Thus, the mean value was based on replicate C only.

3.2.1.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric mean of each replicate. The respective mean values are presented in Table 68 (F0 generation) and Table 69 ((F1 generation).) All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin values in controls were determined to be 7.76E+04 ng VTG/mL for males. The mean VTG values for males were determined to be 8.23E+04, 1.55E+05, 1.93E+04, 2.34E+04 and4.21E+04ng VTG/mL in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively.
The mean VTG values in controls were determined to be 6.79E+07 ng VTG/mL for females. The mean VTG values for females were determined to be 2.94E+07, 1.50E+07, 5.55E+06, 9.85E+06 and 5.78E+06 ng VTG/mL in treatments with 0.220, 0.692, 2.19, 6.38 and 22.5 µg Tamoxifen-Citrate/L (mean measured), respectively.

Statistical analyses revealed a significantly reduced VTG concentration compared to control for female fish at all treatment levels but the lowest one (Step-down Jonckheere-Terpstra Test; $p<0.05$; one-sided smaller). The VTG values in males were not altered by the test substance (Multiple Sequentially-rejective Median Test after Bonferroni-Holm; $\alpha=0.05$; one-sided greater).

Table 68: Study 5, ZEOGRT with Tamoxifen-Citrate: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	**0.220**	**0.692**	**2.19**	**6.38**	**22.5**
Mean VTG concentration, males [ng VTG/mL]	**7.76E+04**	**8.23E+04**	**1.55E+05**	**1.93E+04**	**2.34E+04**	**4.21E+04**
SD	1.03E+05	4.23E+04	2.20E+05	2.44E+04	3.25E+04	3.10E+04
Mean VTG concentration, females [ng VTG/mL]	**6.79E+07**	**2.94E+07**	**1.50E+07)[a]**	**5.55E+06)[a]**	**9.85E+06)[a]**	**5.78E+06)[a]**
SD	4.60E+07	6.13E+06	7.80E+06	2.93E+06	8.97E+06	4.57E+06

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric mean of each replicate.

)[a]: Significantly reduced compared to control; Step-down Jonckheere-Terpstra test; $p<0.05$; one-sided smaller.

In first filial generation (F1), the mean vitellogenin (VTG) values in controls were determined to be 5.21E+03 ng VTG/mL for males. The mean VTG values for males were determined to be 1.03E+04, 9.38E+03, 5.61E+03 and 4.77E+03 ng VTG/mL in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively.

The mean VTG values in controls were determined to be 4.46E+07 ng VTG/mL for females. The mean VTG values for females were determined to be 7.60E+07, 3.19E+07, 3.44E+07 and 3.00E+07 ng VTG/mL in treatments with 0.220, 0.692, 2.19 and 6.38 µg Tamoxifen-Citrate/L (mean measured), respectively.

Statistical analyses revealed no statistically significant difference between controls and treatments, neither for males nor for females of first filial generation (Dunnett`s Multiple t-test; $\alpha=0.05$; one-sided smaller (females), respective one-sided greater (males)).

Table 69: Study 5, ZEOGRT with Tamoxifen-Citrate: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng/mL]

Parameter	Mean measured concentration Tamoxifen-Citrate [µg/L]					
	Control	0.220	0.692	2.19	6.38*	22.5**
Mean VTG concentration, males [ng/mL]	**5.21E+03**	**1.03E+04**	**9.38E+03**	**5.61E+03**	**4.77E+03**	**-**
SD	1.66E+03	3.49E+03	4.78E+03	4.10E+03	1.99E+03	-
Mean VTG concentration, females [ng/mL]	**4.46E+07**	**7.60E+07**	**3.19E+07**	**3.44E+07**	**3.00E+07**	**-**
SD	9.1E+06	1.2E+07	1.4E+07	2.8E+07	-	-

SD = Standard deviation

*: Only one single female fish was present at 6.38 µg test item/L (mean measured concentration).

**: No fish larvae survived at 22.5 µg test item/L (mean measured concentration).

3.2.1.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In chapters 3.2.1.7.1 and 3.2.1.7.2, the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 70 and the results for first filial generation are shown in Table 71.

Neither parental generation (F0) nor first filial generation (F1) showed any treatment-related statistically significant differences compared to control in male or female gonad staging. However, histopathological examination revealed a dose dependent increase shift to a higher testicular stage and a dose dependent increase of hypereosinophilic interstitial cells in the testis of the parental (F0) generation, especially in at treatment level of 22.5 µg Tamoxifen-Citrate/L (mean measured). In the F1 generation, a dose-dependent shift to male fish was seen with almost all male fish in treatment with 6.38 µg Tamoxifen-Citrate/L (mean measured). Further, a dose-dependent cystic degeneration within the liver was seen.

Table 70: Study 5, ZEOGRT with Tamoxifen-Citrate: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Tamoxifen-Citrate [µg/L]					
		Control	**0.220**	**0.692**	**2.19**	**6.38**	**22.5**
Maturation stages, male [1)]		Stage 0 (0/20) Stage 1 (1/20) Stage 2 (19/20) Stage 3 (0/20) Stage 4 (0/20)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (20/20) Stage 3 (0/20) Stage 4 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (19/19) Stage 3 (0/19) Stage 4 (0/19)	Stage 0 (0/17) Stage 1 (1/17) Stage 2 (16/17) Stage 3 (0/17) Stage 4 (0/17)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (19/20) Stage 3 (1/20) Stage 4 (0/20)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (12/18) Stage 3 (6/18) Stage 4 (0/18)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**
	B	**2**	**2**	**2**	**2**	**2**	**3**
	C	**2**	**2**	**2**	**2**	**2**	**3**
	D	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 0 (0/19) Stage 1 (0/19) Stage 2 (6/19) Stage 3 (13/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (2/18) Stage 3 (16/18) Stage 4 (0/18) Stage 5 (0/18)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (3/20) Stage 3 (17/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (4/20) Stage 3 (16/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (5/18) Stage 3 (13/18) Stage 4 (0/18) Stage 5 (0/18)	Stage 0 (0/17) Stage 1 (0/17) Stage 2 (7/17) Stage 3 (10/17) Stage 4 (0/17) Stage 5 (0/17)
Maturation stage, female, median value/ replicate	A	**2**	**3**	**3**	**3**	**3**	**3**
	B	**3**	**3**	**3**	**3**	**3**	**3**
	C	**3**	**3**	**3**	**3**	**3**	**2**
	D	**3**	**3**	**3**	**3**	**3**	**2**

[1)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

[2)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

Table 71: Study 5, ZEOGRT with Tamoxifen-Citrate: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Tamoxifen-Citrate [µg/L]					
		Control	**0.220**	**0.692**	**2.19**	**6.38***	**22.5****
Maturation stages, male [1)]		Stage 0 (0/48) Stage 1 (2/48) Stage 2 (45/48) Stage 3 (1/48) Stage 4 (0/48)	Stage 0 (0/53) Stage 1 (3/53) Stage 2 (47/53) Stage 3 (3/53) Stage 4 (0/53)	Stage 0 (0/41) Stage 1 (4/41) Stage 2 (34/41) Stage 3 (3/41) Stage 4 (0/41)	Stage 0 (0/70) Stage 1 (0/70) Stage 2 (47/70) Stage 3 (23/70) Stage 4 (0/70)	Stage 0 (0/19) Stage 1 (1/19) Stage 2 (10/19) Stage 3 (8/19) Stage 4 (0/19)	-
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**3**	**-**
	B	**2**	**2**	**2**	**3**	**3**	**-**
	C	**2**	**2**	**2**	**2**	**2**	**-**
	D	**2**	**2**	**2**	**2**	**3**	**-**
Maturation stages, female [2)]		Stage 0 (0/25) Stage 1 (1/25) Stage 2 (18/25) Stage 3 (5/25) Stage 4 (1/25) Stage 5 (0/25)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (16/19) Stage 3 (1/19) Stage 4 (2/19) Stage 5 (0/19)	Stage 0 (0/35) Stage 1 (1/35) Stage 2 (22/35) Stage 3 (12/35) Stage 4 (0/35) Stage 5 (0/35)	Stage 0 (0/8) Stage 1 (0/8) Stage 2 (5/8) Stage 3 (2/8) Stage 4 (1/8) Stage 5 (0/8)	Stage 3 (1/1)	-
Maturation stage, female, median value/ replicate	A	**2**	**2**	**2**	**3**	**-**	**-**
	B	**3**	**2**	**2**	**3**	**3**	**-**
	C	**2**	**2**	**3**	**2**	**-**	**-**
	D	**2**	**2**	**2**	**2**	**-**	**-**

[1)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

[2)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

*: Only one single female fish was present at 6.38 µg test item/L (mean measured concentration).

**: No fish larvae survived at 22.5 µg test item/L (mean measured concentration).

3.2.1.7.1 Histopathology results, parental generation (F0)

Control – group 0

Six female fish (6/19) had the ovary stage 2 and thirteen females (13/19) the ovary stage 3. One male fish (1/20) was diagnosed with the testis stage 1 and nineteen male fish (19/20) were diagnosed with the testis stage 2. Additional diagnoses were not observed in the female and male fish of this group.

0.220 µg Tamoxifen-Citrate/L (mean measured) – group 1

Two female fish (2/18) had the ovary stage 2 and sixteen females (16/18) the ovary stage 3. One female fish showed increased egg debris, a granulomatous inflammation and increased post-ovulatory follicles (all grade 1). All male fish (20/20) were diagnosed with the testis stage 2. One male fish (1/20) showed cystic degeneration in the liver (grade 1).

0.692 µg Tamoxifen-Citrate/L (mean measured) – group 2

Three female fish (3/20) had the ovary stage 2 and seventeen females (17/20) the ovary stage 3. One female fish showed egg debris and a granulomatous inflammation (both grade1). One female fish (1/20) showed lymphoma in various tissues, which affected the ovary stage and should therefore be excluded from the analysis of the ovary stage. All male fish (19/19) were diagnosed with the testis stage 2. Additional diagnoses were not observed in the male fish of this group.

2.19 µg Tamoxifen-Citrate/L (mean measured) – group 3

Four female fish (4/20) had the ovary stage 2 and sixteen females (16/20) the ovary stage 3. One male fish was diagnosed with the testis stage 1 (1/17) and sixteen male fish were diagnosed with the testis stage 2 (16/17). Additional diagnoses were not observed in the female and male fish of this group.

6.38 µg Tamoxifen-Citrate/L (mean measured) – group 4

Five female fish (5/18) had the ovary stage 2, thirteen (13/18) the ovary stage 3. One female fish showed increased oocyte atresia, egg debris, and a granulomatous inflammation (all grade 1). Nineteen male fish (19/20) were diagnosed with the testis stage 2 and one male (1/20) with the stage 3. Six male fish (6/20) showed hyper-eosinophilic interstitial cells in the testis (two with grade 1, three with grade 2 and one with grade 3. Five male fish showed cystic degeneration in the liver (two with grade 1, one with grade 2, two with grade 3).

22.5 µg Tamoxifen-Citrate/L (mean measured) – group 4

Seven female fish (7/17) had the ovary stage 2 and ten female fish (10/17) had the ovary stage 3. Two female fish showed increased oocyte atresia (all grade 2), eight females egg debris (two females grade 1, two females grade 3 and four female grade 4), and eight females a granulomatous inflammation (two females grade 1, four females grade 2, one female grade 3 and one female grade 4). Twelve male fish (12/18) were diagnosed with the testis stage 2 and six males (6/18) with the stage 3. One male fish showed increased testicular degeneration and asynchronous germ cell development (both grade 1). In nine male fish (9/18), hyper-eosinophilic interstitial cells were observed in the testis (6 with grade 1, two with grade 2, one with grade 3). In two male fish (2/18), there was cystic degeneration in the liver (both grade 1).

3.2.1.7.2 Histopathology results, first filial generation (F1)

In five fish (three fish from group 0, one fish from group 1, and one fish from group 4) gonads were not detected after processing (including step sections).

Control – group 0

One female fish (1/25) had the ovary stage 1, eighteen females (18/25) the ovary stage 2, five females (5/25) the ovary stage 3, and one female (1/25) the ovary stage 4. One female fish showed increased oocyte atresia (grade 2).

Two male fish (2/48) were diagnosed with the testis stage 1, fourty-five male fish (45/48) were diagnosed with the testis stage 2, and one male fish (1/48) was diagnosed with the testis stage 3. One male fish showed increased testicular degeneration (grade 1) and one male fish was diagnosed with cystic degeneration in the liver (grade 1).

0.220 µg/ Tamoxifen-Citrate/L (mean measured concentration) – group 1

Sixteen female fish (16/19) had the ovary stage 2, one female fish (1/19) the ovary stage 3, and two females (2/19) the ovary stage 4. Additional diagnoses were not observed in the female fish of this group.

Three male fish (3/53) were diagnosed with the testis stage 1, fourty-seven males (47/53) with the stage 2 and three males (3/53) with the stage 3. One male fish showed increased testicular degeneration (grade 1).

0.692 µg/ Tamoxifen-Citrate/L (mean measured concentration) – group 2

One female fish (1/35) had the ovary stage 1, twenty-two females (22/35) the ovary stage 2 and twelve females (12/35) the ovary stage 3. Two females showed egg debris (one female grade 1, one female grade 2) and granulomatous inflammation (both grade 1).

Four male fish (4/41) were diagnosed with the testis stage 1, thirty-four male fish (34/41) were diagnosed with the testis stage 2, and three males (3/41) with the stage 3. One male fish (1/41) showed cystic degeneration in the liver (grade 2).

2.19 µg/ Tamoxifen-Citrate/L (mean measured concentration) – group 3

Five female fish (5/8) had the ovary stage 2, two females (2/8) the ovary stage 3, and one female (1/8) the ovary stage 4. One female fish showed increased oocyte atresia (grade 2), egg debris (grade 2), and a granulomatous inflammation (grade 1).

Fourty-seven male fish (47/70) were diagnosed with the testis stage 2 and twenty three males (23/70) with the testis stage 3. Two male fish showed increased testicular degeneration (one fish grade 1, one fish grade 2). Sixteen (16/70) male fish showed cystic degeneration in the liver (five with grade 1, five with grade 2, five with grade 3, one with grade 4).

6.38 µg/ Tamoxifen-Citrate/L (mean measured concentration) – group 4

There was one female fish (1/1) with the ovary stage 3, showing increased oocyte atresia (grade 2), egg debris (grade 2), and granulomatous inflammation (grade 1). One male fish (1/19) was diagnosed with the testis stage 1, ten male fish (10/19) with the testis stage 2 and eight male fish (8/19) with the stage 3. Two male fish showed increased testicular degeneration (both grade 1). Eleven male fish (11/19) were diagnosed with cystic degeneration in the liver (four with grade 2, six with grade 3, one with grade 4).

3.2.1.8 Summary/Conclusion

All results of the Study 5 (phase II) are summarized in Table 72.

Table 72: Study 5, ZEOGRT with Tamoxifen-Citrate: Summary of NOEC / LOEC determination during the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Tamoxifen-Citrate [µg/L]	NOEC / LOEC Mean measured concentration Tamoxifen-Citrate [µg/L]
F0-Reproduction	Fecundity (Egg number per day and female))[a]	≥26.3 / >26.3	≥22.5 / >22.5
	Fertilization rate	≥26.3 / >26.3	≥22.5 / >22.5
F0-Growth	Wet weight males	≥26.3 / >26.3	≥22.5 / >22.5
	Wet weight females	≥26.3 / >26.3	≥22.5 / >22.5
	Total length males	≥26.3 / >26.3	≥22.5 / >22.5
	Total length females)[b]	**8.3 / 26.3**	**6.38 / 22.5**
F0-Sex ratio	Sex ratio (% males)	Correct setting was confirmed	
	Sex ratio (% females)		
F0-Histopathology	Maturity stage ovary	≥26.3 / >26.3	≥22.5 / >22.5
	Maturity stage testis	≥26.3 / >26.3	≥22.5 / >22.5
	Histopathology (females)	No treatment-related differences in the occurrence of the investigated lesions.	
	Histopathology (males)	**Dose dependent increase of hypereosinophilic interstitial cells in the testis of the parental (F0) generation.**	
F0-Biomarker	Vitellogenin males	≥26.3 / >26.3	≥22.5 / >22.5
	Vitellogenin females)[c]	**0.29 / 0.92**	**0.220 / 0.692**
F1-ELS	**Hatching success)[d]**	**8.3 / 26.3**	**6.38 / 22.5**
	Survival day 21)[e]	**2.92 / 8.3**	**2.19 / 6.38**
	Survival day 35)[e]	**2.92 / 8.3**	**2.19 / 6.38**
	Total length day 35	≥8.3 / >8.3	≥6.38 / >6.38
F1-Juveniles	Survival day 35 – day 63	≥8.3 / >8.3	≥6.38 / >6.38
	Total length day 63	≥8.3 / >8.3	≥6.38 / >6.38
F1-Reproduction	**Time to first spawning)[f]**	**0.29 / 0.92**	**0.220 / 0.692**
	Fecundity (Egg number per day and female))[g]	**2.92 / 8.3**	**2.19 / 6.38**

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Tamoxifen-Citrate [µg/L]	NOEC / LOEC Mean measured concentration Tamoxifen-Citrate [µg/L]
	Fertilization rate)[g]	**2.92 / 8.3**	**2.19 / 6.38**
F1-Termination *	Survival (test end)	≥8.3 / >8.3	≥6.38 / >6.38
	Total length males)[h]	**<0.29 / ≤0.29**	**<0.220 / ≤0.220**
	Total length females	≥8.3 / >8.3	≥6.38 / >6.38
	Wet weight males)[h]	**<0.29 / ≤0.29**	**<0.220 / ≤0.220**
	Wet weight females	≥8.3 / >8.3	≥6.38 / >6.38
	Sex ratio (% males))[i]	**2.92 / 8.3**	**2.19 / 6.38**
	Sex ratio (% females)	≥8.3 / >8.3	≥6.38 / >6.38
F1-Biomarker *	Vitellogenin males	≥8.3 / >8.3	≥6.38 / >6.38
	Vitellogenin females	≥8.3 / >8.3	≥6.38 / >6.38
F1-Histopathology *	Maturity stage ovary	≥8.3 / >8.3	≥6.38 / >6.38
	Maturity stage testis	≥8.3 / >8.3	≥6.38 / >6.38
	Histopathology (females)	No treatment-related differences in the occurrence of the investigated lesions.	
	Histopathology (males)	A dose dependent cystic degeneration within the liver was seen.	
F2-Embryo *	Hatch day 4	≥8.3 / >8.3	≥6.38 / >6.38

)[a]: Significantly reduced compared to control. only at nominal 0.29 µg/L; Dunnett`s Multiple t-test, p<0.05, one-sided smaller.

)[b]: Significantly increased compared to control. at nominal 26.3 µg/L; Williams Multiple t-test, p<0.05, two-sided.

)[c]: Significantly reduced compared to control; Step-down Jonckheere-Terpstra Test; p<0.05; one-sided smaller.

)[d]: Significantly higher mortality compared to ctr.; Chi^2 2x2 Table Test with Bonferroni Correction; p<0.05; one-sided greater.

)[e]: Significantly higher mortality compared to ctr.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[f]: First spawning was delayed compared to ctr.; Williams Multiple t-test; p<0.05; one-sided greater.

)[g]: Reduced compared to ctr.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[h]: Significantly reduced at all treatments compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[i]: Significantly increased compared to control.; Step-down Jonckheere-Terpstra test; p<0.05; two-sided.

*: No fish larvae survived at nominal 26.3 µg test item/L, all larvae were dead before day 35 pf. Furthermore, only one single female fish present at nominal 8.30 µg Tamoxifen-Citrate/L.

3.2.2 Phase II, Study 6: ZEOGRT with Prochloraz

3.2.2.1 Test conditions

The mean water temperatures per replicate in controls and treatments were calculated to be between 25.9 and 26.2 °C and thus in line with the defined acceptance criteria. The single measurements in all test vessels were between 24.7 and 27.2°C and thus in line with the defined acceptance criteria of 26 °C ± 2 °C.
The mean oxygen concentrations per replicate throughout the test period were determined to be between 88 and 96 %, based on single measurement between 72 and 104 %. The mean pH levels on replicate basis were between 7.70 and 7.85 in controls and treatments. The single pH measurements were between 7.50 and 8.13. There was no impact of the substance application on the pH level.

Summing up, stable conditions of exposure could be confirmed.

All mean values and standard deviations are summarized in Table 73 to Table 75. For more details, please refer to the appendix report.

Table 73: Study 6, ZEOGRT with Prochloraz: Water temperature [°C]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	26.1	0.4	24.8	26.9
	B	26.1	0.4	24.8	27.1
	C	26.2	0.4	24.9	26.9
	D	26.1	0.4	24.9	27.0
3.2	**A**	26.1	0.4	24.8	27.0
	B	26.1	0.4	24.9	27.0
	C	26.1	0.4	24.8	27.2
	D	26.1	0.5	24.8	27.2
10	**A**	26.0	0.4	25.0	26.9
	B	25.9	0.4	24.8	26.8
	C	26.0	0.4	24.7	27.0
	D	25.9	0.4	24.8	27.0
32	**A**	25.9	0.4	24.9	26.8
	B	26.0	0.4	24.9	26.9
	C	26.1	0.4	25.0	26.9
	D	26.0	0.4	24.8	26.8
100	**A**	26.0	0.4	24.8	26.8
	B	26.0	0.4	25.1	26.9

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
	C	26.0	0.4	24.8	26.8
	D	26.0	0.4	25.0	27.0
320	**A**	26.0	0.3	25.2	27.0
	B	25.9	0.4	25.0	26.8
	C	26.0	0.4	25.2	26.8
	D	25.9	0.4	24.9	26.8

SD = Standard deviation

Table 74: Study 6, ZEOGRT with Prochloraz: Oxygen concentration [%]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	96	5	86	102
	B	91	6	78	99
	C	94	6	83	103
	D	90	5	76	99
3.2	**A**	94	8	77	104
	B	90	5	80	99
	C	93	7	79	104
	D	89	5	79	98
10	**A**	92	7	75	99
	B	90	6	80	99
	C	91	7	75	98
	D	89	6	77	98
32	**A**	92	7	75	100
	B	90	7	75	99
	C	91	7	76	99
	D	88	7	75	98

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	90	9	76	98
	B	88	8	72	99
	C	91	8	75	101
	D	89	6	79	99
320	**A**	94	7	78	101
	B	92	6	79	100
	C	94	7	79	102
	D	91	6	80	100

SD = Standard deviation

Table 75: Study 6, ZEOGRT with Prochloraz: pH-values

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.83	0.08	7.70	7.96
	B	7.75	0.14	7.50	8.09
	C	7.81	0.10	7.68	7.96
	D	7.73	0.11	7.58	7.98
3.2	**A**	7.84	0.09	7.71	8.01
	B	7.77	0.13	7.59	8.13
	C	7.81	0.10	7.66	7.97
	D	7.73	0.12	7.56	8.00
10	**A**	7.85	0.08	7.68	8.00
	B	7.76	0.13	7.61	8.11
	C	7.81	0.08	7.69	7.95
	D	7.72	0.12	7.53	7.99
32	**A**	7.82	0.09	7.69	7.99
	B	7.74	0.13	7.53	8.08
	C	7.80	0.08	7.68	7.92
	D	7.71	0.12	7.51	7.97

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
100	**A**	7.82	0.09	7.68	7.97
	B	7.73	0.13	7.55	8.06
	C	7.79	0.08	7.67	7.93
	D	7.70	0.12	7.55	7.96
320	**A**	7.82	0.09	7.66	7.97
	B	7.74	0.12	7.57	8.04
	C	7.80	0.08	7.70	7.93
	D	7.70	0.12	7.56	7.95

SD = Standard deviation

3.2.2.2 Chemical analysis

The concentrations of Prochloraz were measured in regular intervals. The mean measured concentrations were calculated to be 2.86, 9.48, 28.0, 85.0, and 277 µg Prochloraz/L. These corresponded to 89.4, 94.8, 87.5, 85 and 86.6 % of the nominal concentrations, respectively.

3.2.2.3 Biological results, parental generation (F0)

The parental group was initiated with spawning groups of approx. 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and for each treatment concentration. Notably, 13 fishes of F0 died during the study. One female fish died during recording reproduction and was considered for calculation of mean egg number per day and female (i.e. one female in replicate B at 28.0 µg Prochloraz/L (mean measured) at test day 19). Since three fish died in control replicate A, one fish in control replicate B and one fish in control replicate C, the acceptance criterion of ≥90 % survival rate of adult fish in the controls was not met, as 5 out 40 fish in that replicate died (see Table 76 for more details).

Reproduction was recorded in terms of egg numbers and fertilization rates in the period from test day 1 to test day 20. For the controls, a mean of 47 eggs per female and day was calculated. The mean egg numbers per female and day in treatments were determined to be 42, 40, 37, 28 and 31 at 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean egg numbers and standard deviation are shown in Table 77. The mean fertilization rate for the controls was calculated to be 96.9 %. For the exposed groups, the fertilization rates were determined to be between 96.3 % (at 28.0 µg Prochloraz/L (mean measured)) and 91.6 % (at 277 µg Prochloraz/L (mean measured)). The statistical evaluation revealed a statistically significant reduction of eggs per day and female compared to control in treatments with 85.0 and 277 µg Prochloraz/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller). In terms of fertility, a significant reduction compared to control was determined in the highest treatment level of 277 µg Prochloraz/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller). However, since fertility was 91.6 %, and thus higher than the defined validity criterion of 90 % for the controls, this effect seems to be of minor biological relevance.

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads and verified by histopathological analysis. The inspection revealed

that the groups were set correctly, i.e. approx. 5 males and 5 females were present (see Table 76 for details). Only three fish, formerly judged to be male, were corrected to female (i.e. fish no. 9 in control replicate C, fish no. 10 in replicate B at 2.86 µg Prochloraz/L (mean measured), fish no. 6 in replicate C at 85.0 µg Prochloraz/L (mean measured)).

The mean fish total lengths of males were determined to be 4.7 cm in the controls and 4.7, 4.7, 4.8, 4.8 and 4.8 cm in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean fish total lengths of females were determined to be 4.9 cm in controls and 4.9, 5.0, 5.1, 5.0 and 5.2 cm in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean wet weights of males were determined to be 0.813 g in controls and 0.847, 0.821, 0.887, 0.869 and 0.938 g in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean wet weights in females were determined to be 1.078 g in controls and 1.050, 1.106, 1.152, 1.186 and 1.427 g in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. Statistical evaluation revealed that an induced growth (individual total length and wet weight) compared to control was determined in the highest treatment level of 277 µg Prochloraz/L (mean measured) for both, male and female (Williams Multiple t-test; $p<0.05$; two-sided).

All mean values and standard deviation of all biological parameters are summarized in Table 77.

For more details, please refer to the appendix report.

Table 76: Study 6, ZEOGRT with Prochloraz: Overview fish group setting (verified by histopathological analysis) and mortality, F0 generation

	Nominal concentration Prochloraz [µg/L]																							
	Control				3.2				10				32				100				320			
	Mean measured concentration Prochloraz [µg/L]																							
	Control				2.86				9.48				28.0				85.0				277			
Replicate	A	B	C *	D	A	B *	C	D	A	B	C *	D	A	B	C	D	A	B	C	D	A	B	C	D
Male (m) [n]	3	4	4	5	5	4	5	5	5	5	5	5	5	5	4	5	5	5	4	5	4	5	5	5
Female (f) [n]	4	5	5	5	4	6	5	5	5	4	5	5	4	4	4	5	5	5	5	5	5	5	5	5
Mortality (no. and sex) [n]	1 m 2 ?	1 m	1	-	1	-	-	-	-	1	-	-	1	1 f	2	-	-	-	1 m	-	1 m	-	-	-

Remark:

*Three fish, formerly judged by macroscopic inspection to be male, were corrected to female (i.e. fish no. 9 in control replicate C, fish no. 10 in replicate B at 2.86 µg Prochloraz/L (mean measured), fish no. 6 in replicate C at 85.0 µg Prochloraz/L (mean measured)).

One female fish died during recording reproduction and was considered for calculation of mean egg number per day and female (i.e. one female in replicate B at 28.0 µg Prochloraz/L (mean measured) at test day 19).

One male fish died in control. replicate A at test day 13; one male fish died in control. Replicate B at test day 13; one male fish died in replicate C at 85.0 µg Prochloraz/L (mean measured) at test day 12; one male fish died in replicate A at 277 µg Prochloraz/L (mean measured) at test day 5.

The numbers for dead fish were calculated based on the recorded findings of remaining fish.

Table 77: Study 6, ZEOGRT with Prochloraz: Biological results for parental generation (F0)

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	2.86	9.48	28.0	85.0	277
Mean egg number per day and female [n]	47	42	40	37	28)[a]	31)[a]
SD	13	17	22	2	9	6
Mean fertilization rate [%]	96.9	95.7	95.2	96.3	95.2	91.6)[b]
SD	0.8	1.7	1.0	0.4	1.6	2.9
Total length, males [cm]	4.7	4.7	4.7	4.8	4.8	4.8)[c]
SD	0.1	0.0	0.1	0.0	0.1	0.1
Total length, females [cm]	4.9	4.9	5.0	5.1	5.0	5.2)[c]
SD	0.1	0.1	0.1	0.1	0.1	0.1

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	**2.86**	**9.48**	**28.0**	**85.0**	**277**
Wet weight, males [g]	**0.813**	**0.847**	**0.821**	**0.887**	**0.869**	**0.938)[c]**
SD	0.020	0.012	0.060	0.036	0.069	0.108
Wet weight, females [g]	**1.078**	**1.050**	**1.106**	**1.152**	**1.186**	**1.427)[c]**
SD	0.138	0.050	0.060	0.057	0.097	0.151
Mean sex ratio, [% males]	**45**	**49**	**51**	**53**	**49**	**49**
SD	3	6	3	3	3	3
Mean sex ratio, [% females]	**55**	**51**	**49**	**47**	**51**	**51**
SD	3	6	3	3	3	3

SD = Standard Deviation

)[a]: Significantly reduced egg no. per day and female compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[b]: Significantly reduced fertility compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[c]: Significantly induced growth parameter compared to control.; Williams Multiple t-test; p<0.05; two-sided.

3.2.2.4 Biological results, first filial generation (F1)

The filial 1 (F1) generation was initiated by keeping fertilized eggs from the parental group and placing them in separate brood chambers placed in each test vessel.

Hatch was sufficient to obtain enough larvae to proceed and was observed to be >80 % in all test vessels (related to the number of eggs introduced). The mean hatching rate was 100 % in the controls and 99.3, 97.9, 99.3, 95.1 and 81.9 % in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. A significantly lower number of hatched eggs compared to control was found at 85.0 and 277 µg Prochloraz/L (mean measured; Step-down Cochrane-Armitage Test; $p<0.05$; one-sided greater).

The number of surviving fish larvae were determined on day 21 and 35 pf. For this, all remaining larvae were catched and photographed. The photos from day 35 pf were also evaluated for total fish length. After each evaluation the fish were carefully re-introduced to the respective test vessel. The post-hatch survival rate in controls was calculated to be 87.5 % at day 21 and 35 pf, respectively. The acceptance criterion was thus fulfilled. In the exposed groups, the mean post-hatch survival rates were determined to be 81.8, 90.1, 79.7, 49.2 and 14.3 % at day 21 pf in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. At day 35 pf the mean post-hatch survival rates were determined to be 79.8, 87.9, 79.0, 49.2 and 14.3 % in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. Statistical analyses revealed a significant reduction of larval survival compared to controls at 85.0 and 277 µg Prochloraz/L (mean measured) for either day 21 pf and day 35 pf (Step-down Rao-Scott-Cochran-Armitage Test Procedure; $p<0.05$; one-sided greater).

On day 35 pf, the total length of all remaining fish larvae was measured computer aided. The mean total length was 1.5 cm in the controls and all treatment levels but the highest one, i.e. at 277 µg Prochloraz/L (mean measured) the mean total length at day 35 pf was determined to be 1.6 cm. The statistical evaluation revealed no significant effect compared to control (Williams Multiple t-test; $\alpha=0.05$; one-sided smaller).

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish. On day 65 pf, the fish groups were again photographed, revealing a surviving rate of 100 % across all treatment levels. The mean total length at day 65 pf was measured to be 3.8 cm in the controls and 3.8, 3.7, 3.8, 3.7 and 3.8 cm in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. There was no significant effect compared to control in terms of total length at day 65 pf (Dunnett`s Multiple t-test; $\alpha=0.05$; two-sided).

At day 63 pf spawning trays were introduced to record first spawning events. Since there were no female but only male fish present at the highest treatment level of 277 µg Prochloraz/L (mean measured) no egg production was possible at all. In the controls, the time to first spawn was in the range of day 65 to day 79 pf , while spawning onset was found to be in the range from day 67 to day 74, from day 65 to day 75, from day 65 to day 75 and from day 69 to day 77 pf in treatments with 2.86, 9.48, 28.0 and 85.0 µg Prochloraz/L (mean measured), respectively. Statistical analysis did not reveal a significant effect compared to control on first spawning (Dunnett`s Multiple t-test; $\alpha=0.05$; one-sided greater).

From day 98 pf onwards, reproduction was recorded in terms of egg numbers and fertilization rates. For the controls, a mean of 33 eggs per female and day was calculated. The mean egg numbers per female and day in the exposed groups were determined to be 30, 28, 29 and 28 in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean fertilization rate was calculated to be 88.7 % in the controls and 91.9, 91.7, 91.5 and 92.0 % in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured),

respectively. Neither for fecundity nor for fertility a significant effect compared to control was found (fecundity and fertility: Dunnett`s Multiple t-test; α=0.05; one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The mean sex ratio in the controls was determined to be 30.1 % males and 68.6 % females and 1.3 % unidentified sex (no gonads), and thus in line with the validity criterion of 30 to 70 % males or females.

In the exposed groups mean sex ratios of 33.8 %, 50.4 %, 53.0 %, 64.3 % and 100 %males were determined in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively.

Mean sex ratios of 65.0 %, 49.6 %, 47.0 %, 35.7 % and 0 % females were determined in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively.

Statistical evaluation revealed a significant test item induced shift in sex ratio towards an increased number of males at all treatment levels but the lowest one, respective fewer females at all treatment levels (Williams Multiple t-test; $p<0.05$; two-sided).

The mean fish total lengths in males were determined to be 4.6 cm in the controls and 4.6, 4.6, 4.8, 4.6 and 4.7 cm in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. No significant effect compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean fish total lengths in females were determined to be 4.6 cm in the controls and 4.7, 4.6, 4.9 and 4.6 in treatments with 2.86, 9.48, 28.0 and 85.0 µg Prochloraz/L (mean measured), respectively. Length was found to be significantly induced in treatment with 28.0 µg Prochloraz/L (mean measured), only (Dunnett`s Multiple t-test; $p<0.05$; two-sided).

The mean fish wet weights of males were determined to be 0.796 in the controls and 0.828, 0.774, 0.879, 0.823 and 0.875 g in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. No test item induced effect on male weight compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean wet weights in females were determined to be 0.922 g for the controls and 0.994, 0.935, 1.131 and 0.946 g in treatments with 2.86, 9.48, 28.0 and 85.0 µg Prochloraz/L (mean measured), respectively. Female weight was found to be significantly induced in treatment with 28.0 µg Prochloraz/L (mean measured), only (Dunnett`s Multiple t-test; $p<0.05$; two-sided).

All results, i.e. mean values and standard deviations, are shown in Table 78. For more details, please refer to the appendix report.

Table 78: Study 6, ZEOGRT with Prochloraz: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	2.86	9.48	28.0	85.0	277*
Mean hatching rate [%]	**100**	**99.3**	**97.9**	**99.3**	**95.1)[a]**	**81.9)[a]**
SD	0	1.4	4.2	1.4	6.6	9.2
Mean post-hatch survival at day 21 pf [%]	**87.5**	**81.8**	**90.1**	**79.7**	**49.2)[b]**	**14.3)[b]**
SD	8.0	12.3	4.8	12.1	16.1	13.1
Mean post-hatch survival at day 35 pf [%]	**87.5**	**79.8**	**87.9**	**79.0**	**49.2)[b]**	**14.3)[b]**
SD	8.0	13.2	4.4	13.0	16.1	13.1
Mean total length, day 35 pf (all fish pre-reduction) [cm]	**1.5**	**1.5**	**1.5**	**1.5**	**1.5**	**1.6**
SD	0.1	0.1	0.0	0.1	0.1	0.1
Mean survival day 65 pf (compared to no. post-reduction at day 35 pf) [%]	**100**	**100**	**100**	**100**	**100**	**100**
SD	0	0	0	0	0	0
Mean total length, day 65 pf [cm]	**3.8**	**3.8**	**3.7**	**3.8**	**3.7**	**3.8**
SD	0.1	0.1	0.2	0.1	0.1	0.4
Time to first spawning [day pf]	**A** 79	67	74	72	69	-
	B 72	73	73	65	75	-
	C 65	74	75	71	71	-
	D 65	73	65	75	77	-
Mean egg number per day and female [n]	**33**	**30**	**28**	**29**	**28**	**-**
SD	11	8	15	16	9	-
Mean fertilization rate [%]	**88.7**	**91.9**	**91.7**	**91.5**	**92.0**	**-**
SD	6.9	1.8	2.4	3.0	2.5	-
Mean survival, adult stage (day 65 pf	**92.5**	**96.3**	**92.5**	**85.0**	**100**	**41.7**

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	**2.86**	**9.48**	**28.0**	**85.0**	**277***
until testend) [%]						
SD	6.5	4.8	2.9	7.1	0	50.0
Mean total length, males, adult stage [cm]	**4.6**	**4.6**	**4.6**	**4.8**	**4.6**	**4.7 ****
	0.2	0.1	0.2	0.1	0.2	0.0
Mean total length, females, adult stage [cm]	**4.6**	**4.7**	**4.6**	**4.9)[c]**	**4.6**	**-**
SD	0.2	0.2	0.1	0.1	0.2	
Mean wet weight, males, adult stage [g]	**0.796**	**0.828**	**0.774**	**0.879**	**0.823**	**0.875****
SD	0.104	0.066	0.127	0.048	0.091	0.010
Mean wet weight, females, adult stage [g]	**0.922**	**0.994**	**0.935**	**1.131)[c]**	**0.946**	**-**
SD	0.084	0.137	0.060	0.096	0.125	-
Mean sex ratio, [% males]	**30.1**	**33.8**	**50.4)[d]**	**53.0)[d]**	**64.3)[d]**	**100**)[d]**
SD	12.1	10.0	17.6	12.8	7.8	0.0
Mean sex ratio, [% females]	**68.6**	**65.0)[d]**	**49.6)[d]**	**47.0)[d]**	**35.7)[d]**	**0)[d]**
SD	13	10	18	13	8	0
Unidentified (no gonads) [%]	**1.3**	**1.3**	**0**	**0**	**0**	**0**
SD	2.6	2.5	0	0	0	0

SD = Standard deviation

*: Only male fish present at 277 µg Prochloraz/L (mean measured) in F1 generation.

**: Remark: mean derives from two replicates only as no surviving fish in replicate A and C.

)[a]: Decrease compared to control.; Step-down Cochrane-Armitage Test; p<0.05; one-sided greater.

)[b]: Decrease compared to control.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[c]: Significant increase compared to control.; Dunnett`s Multiple t-test; p<0.05; two-sided.

)[d]: Significant increase (males), respective decrease (females) compared to control.; Williams Multiple t-test; p<0.05; two-sided.

3.2.2.5 Biological results, second filial generation (F2)

The mean hatching success in the controls was 100 % and 97.5, 100, 93.8 and 96.3 % in treatments 2.86, 9.48, 28.0 and 85.0 µg Prochloraz/L (mean measured), respectively.

For more details, please refer to the appendix report.

Statistical evaluation revealed that compared to control the test item Prochloraz had no influence on hatching success (Chi^2 2x2 Table Test with Bonferroni Correction; one-sided greater α=0.05; one-sided greater).

Table 79: Study 6, ZEOGRT with Prochloraz: Biological results for F2 generation

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	**2.86**	**9.48**	**28.0**	**85.0**	**277 ***
Mean hatching rate [%]	**100**	**97.5**	**100**	**93.8**	**96.3**	**-**
SD	0	2.9	0	6.3	7.5	-

SD = Standard deviation

*: Only male fish were present at 277 µg test Prochloraz/L (mean measured), thus no reproduction in F1 was possible.

3.2.2.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 80 (F0 generation) and Table 81 (F1 generation). All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin values for males were determined to be 6.33E+03 ng VTG/mL in controls and 3.54E+03, 7.96E+03, 1.55E+03, 6.44E+03 and 1.00E+04 ng VTG/mL in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean VTG values for females were determined to be 3.40E+07 ng VTG/mL in the controls and 3.56E+07, 3.19E+07, 2.00E+07, 1.05E+07 and 1.44E+06 ng VTG/mL in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively.

Statistical analyses revealed no significant difference between controls and treatments for male fish (Multiple Sequentially-rejective U-test after Bonferroni-Holm; α=0.05; one-sided greater), while female fish displayed a significant reduced vitellogenin value compared to control at 85.0 and 277 µg Prochloraz/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller).

Table 80: Study 6, ZEOGRT with Prochloraz: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Mean measured concentration Prochloraz [µg/L]					
	Control	2.86	9.48	28.0	85.0	277
Mean VTG concentration, males [ng VTG/mL]	**6.33E+03**	**3.54E+03**	**7.96E+03**	**1.55E+03**	**6.44E+03**	**1.00E+04**
SD	7.24E+03	1.40E+03	5.86E+03	2.93E+02	5.18E+03	1.18E+04
Mean VTG concentration, females [ng VTG/mL]	**3.40E+07**	**3.56E+07**	**3.19E+07**	**2.00E+07**	**1.05E+07)[a]**	**1.44E+06)[a]**
SD	1.84E+07	1.79E+07	1.03E+07	6.38E+06	1.04E+07	6.94E+05

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

)[a]: Significantly reduced compared to control.; Williams Multiple t-test; $p<0.05$; one-sided.

In first filial generation (F1) the mean vitellogenin (VTG) values for males were determined to be 5.45E+04 ng VTG/mL in controls and 7.01E+04, 8.94E+03, 6.52E+03, 4.24E+03 and 4.08E+03 ng VTG/mL in treatments with 2.86, 9.48, 28.0, 85.0 and 277 µg Prochloraz/L (mean measured), respectively. The mean VTG values for females were determined to be 2.82+07 ng VTG/mL in controls and 2.52E+07, 3.13E+07, 2.16E+07 and 6.42E+06 ng VTG/mL in treatments with 2.86, 9.48, 28.0 and 85.0 µg Prochloraz/L (mean measured), respectively.

For male fish, no statistically significant effect on vitellogenin values was found compared to control (Multiple Sequentially-rejective Median test; α=0.05; one-sided greate). The statistical evaluation of the female vitellogenin values revealed a reduction compared to control at 85.0 µg Prochloraz/L (mean measured) (please remark: no females present at 277 µg Prochloraz/L; Williams Multiple Sequential t-test; p<0.05; one-sided smaller).

Table 81: Study 6, ZEOGRT with Prochloraz: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng VTG/mL]

Parameter	Nominal concentration Prochloraz [µg/L]					
	Control	2.86	9.48	28.0	85.0	277*
Mean VTG concentration, males [ng VTG/mL]	**5.45E+04**	**7.01E+04**	**8.94E+03**	**6.52E+03**	**4.24E+03**	**4.08E+03**
SD	7.18E+04	8.11E+04	7.81E+03	6.11E+03	8.95E+02	4.96E+02
Mean VTG concentration, females [ng VTG/mL]	**2.82E+07**	**2.52E+07**	**3.13E+07**	**2.16E+07**	**6.42E+06)[a]**	**-**
SD	2.75E+07	1.22E+07	1.38E+07	5.81E+06	6.28E+06	-

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

*: Only male fish present at 277 µg Prochloraz/L (mean measured).

)[a]: Significantly reduced compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

3.2.2.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in chapter 2.1.12.2

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In chapters 3.2.2.7.1 and 3.2.2.7.2, the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 82 and the results for first filial generation are shown in Table 83.

Neither parental generation (F0) nor first filial generation (F1) showed any treatment-related statistically significant differences compared to control in male or female gonad staging. Furthermore, an increased atresia, egg debris and granulomatous inflammation in the ovaries was observed in female fish of the parental generation especially in treatments with 100 and 320 µg Prochloraz/L. In addition, in the first filial generation F1, the number of female fish was decreased in treatment with 100 µg Prochloraz/L. In treatment with 320 µg Prochloraz/L no females were present at all.

Table 82: Study 6, ZEOGRT with Prochloraz: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Prochloraz [µg/L]					
		Control	**2.86**	**9.48**	**28.0**	**85.0***	**277**
Maturation stages, male [1)]		Stage 0 (0/16) Stage 1 (0/16) Stage 2 (16/16) Stage 3 (0/16) Stage 4 (0/16)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (17/19) Stage 3 (2/19) Stage 4 (0/19)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (15/20) Stage 3 (5/20) Stage 4 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (17/19) Stage 3 (2/19) Stage 4 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (12/19) Stage 3 (7/19) Stage 4 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (13/19) Stage 3 (6/19) Stage 4 (0/19)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**2**
	B	**2**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**3**
	D	**2**	**2**	**2**	**2**	**3**	**2**
Maturation stages, female [2)]		Stage 0 (0/19) Stage 1 (1/19) Stage 2 (9/19) Stage 3 (9/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (10/20) Stage 3 (10/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (7/19) Stage 3 (12/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/17) Stage 1 (0/17) Stage 2 (4/17) Stage 3 (12/17) Stage 4 (1/17) Stage 5 (0/17)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (6/19) Stage 3 (13/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (7/20) Stage 3 (13/20) Stage 4 (0/20) Stage 5 (0/20)
Maturation stage, female, median value/ replicate	A	**3**	**2**	**3**	**3**	**3**	**2**
	B	**2**	**3**	**3**	**3**	**3**	**3**
	C	**2**	**3**	**2**	**3**	**3**	**3**
	D	**3**	**2**	**3**	**3**	**3**	**3**

[1)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

[2)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

*: One female fish (1/20) showed lymphoma in various tissues, which affected the ovary stage, this female fish was thus excluded from the analysis of the ovary stage.

Table 83: Study 6, ZEOGRT with Prochloraz: Median maturation stages for F1 generation

Parameter		Nominal concentration Prochloraz [µg/L]					
		Control	**2.86**	**9.48**	**28.0**	**85.0**	**277***
Maturation stages, male [1)]		Stage 0 (0/22) Stage 1 (4/22) Stage 2 (18/22) Stage 3 (0/22) Stage 4 (0/22)	Stage 0 (0/26) Stage 1 (2/26) Stage 2 (24/26) Stage 3 (0/26) Stage 4 (0/26)	Stage 0 (0/37) Stage 1 (4/37) Stage 2 (29/37) Stage 3 (4/37) Stage 4 (0/37)	Stage 0 (0/36) Stage 1 (1/36) Stage 2 (33/36) Stage 3 (2/36) Stage 4 (0/36)	Stage 0 (1/42) Stage 1 (1/42) Stage 2 (29/42) Stage 3 (11/42) Stage 4 (0/42)	Stage 0 (0/13) Stage 1 (0/13) Stage 2 (11/13) Stage 3 (2/13) Stage 4 (0/13)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**2**	**2**	**-**
	B	**2**	**2**	**2**	**2**	**2**	**2**
	C	**2**	**2**	**2**	**2**	**2**	**-**
	D	**2**	**2**	**2**	**2**	**2**	**2**
Maturation stages, female [2)]		Stage 0 (4/51) Stage 1 (2/51) Stage 2 (25/51) Stage 3 (20/51) Stage 4 (0/51) Stage 5 (0/51)	Stage 0 (1/50) Stage 1 (3/50) Stage 2 (23/50) Stage 3 (22/50) Stage 4 (1/50) Stage 5 (0/50)	Stage 0 (2/37) Stage 1 (3/37) Stage 2 (19/37) Stage 3 (13/37) Stage 4 (0/37) Stage 5 (0/37)	Stage 0 (0/32) Stage 1 (0/32) Stage 2 (17/32) Stage 3 (15/32) Stage 4 (0/32) Stage 5 (0/32)	Stage 0 (1/22) Stage 1 (0/22) Stage 2 (15/22) Stage 3 (6/22) Stage 4 (0/22) Stage 5 (0/22)	-
Maturation stage, female, median value/ replicate	A	**3**	**2**	**2**	**3**	**2**	**-**
	B	**2**	**2**	**2**	**2**	**3**	**-**
	C	**2**	**3**	**2**	**2**	**2**	**-**
	D	**3**	**2**	**2**	**2**	**2**	**-**

[1)] Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

[2)] Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

*: Only male fish present at 277 µg Prochloraz/L (mean measured).

3.2.2.7.1 Histopathology results, parental generation (F0)

Control – group 0

One female fish (1/19) had the ovary stage 1, nine female fish (9/19) had the ovary stage 2 and nine females (9/19) the ovary stage 3. One female fish showed increased oocyte atresia (grade 2), and two female fish showed egg debris and granulomatous inflammation (all grade 1).

All male fish (16/16) were diagnosed with the testis stage 2.

Additional diagnoses were not observed in male fish of this group.

2.86 µg Prochloraz/L (mean measured) – group 1

Ten female fish (10/20) had the ovary stage 2 and ten females (10/20) the ovary stage 3.

Seventeen male fish (17/19) were diagnosed with the testis stage 2. Two male fish (2/19) had the testis stage 3. One male fish showed increased proportion of spermatogonia (grade 1) and one male fish showed asynchronous germ cell development (grade 1).

9.48 µg Prochloraz/L (mean measured) – group 2

Seven female fish (7/19) had the ovary stage 2 and twelve females (12/19) the ovary stage 3. Two female fish showed increased oocyte atresia (grade 1). Three female fish showed egg debris (one female grade 1, one female grade 2, one female grade 3). Three female fish showed granulomatous inflammation (one female grade 1, one female grade 2, one female grade 3). Increased post-ovulatory follicles were observed in one female fish (grade 3).

Fifteen male fish (15/20) were diagnosed with the testis stage 2 and five (5/20) male fish had the testis stage 3. Four male fish showed interstitial cell hypertrophy/hyperplasia (grade 1).

28.0 µg Prochloraz/L (mean measured) – group 3

Four female fish (4/17) had the ovary stage 2, twelve females (12/17) the ovary stage 3, and one female (1/17) the ovary stage 4. Two females showed increased oocyte atresia (one female grade 1, one female grade 2). Egg debris and granulomatous inflammation were observed in three females (two females grade 1, one female grade 2).

Seventeen male fish (17/19) were diagnosed with the testis stage 2 and two male fish (2/19) were diagnosed with the testis stage 3. Two male fish showed increased testicular degeneration (grade 1) and one fish had asynchronous germ cell development (grade 1).

85.0 µg Prochloraz/L (mean measured) – group 4

Six female fish (6/20) had the ovary stage 2, thirteen (13/20) the ovary stage 3. One female fish (1/20) showed lymphoma in various tissues, which affected the ovary stage and should therefore be excluded from the analysis of the ovary stage. Six female fish showed increased oocyte atresia (three females grade 1, two females grade 2, one female grade 3). Six females showed egg debris (two females grade 1, three females grade 2, one female grade 3), and a granulomatous inflammation (three females grade 1, one female grade 2, two females grade 3).

Twelve male fish (12/19) were diagnosed with the testis stage 2 and seven male fish (7/19) with the stage 3. Two male fish showed increased testicular degeneration (grade 1) and one fish had increased proportion of spermatogonia (grade 1).

277 µg Prochloraz/L (mean measured) – group 5

Seven female fish (7/20) had the ovary stage 2 and thirteen female fish (13/20) had the ovary stage 3. Fifteen female fish showed increased oocyte atresia (ten females grade 1, five females grade 2), ten females had egg debris (five females grade 1, three females grade 2, two females grade 3), and ten females showed a granulomatous inflammation (eight females grade 1, two females grade 2).

Thirteen male fish (13/19) were diagnosed with the testis stage 2 and six males (6/19) with the stage 3. Four male fish showed increased testicular degeneration (grade 1) and one male showed interstitial cell hypertrophy/hyperplasia (grade 1).

3.2.2.7.2 Histopathology results, first filial generation (F1)

In two fish (one fish from group 0, replicate B fish no. 18), one fish from group 1, replicate A fish no. 4) gonads were not detected after processing (including step sections). Therefore, the gonads were interpreted to be degenerated in these animals.

Control – group 0

Four female fish (4/51) had the ovary stage 0, two female fish (2/51) the ovary stage 1, twenty-five females (25/51) the ovary stage 2, and twenty females (20/51) the ovary stage 3. Three female fish showed increased oocyte atresia (two females grade 1, one female grade 2). Seven females had egg debris (six females grade 1, one female grade 2), and six females showed granulomatous inflammation (five females grade 1, one female grade 2).

Four male fish (4/22) were diagnosed with the testis stage 1 and eighteen male fish (18/22) were diagnosed with the testis stage 2.
Additional diagnoses were not observed in male fish of this group

2.86 µg Prochloraz/L (mean measured concentration) – group 1

One female fish (1/50) had the ovary stage 0, three female fish (3/50) had the ovary stage 1, twenty-three female fish (23/50) had the ovary stage 2, twenty-two female fish (22/50) the ovary stage 3, and one female (1/50) the ovary stage 4. Eleven females had increased oocyte atresia (six females grade 1, five females grade 2). Egg debris was detected in seven females (three females grade 1, three females grade 2, one female grade 3), accompanied by granulomatous inflammation (six females grade 1, one female grade 2).

Two male fish (2/26) were diagnosed with the testis stage 1, and twenty-four males (24/26) with the stage 2. Two male fish showed increased testicular degeneration (grade 1).

9.48 µg Prochloraz/L (mean measured) – group 2

Two female fish (2/37) had the ovary stage 0, three female fish (3/37) had the ovary stage 1, nineteen females (19/37) the ovary stage 2 and thirteen females (13/37) the ovary stage 3. One female showed increased oocyte atresia (grade 1), egg debris (grade 2) and granulomatous inflammation (grade 2).

Four male fish (4/37) were diagnosed with the testis stage 1, twenty-nine male fish (29/37) were diagnosed with the testis stage 2, and four males (4/37) with the stage 3. One male fish (1/37) showed testis-ova (grade 1). Seven male fish had increased testicular degeneration (grade 1), and two male fish exhibited increased proportion of spermatogonia (grade 1).

28.0 µg Prochloraz/L (mean measured concentration) – group 3

Seventeen female fish (17/32) had the ovary stage 2, and fifteen females (15/32) the ovary stage 3. Two female fish showed increased oocyte atresia (grade 1).

One male fish (1/36) was diagnosed with the testis stage 1, thirty-three male fish (33/36) with the testis stage 2, and two males (2/36) with the testis stage 3. One male fish showed increased testicular degeneration (grade 1), one male fish had increased proportion of spermatogonia (grade 1), and in one male, there was asynchronous germ cell development (grade 1).

85.0 µg Prochloraz/L (mean measured) – group 4

There was one female fish (1/22) with the ovary stage 0, fifteen females (15/22) with the ovary stage 2, and six females (6/22) with the ovary stage 3. Two females showed increased oocyte atresia, three females showed egg debris, and three females had granulomatous inflammation (all grade 1).

One male fish (1/42) was diagnosed with the testis stage 0, one (1/42) with the testis stage 1, twenty-nine male fish (29/42) with the testis stage 2 and eleven males (11/42) with the stage 3. One male fish showed testis-ova (grade 1), six male fish had increased testicular degeneration (grade 1), and two fish had increased proportion of spermatogonia (grade 1).

277 µg Prochloraz/L (mean measured) – group 5

This group was represented by male fish only, of which twelve (11/13) were diagnosed with the testis stage 2, and three (2/13) with the testis stage 3. Two male fish showed increased testicular degeneration (grade 1).

3.2.2.8 Summary/Conclusion

All results of Study 6 (phase II) are summarized in Table 84.

Table 84: Study 6, ZEOGRT with Prochloraz: Summary of NOEC / LOEC determination during the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
F0-Reproduction	**Fecundity (Egg number per day and female))[a]**	**32 / 100**	**28.0 / 85.0**
	Fertilization rate)[a]	**100 / 320**	**85.0 / 277**
F0-Growth	**Wet weight males)[b]**	**100 / 320**	**85.0 / 277**
	Wet weight females)[b]	**100 / 320**	**85.0 / 277**
	Total length males)[b]	**100 / 320**	**85.0 / 277**
	Total length females)[b]	**100 / 320**	**85.0 / 277**
F0-Sex ratio	Sex ratio (% males)	correct setting was confirmed	
	Sex ratio (% females)		
F0-Histopathology	Maturity stage ovary	≥320 / >320	≥277 / >277
	Maturity stage testis	≥320 / >320	≥277 / >277
	Histopathology (females)		
	Histopathology (males)		
F0-Biomarker	Vitellogenin males	≥320 / >320	≥277 / >277
	Vitellogenin females)[a]	**32 / 100**	**28.0 / 85.0**
F1-ELS	**Hatch, total)[c]**	**32 / 100**	**28.0 / 85.0**
	Survival day 21)[d]	**32 / 100**	**28.0 / 85.0**
	Survival day 35)[d]	**32 / 100**	**28.0 / 85.0**
	Total length day 35	≥320 / >320	≥277 / >277
F1-Juveniles	Survival day 35 – day 63	≥320 / >320	≥277 / >277
	Total length day 63	≥320 / >320	≥277 / >277
F1-Reproduction*	Time to first spawning*	≥100 / >100	≥85.0 / >85.0
	Fecundity (Egg number per day and female)*	≥100 / >100	≥85.0 / >85.0
	Fertilization rate*	≥100 / >100	≥85.0 / >85.0
F1-Termination*	Survival (test end)	≥320 / >320	≥277 / >277
	Total length males	≥320 / >320	≥277 / >277

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
	Total length females*)[e]	≥100 / >100	≥85.0 / >85.0
	Wet weight males	≥320 / >320	≥277 / >277
	Wet weight females*)[e]	≥100 / >100	≥85.0 / >85.0
	Sex ratio (% males))[f]	**3.2 / 10**	**2.86 / 9.48**
	Sex ratio (% females))[f]	**<3.2 / ≤ 3.2**	**<2.86 / ≤ 2.86**
F1-Biomarker*	Vitellogenin males	≥320 / >320	≥277 / >277
	Vitellogenin females*)[a]	**32 / 100**	**28.0 / 85.0**
F1-Histopathology*	Maturity stage ovary*	≥100 / >100	≥85.0 / >85.0
	Maturity stage testis	≥320 / >320	≥277 / >277
F2-Embryo**	Hatch day 4**	≥100 / >100	≥85.0 / >85.0

)[a]: Significantly reduced compared to control.; Williams Multiple t-test; p<0.05; one-sided smaller.

)[b]: Significantly induced growth parameter compared to control.; Williams Multiple t-test; p<0.05; two-sided.

)[c]: Decrease compared to control.; Step-down Cochrane-Armitage Test; p<0.05; one-sided greater.

)[d]: Decrease compared to control.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[e] Significant increase compared to control. at 28.0 µg Prochloraz/L (mean measured), only; Dunnett`s Multiple t-test; p<0.05; two-sided.

)[f]: Significant increase (males), respective decrease (females) compared to control.; Williams Multiple t-test; p<0.05; two-sided.

*: Only male fish present at 277 µg Prochloraz/L (mean measured) in F1 generation.
**Subsequently, no eggs for starting second filial generation could be produced at this treatment level.

3.2.3 Phase II, Study 7: ZEOGRT with Prochloraz

3.2.3.1 Test conditions

The mean water temperatures per replicate in control and treatments were calculated to be between 26.1 and 26.5 °C. The single measurements in all test vessels were between 23.6 and 27.9°C, single values were found to be outside the defined acceptance criteria. Furthermore, temperature was not measured daily, but once a week.
The mean oxygen concentrations per replicate throughout the test period were determined to be between 77 and 92 %. The corresponding single values were between 27 and 106 %, thus were found to be outside the acceptance criteria of >60 %. The mean pH levels on replicate basis were between 7.27 and 7.58 in controls and treatments. The single pH measurements were between 6.34 and 8.10. There was no impact of the substance application on the pH level.

All mean values and standard deviations are summarized in Table 85 to Table 87. For more details, please refer to the appendix report.

Table 85: Study 7, ZEOGRT with Prochloraz: Water temperature [°C]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	26.4	0.5	24.9	27.0
	B	26.4	0.5	24.7	27.0
	C	26.3	0.5	25.2	26.8
	D	26.4	0.6	24.8	26.9
Solvent control	**A**	26.5	0.5	24.8	27.1
	B	26.4	0.4	25.1	27.0
	C	26.3	0.5	24.7	27.0
	D	26.3	0.5	24.9	27.0
3.2	**A**	26.3	0.6	24.2	26.9
	B	26.2	0.7	24.4	27.9
	C	26.2	0.6	24.5	26.9
	D	26.2	0.6	23.9	26.8
10	**A**	26.2	0.5	25.0	26.8
	B	26.1	0.5	24.7	26.7
	C	26.2	0.6	24.9	26.9
	D	26.3	0.5	24.9	26.9
32	**A**	26.3	0.5	24.4	26.8
	B	26.2	0.5	24.5	26.7
	C	26.2	0.5	25.0	26.8

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
	D	26.1	0.6	24.3	26.8
100	A	26.2	0.6	23.6	26.7
	B	26.2	0.6	24.3	26.9
	C	26.2	0.6	24.8	26.9
	D	26.2	0.6	24.6	26.8
320	A	26.3	0.5	25.1	26.9
	B	26.2	0.6	24.8	26.9
	C	26.3	0.5	25.2	26.9
	D	26.3	0.6	24.8	26.9

SD = Standard deviation

Table 86: Study 7, ZEOGRT with Prochloraz: Oxygen concentration [%]

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	A	91	7	73	105
	B	92	6	73	103
	C	91	6	73	102
	D	91	5	73	102
Solvent control	A	83	12	45	106
	B	86	8	64	102
	C	83	10	58	106
	D	83	11	45	103
3.2	A	82	10	54	105
	B	82	8	64	98
	C	81	9	56	94
	D	81	9	58	95
10	A	83	8	65	103
	B	83	9	45	97
	C	81	7	61	93
	D	83	7	60	97
32	A	83	9	60	106

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
	B	82	8	63	97
	C	80	9	49	94
	D	80	7	66	92
100	**A**	83	9	60	105
	B	81	10	54	97
	C	78	10	53	96
	D	77	10	46	91
320	**A**	81	10	48	95
	B	83	9	50	95
	C	82	9	53	96
	D	81	13	27	98

SD = Standard deviation

Table 87: Study 7, ZEOGRT with Prochloraz: pH values

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
Control	**A**	7.49	0.22	6.69	7.92
	B	7.54	0.18	6.86	7.88
	C	7.54	0.17	6.91	7.87
	D	7.58	0.17	7.04	7.89
Solvent control	**A**	7.27	0.29	6.53	8.10
	B	7.30	0.26	6.34	8.06
	C	7.32	0.24	6.47	7.96
	D	7.34	0.22	6.61	7.89
3.2	**A**	7.41	0.21	7.05	8.01
	B	7.41	0.20	7.11	8.02
	C	7.41	0.19	7.11	8.01
	D	7.42	0.20	7.09	8.01
10	**A**	7.44	0.19	7.09	7.94
	B	7.43	0.19	6.99	7.97
	C	7.41	0.16	7.09	7.83

Nominal concentration Prochloraz [µg/L]	Replicate	Mean	SD	Min value	Max value
	D	7.44	0.16	7.11	7.95
32	**A**	7.45	0.19	7.16	7.95
	B	7.43	0.18	7.15	7.95
	C	7.40	0.17	7.07	7.96
	D	7.40	0.17	7.17	7.97
100	**A**	7.43	0.21	7.05	7.98
	B	7.40	0.20	7.06	7.99
	C	7.36	0.20	7.02	7.99
	D	7.35	0.19	7.02	7.99
320	**A**	7.43	0.18	7.00	7.88
	B	7.43	0.19	7.02	7.92
	C	7.44	0.19	7.04	7.98
	D	7.46	0.19	6.98	7.91

SD = Standard deviation

3.2.3.2 Chemical analysis

The preparation approach was suitable to apply the test substance Prochloraz in stable concentrations. Decrease of test substance concentration, e.g. due to degradation or adsorption of the substance could not be observed. Due to the given technical devices, the use of a solvent carrier was necessary. Thus, a solvent control was applied.

The mean measured concentration were calculated to be at 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L. These concentrations correspond to 105, 108, 103, 112 and 103 % of the nominal concentrations. For more details, please refer to the appendix report.

3.2.3.3 Biological results, parental generation (F0)

The parental group was initiated with spawning groups of 5 male and 5 female fish per test vessel, i.e. 4 spawning groups per control and solvent control, and for each treatment concentration. One fish (at 3.36 µg Prochloraz/L (mean measured), replicate D, no. 8) was missing for the histopathological examination and the respective fish was excluded from any further evaluation. In five fish (one fish in control replicate C; one fish at 3.36 µg Prochloraz/L (mean measured) replicate A; one fish at 112 µg Prochloraz/L (mean measured) in replicate C; two fish at 331 µg Prochloraz/L (mean measured), one in replicate B, another one in replicate D) gonads were not detected after processing (including step sections), subsequently those fish were excluded from any further evaluation. Eleven fish died during the course of the study, i.e. five females (one female in control replicate C at test day 21; one female in solvent control D at test day 10; one female at 3.36 µg Prochloraz/L (mean measured) at test day 10; one female at 10.8 µg Prochloraz/L (mean measured) at test day 3; one female at 331 µg Prochloraz/L (mean measured) at test day 39), two males (i.e. one male at 33.0 µg Prochloraz/L (mean measured) at test day 10; one male at 331 µg Prochloraz/L (mean measured) at test day 44), four fish without sex determination (one fish in solvent control replicate D; one fish at 3.36 µg Prochloraz/L (mean measured) replicate C was missed at termination at test day 142; one fish at 10.8 µg Prochloraz/L (mean measured) replicate B; one fish at 112 µg Prochloraz/L (mean measured) replicate A). Female fish that died during reproduction recording (test day 1 to test day 21) were considered for calculation of fecundity. For more details, please refer to Table 88 and Table 89.

Reproduction was recorded in terms of egg numbers and fertilization rates in the period from day 1 pf to day 21 pf. For the solvent controls, a mean of 58 eggs per female and day was calculated. The mean egg numbers per female and day in the treatments were determined to be 47, 58, 51, 55 and 44 at 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean egg numbers and standard deviation are shown in Table 90. The mean fertilization rate for the solvent controls was calculated to be 82.7 %. For the exposed groups, the fertilization rates were determined to be between 80.6 % (at 3.36 µg Prochloraz/L (mean measured)) and 93.7 % (at 112 µg Prochloraz/L (mean measured)). Neither egg numbers per female and day nor fertility rate showed any significant difference compared to solvent control (fecundity: Dunnett`s Multiple t-test; α=0.05; one-sided smaller; fertility: Williams Multiple t-test; α=0.05; one-sided smaller).

After successful initiation of the first filial generation (see below for details), the adult fish groups were sacrificed. The phenotypic sex of each fish was examined by macroscopic inspection of the fish gonads and verified by histopathological analysis. The inspection revealed that the groups were nearly set correctly, i.e. approx. 5 males and females were present.

The mean fish total lengths of males were determined to be 3.9 cm in the solvent controls and 3.9, 3.9, 3.9, 4.0 and 4.0 cm in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean fish total lengths of females were determined to be 4.2 cm in solvent controls and 4.1, 4.1, 4.2, 4.2 and 4.4 cm in treatments 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean wet weights of males were determined to be 0.602 g in solvent controls and 0.574, 0.570, 0.544, 0.605 and 0.654 g in in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean wet weights in females were determined to be 0.840 g in solvent controls and 0.778, 0.798, 0.864 0.851 and 1.100 in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. Statistical evaluation revealed that male total length was not significantly altered by the test item Prochloraz (Dunnett`s Multiple t-test; α=0.05; two-sided). In contrast to that, female total length as well as male and female weight were significantly induced

at the highest treatment level of 331 µg Prochloraz/L (mean measured) compared to solvent control (Williams Multiple t-test, $p < 0.05$, two-sided).

All mean values and standard deviation of all biological parameters are summarized in Table 90.

For more details, please refer to the appendix report.

Table 88: Study 7, ZEOGRT with Prochloraz: Overview fish group setting (verified by histopathological analysis), F0: control, solvent control and 3.2 µg Prochloraz/L (nominal concentration)

	Nominal concentration Prochloraz [µg/L]											
	Control				Solvent control				3.2			
	Mean measured concentration Prochloraz [µg/L]											
	Control				Solvent control				3.36			
Replicate	A	B	C	D	A	B	C	D	A	B	C	D
Male (m) [n]	5	5	4	5	5	4	5	5	4	5	4	5
Female (f) [n]	5	5	4	5	5	5	5	4	5	5	4	4
Unidentified [n]	-	-	1	-	-	-	-	-	1	-	-	-
Mortality (no. and sex) [n]	-	-	1f	-	-	1*	-	1f	-	-	1f 1*	1**

Remark: Three female fish died during recording reproduction and were considered for calculation of mean egg number per day and female (i.e. one female in control replicate C at test day 21, one female in solvent control replicate D at test day 10, one female in replicate C at 3.2 µg Prochloraz/L at test day 10).

*: The sex of the dead fish could not be assessed.

**: Missing fish, not delivered to histopathological examination.

Table 89: Study 7, ZEOGRT with Prochloraz: Overview fish group setting (verified by histopathological analysis), F0: 10, 32, 100 and 320 µg Prochloraz/L (nominal concentration)

	Nominal concentration Prochloraz [µg/L]															
	10				32				100				320			
	Mean measured concentration Prochloraz [µg/L]															
	10.8				33.0				112				331			
Replicate	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Male (m) [n]	5	5	5	5	5	5	5	4	4	5	5	5	3	4	6	6
Female (f) [n]	5	4	4	5	5	5	5	5	5	4	5	5	6	4	4	3
Unidentified [n]	-	-	-	-	-	-	-	-	-	1	-	-	-	1		1
Mortality (no. and sex) [n]	-	1 *	-	-	-	-	-	1 m	1*	-	-	-	1 f	-	-	-

One female fish died during recording reproduction and was considered for calculation of mean egg number per day and female (i.e. one female at test day 3 in replicate C at 10 µg Prochloraz/L). Another female died at test day 39, thus after recording reproduction. *: The sex of the dead/missing fish could not be assessed.

Table 90: Study 7, ZEOGRT with Prochloraz: Biological results for parental generation (F0)

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control	Solvent control	3.36	10.8	33.0	112	331
Mean egg number per day and female [n]	**49**	**58**	**47**	**58**	**51**	**55**	**44**
SD	4	16	13	15	2	14	19
Mean fertilization rate [%]	**83.3**	**82.7**	**80.6**	**86.7**	**93.0**	**93.7**	**89.7**
SD	2.4	3.3	8.9	4.4	2.8	2.3	4.9
Total length, males [cm]	**3.9**	**3.9**	**3.9**	**3.9**	**3.9**	**4.0**	**4.0**
SD	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Total length, females [cm]	**4.1**	**4.2**	**4.1**	**4.1**	**4.2**	**4.2**	**4.4)[a]**
SD	0.1	0.0	0.2	0.0	0.1	0.1	0.1
Wet weight, males [g]	**0.624**	**0.602**	**0.574**	**0.570**	**0.544**	**0.605**	**0.654)[a]**
SD	0.039	0.022	0.026	0.024	0.030	0.022	0.052
Wet weight, females [g]	**0.807**	**0.840**	**0.778**	**0.798**	**0.864**	**0.851**	**1.10)[a]**
SD	0.033	0.052	0.068	0.050	0.088	0.055	0.08
Mean sex ratio, [% males]	**48.6**	**50.0**	**48.9**	**52.8**	**48.6**	**48.6**	**49.4**
SD	2.8	4.5	6.5	3.2	2.8	2.8	13.0
Mean sex ratio, [% females]	**48.6**	**50.0**	**48.6**	**47.2**	**51.4**	**48.9**	**45.3**
SD	2.8	4.5	2.8	3.2	2.8	6.5	15.5
Unidentified sex [%]	**2.8**	**0.0**	**2.5**	**0.0**	**0.0**	**2.5**	**5.3**
SD	5.6	0.0	5.0	0.0	0.0	5.0	6.1

SD = Standard Deviation
)a: Significantly induced compared to solvent control.; Williams Multiple t-test; p<0.05; two-sided.

3.2.3.4 Biological results, first filial generation (F1)

Hatch was sufficient to obtain enough larvae to proceed and was observed to be >80 % in almost in all test vessels (related to the number of eggs introduced). However, for the solvent control, the mean hatching rate was 77.1 %, and thus slightly below the defined acceptance criterion.

Under exposure, a mean hatching rate of 86.1, 79.9, 98.6, 100 and 97.9 % was determined in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. First larvae hatched at day 1 pf and hatch was completed at day 5 pf. There was no statistically significant difference compared to solvent control in treatments with Prochloraz (Step-down Rao-Scott-Cochran-Armitage Test; α=0.05; one-sided greater).

The number of surviving fish larvae were determined at day 35 pf. For this, all remaining larvae were catched and photographed. The photos from day 35 pf were also evaluated for total fish length. After each evaluation, the fish were carefully re-introduced to the respective test vessel. The post-hatch survival rates in the controls were calculated to be 74.0 and 73.9 % at day 35 pf for the dilution water and the solvent control, respectively. Unfortunately, this was slightly below the acceptance criteria of ≥75 %. In the exposed groups, the mean post-hatch survival rates at day 35 pf were determined to be 65.9, 64.4, 62.7, 69.4 and 28.0 % in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively.
Statistical analysis revealed a significant higher mortality between solvent controls and treatment with 331 µg Prochloraz/L (mean measured; Step-down Rao-Scott-Cochran-Armitage Test Procedure; $p<0.05$; one-sided greater).

On day 35 pf, the total length of all remaining fish larvae was measured. The mean total length in solvent controls was calculated to be 1.1 cm. The fish lengths in the exposed groups were determined to be 1.1, 1.0, 1.1, 1.0 and 0.9 cm in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The statistical evaluation revealed a significantly reduced length compared to control at the highest treatment level, i.e. 331 µg Prochloraz/L (mean measured; Williams Multiple t-test; $p<0.05$; one-sided smaller).

After the measurement of fish length, the fish groups were randomly reduced to 20 fish each per test vessel, i.e. no selective selection e.g. based on fish. On day 63 pf, the fish groups were again photographed, revealing a surviving rate of 100 % across almost all treatment levels. Only at 112 µg Prochloraz/L (mean measured) one fish died, leading to a surviving rate of 98.8 %. The mean total length at day 63 pf was measured to be 2.4 cm in the solvent controls and 2.5, 2.5, 2.5, 2.4 and 2.3 cm in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. No statistically significant difference between solvent controls and treatments were obtained in terms of fish total length at day 63 pf (Williams Multiple t-test; α=0.05; two-sided).

At day 55 pf, spawning trays were introduced to assess first spawning events. Since there were no female but only male fish present at the highest treatment level of 331 µg Prochloraz/L (mean measured) no egg production was possible at all. Furthermore, no females but only males were present in replicate D at 3.36 µg Prochloraz/L (mean measured). Subsequently, the respective mean value is based on three replicates only.
In the solvent controls, the time to first spawning was in the range from day 71 to day 76 pf, while spawning onset was found to be in the range from day 71 to day 72 pf, from day 71 to day 73 pf, from day 71 to day 80 pf and from day 71 to day 79 pf in treatments with 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively. Statistical analysis revealed no significantly delayed spawning onset in treatments with (Dunnett`s Multiple t-test; α=0.05; one-sided greater).

Reproduction was recorded in terms of egg numbers and fertilization rates for 20 days. For the solvent controls, a mean of 26 eggs per female and day was calculated. The mean egg numbers per female and day in the exposed groups were determined to be 19, 17, 25 and 21 in treatments with 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively. Since there were no female but only male fish present at the highest treatment level of 331 µg Prochloraz/L

(mean measured) no egg production was possible at all. The mean fertilization rate was calculated to be 83.2 % in the solvent controls and 95.1, 94.9, 93.3 and 96.0 % in treatments with 3.2, 10, 32 and 112 µg Prochloraz/L (mean measured), respectively. Neither for fecundity nor for fertility a significant effect compared to control was found (fecundity: Dunnett`s Multiple t-test; α=0.05; one-sided smaller; fertility: Williams Multiple t-test; α=0.05; one-sided smaller).

After successful initiation of the second filial generation, the adult fish groups were sacrificed. The sex ratio was first calculated based on the macroscopic inspection of fish gonads and afterwards verified by histopathological analysis. The mean sex ratio in the solvent controls was determined to be 66.0 % males / 34.0 % females, and thus in line with the validity criterion of 30 to 70 % males or females. In the exposed groups mean sex ratios of 66.6 % males / 33.4 % females, 68.6 % males / 31.4 % females, 63.4 % males / 36.6 % females, 87.9 % males / 12.1 % females and 100 % males / 0 % females were determined in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. Statistical evaluation revealed a test item induced shift in sex ratio towards an increased number of males, respective a decreased number of females (Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm; $p<0.05$; two-sided).

The mean fish total lengths in males were determined to be 3.6 cm in the solvent controls and 3.4, 3.4, 3.4, 3.4 and 3.3 cm in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. No significantly test item induced effect on length compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean fish total lengths in females were determined to be 3.6 cm in the solvent controls and 3.6, 3.6, 3.4 and 3.5 cm in treatments with 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively. No significant effect on length was found compared to control (Dunnett`s Multiple t-test; α=0.05; two-sided).

The mean fish wet weights of males were determined to be 0.434 g in the solvent controls and 0.401, 0.407, 0.411, 0.443 and 0.404 g in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. A statistically significant increase compared to control was not found (Dunnett`s Multiple t-test; α=0.05; two-sided). The mean wet weights in females were determined to be 0.481 g for the solvent controls and 0.534, 0.575, 0.523 and 0.561 g in treatments 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively. No significant effect compared to control was found (Dunnett`s Multiple t-test; α=0.05; two-sided).

All results, i.e. mean values and standard deviations, are shown in Table 91. For more details, please refer to the appendix report.

Table 91: Study 7, ZEOGRT with Prochloraz: Biological results for first filial generation (F1)

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control	Solvent control	3.36	10.8	33.0	112	331****
Mean hatching rate [%]	**88.2**	**77.1***	**86.1**	**79.9**	**98.6**	**100**	**97.9**
SD	10.7	9.7	9.6	31.2	1.6	0.0	4.2
Mean post-hatch survival at day 21 pf [%]	**78.4**	**74.7****	**65.9**	**68.6**	**63.4**	**71.5**	**28.0)[a]**
SD	17.7	13.0	10.6	9.1	19.6	31.9	26.8
Mean post-hatch survival at day 35 pf [%]	**74.0****	**73.9****	**65.9**	**64.4**	**62.7**	**69.4**	**28.0)[a]**
SD	15.6	14.4	10.6	15.3	18.7	30.5	26.8
Mean total length, day 35 pf (all fish pre-reduction) [cm]	**1.0**	**1.1**	**1.1**	**1.0**	**1.1**	**1.0**	**0.9)[b]**
SD	0.1	0.1	0.1	0.1	0.1	0.2	0.1
Mean survival day 63 pf (compared to no. post-reduction at day 35 pf) [%]	**100**	**100**	**100**	**100**	**100**	**98.8**	**100**
SD	0	0	0	0	0	2.5	0
Mean total length, day 63 pf [cm]	**2.5**	**2.4**	**2.5**	**2.5**	**2.5**	**2.4**	**2.3**
SD	0.0	0.1	0.1	0.0	0.1	0.1	0.2

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control	Solvent control	3.36	10.8	33.0	112	331****
Time to first spawning [day pf]	**A** 71	71	72	73	80	73	-
	B 73	71	71	71	77	71	-
	C 73	76	72	71	71	71	-
	D 76	73	-***	71	71	79	-
Mean egg number per day and female [n]	**17**	**26**	**19*** **	**17**	**25**	**21**	**-**
SD	5	13	7	2	12	13	-
Mean fertilization rate [%]	**86.0**	**83.2**	**95.1*** **	**94.9**	**93.3**	**96.0**	**-**
SD	3.5	5.1	1.4	2.2	3.8	2.6	-
Mean survival, adult stage (day 63 pf until testend) [%]	**97.5**	**98.8**	**98.8**	**96.9**	**100**	**97.4**	**100**
SD	2.9	2.5	2.5	6.3	0	3.0	0
Mean total length, males, adult stage [cm]	**3.6**	**3.6**	**3.4**	**3.4**	**3.4**	**3.4**	**3.3**
	0.1	0.1	0.3	0.3	0.3	0.3	0.2
Mean total length, females, adult stage [cm]	**3.7**	**3.6**	**3.6*** **	**3.6**	**3.4**	**3.5**	**-**
SD	0.2	0.2	0.3	0.5	0.3	0.3	-
Mean wet weight, males, adult stage [g]	**0.414**	**0.434**	**0.401**	**0.407**	**0.411**	**0.443**	**0.404**
SD	0.026	0.014	0.049	0.039	0.042	0.041	0.056
Mean wet weight, females, adult stage [g]	**0.554**	**0.481**	**0.534*** **	**0.575**	**0.523**	**0.561**	**-**
SD	0.086	0.056	0.080	0.150	0.042	0.187	-
Mean sex ratio, [% males]	**69.3**	**66.0**	**66.6**	**68.6**	**63.4**	**87.9**	**100)[c]**
SD	18.4	23.1	30.6	17.8	34.0	2.5	0

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control	Solvent control	3.36	10.8	33.0	112	331****
Mean sex ratio, [% females]	**30.7**	**34.0**	**33.4**	**31.4**	**36.6**	**12.1**	**0)[c]**
SD	18.4	23.1	30.6	17.8	34.0	2.5	0

SD = Standard Deviation

*: Value slightly below the defined validity criterion of 80 % (refers to solvent control).

**: Value slightly below the defined validity criterion of 75 % (refers to both controls).

***: Only male fish present in replicate D at 3.36 µg/L (mean measured). Thus, mean value is based on three replicates only (i.e. replicates A, B and C).

****: Only male fish present at 331 µg Prochloraz/L (mean measured).

)[a]: Significantly higher mortality compared to solvent control.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[b]: Significantly reduced compared to solvent control; Williams Multiple t-test; p<0.05; one-sided smaller

)[c]: Significant increase (males), respective decrease (females) compared to solvent control; Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm; p<0.05; two-sided.

3.2.3.5 Biological results, second filial generation (F2)

The mean hatching success in the solvent controls was 98.8 % and 95.0; 97.5, 93.8 and 96.0 % in treatments with 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively.

For more details, please refer to the appendix report.

Statistical evaluation revealed that compared to the solvent control Prochloraz had no influence on hatching success (Chi^2 2x2 Table Test with Bonferroni Correction; one-sided greater α=0.05; one-sided greater).

Table 92: Study 7, ZEOGRT with Prochloraz: Biological results for second filial generation (F2)

Parameter	Mean measured concentration Prochloraz [µg/L]						
	Control	Solvent control	3.36*	10.8	33.0	112	331**
Mean hatching rate [%]	**78.8**	**98.8**	**95.0**	**97.5**	**93.8**	**96.0**	**-**
SD	14.4	2.5	8.7	5.0	12.5	5.3	-

SD = Standard deviation

*: Only male fish present in replicate D at 3.36 µg/L (mean measured). Subsequently, in this specific replicate no eggs were produced, and the mean value ist based on three replicates only (i.e. replicates A, B and C).

**: Only male fish present at 331 µg Prochloraz/L (mean measured), thus no reproduction in F1 was possible.

3.2.3.6 Biomarker results

The mean vitellogenin (VTG) concentrations in controls and treatments were calculated based on the geometric means of each replicate. The respective mean values are presented in Table 93 (F0) and Table 94 (F1). All single values are given in the appendix report.

In parental generation (F0) the mean vitellogenin values for males were determined to be 1.10E+03 ng VTG/mL in the solvent controls and 7.69E+02, 8.76E+02, 5.00E+02, 2.04E+02 and 8.70E+01ng VTG/mL in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean VTG values for females were determined to be 2.74E+06 ng VTG/mL in the solvent controls and 2.93E+06, 2.57E+06, 1.56E+06, 1.36E+06 and 1.49E+05 ng VTG/mL in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively.

Compared to solvent control neither males nor females showed any statistically significant effect on vitellogenin values (Multiple Sequentially-rejective Welsh t-test After Bonferroni-Holm; α=0.05; one-sided greater (males), one-sided smaller (females)).

Table 93: Study 7, ZEOGRT with Prochloraz: Biomarker results for parental generation (F0); vitellogenin concentration in blood plasma [ng VTG/mL]

	Mean measured concentration Prochloraz [µg/L]						
	Control	**Solvent control**	**3.36**	**10.8**	**33.0**	**112**	**331**
Mean VTG concentration, males [ng VTG/mL]	**9.63E+02**	**1.10E+03**	**7.69E+02**	**8.76E+02**	**5.00E+02**	**2.04E+02**	**8.70E+01**
SD	3.09E+02	5.69E+02	3.36E+02	3.13E+02	1.68E+02	7.74E+01	6.76E+01
Mean VTG concentration, females [ng VTG/mL]	**2.49E+06**	**2.74E+06**	**2.93E+06**	**2.57E+06**	**1.56E+06**	**1.36E+06**	**1.49E+05**
SD	2.41E+06	2.78E+06	3.74E+06	2.17E+06	9.36E+05	3.54E+05	6.87E+04

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

In first filial generation (F1), the mean vitellogenin (VTG) values for males were determined to be 2.05E+02 ng VTG/mL in controls and 1.67E+03, 2.45E+02, 1.68E+02, 1.82E+02 and 1.93E+02 ng VTG/mL in treatments with 3.36, 10.8, 33.0, 112 and 331 µg Prochloraz/L (mean measured), respectively. The mean VTG values for females were determined to be 8.23E+05 ng VTG/mL in controls and 9.72E+05, 4.95E+06, 1.19E+06 and 1.10E+06 ng VTG/mL in treatments with 3.36, 10.8, 33.0 and 112 µg Prochloraz/L (mean measured), respectively.

Compared to solvent control neither males nor females showed any statistically significant effect on the vitellogenin value (males: Multiple Sequentially-rejective Median (2x2-Table) Test After Bonferroni-Holm; α=0.05; one-sided greater; females: Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm; α=0.05; one-sided smaller).

Table 94: Study 7, ZEOGRT with Prochloraz: Biomarker results for first filial generation (F1); vitellogenin concentration in blood plasma [ng VTG/mL]

	Mean measured concentration Prochloraz [µg/L]						
	Control	**Solvent control**	**3.36**	**10.8**	**33.0**	**112**	**331***
Mean VTG concentration, males [ng VTG/mL]	**9.24E+01**	**2.05E+02**	**1.67E+03**	**2.45E+02**	**1.68E+02**	**1.82E+02**	**1.93E+02**
SD	6.55E+01	1.62E+02	3.10E+03	3.05E+02	1.30E+02	9.27E+01	1.43E+02
Mean VTG concentration, females [ng VTG/mL]	**3.16E+05**	**8.23E+05**	**9.72E+05****	**4.95E+06**	**1.19E+06**	**1.10E+06**	**-**
SD	1.69E+05	6.74E+05	5.28E+05	3.30E+06	1.74E+06	8.90E+05	-

SD = Standard deviation

Remark: The mean VTG concentrations in controls and treatments were calculated based on the geometric means of each replicate.

*: Only male fish present at 331 µg Prochloraz/L (mean measured).

**: Mean is based on three replicates only (i.e. relicates A, B and C), since there were no females present in replicate D at 3.36 µg Prochloraz/L (mean measured).

3.2.3.7 Histopathology of fish gonads

The histopathological evaluation of the fish gonads was performed according to the OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads (OECD 2010). Criteria for staging and grading are given in detail in 2.1.12.2.

The obtained results for each individual fish and the evaluation of these data on replicate and treatment basis are presented in the appendix report. In 3.2.3.7.1 and in 3.2.3.7.2 the results are sorted according to the corresponding control or treatment level for parental generation (F0) and first filial generation (F1), respectively. Based on these data, the median gonadal stage for each test vessel was calculated. The results for parental generation are presented in Table 95 and the results for first filial generation are shown in Table 96.

Summarizing, in parental generation (F0) a statistically significant shift to a higher testicular stage compared to solvent control at treatment with 331 µg Prochloraz/L (mean measured) was determined (Step-down Jonckheere-Terpstra Test; $p<0.05$; two-sided). Furthermore, a dose-dependent increase of hyper-eosinophilic interstitial cells in the testis could be observed with a peak in fish in treatment of 33.0 µg Prochloraz/L (mean measured).

In the F1 generation, a dose-dependent shift to male fish was seen with all male fish in treatment of 331 µg Prochloraz/L (mean measured) and a statistically significant shift to a higher testicular stage compared to solvent control at treatment with 331 µg Prochloraz/L (mean measured) was determined (Step-down Jonckheere-Terpstra Test; $p<0.05$; two-sided). Furthermore, a dose-dependent increase of hyper-eosinophilic interstitial cells in the testis was seen with a peak in treatment of 33.0 µg Prochloraz/L (mean measured).

Table 95: Study 7, ZEOGRT with Prochloraz: Median gonad maturation stages for F0 generation

Parameter		Mean measured concentration Prochloraz [µg/L]						
		Control	**Solvent control**	**3.36**	**10.8**	**33.0**	**112**	**331**
Maturation stages, male [1)]		Stage 0 (0/19) Stage 1 (2/19) Stage 2 (17/19) Stage 3 (0/19) Stage 4 (0/19)	Stage 0 (0/19) Stage 1 (4/19) Stage 2 (15/19) Stage 3 (0/19) Stage 4 (0/19)	Stage 0 (0/18) Stage 1 (3/18) Stage 2 (14/18) Stage 3 (1/18) Stage 4 (0/18)	Stage 0 (0/20) Stage 1 (5/20) Stage 2 (15/20) Stage 3 (0/20) Stage 4 (0/20)	Stage 0 (0/19) Stage 1 (3/19) Stage 2 (16/19) Stage 3 (0/19) Stage 4 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (13/19) Stage 3 (6/19) Stage 4 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (7/19) Stage 3 (12/19) Stage 4 (0/19)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**2**	**1**	**2**	**3**	**3**
	B	**2**	**2**	**2**	**2**	**2**	**3**	**3**
	C	**2**	**2**	**2**	**2**	**2**	**2**	**3**
	D	**2**	**2**	**2**	**2**	**2**	**2**	**3**
Treatment found to be significantly different compared to solvent control.		-	-	-	-	-	-	**)[a]**
Maturation stages, female [2)]		Stage 0 (0/19) Stage 1 (2/19) Stage 2 (12/19) Stage 3 (5/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (5/19) Stage 3 (13/19) Stage 4 (1/19) Stage 5 (0/19)	Stage 0 (1/18) Stage 1 (0/18) Stage 2 (7/18) Stage 3 (10/18) Stage 4 (0/18) Stage 5 (0/18)	Stage 0 (0/18) Stage 1 (0/18) Stage 2 (8/18) Stage 3 (10/18) Stage 4 (0/18) Stage 5 (0/18)	Stage 0 (0/20) Stage 1 (0/20) Stage 2 (12/20) Stage 3 (8/20) Stage 4 (0/20) Stage 5 (0/20)	Stage 0 (0/19) Stage 1 (0/19) Stage 2 (7/19) Stage 3 (12/19) Stage 4 (0/19) Stage 5 (0/19)	Stage 0 (0/17) Stage 1 (0/17) Stage 2 (7/17) Stage 3 (10/17) Stage 4 (0/17) Stage 5 (0/17)
Maturation stage, female, median value/ replicate	A	**2**	**3**	**2**	**3**	**2**	**3**	**3**
	B	**3**	**3**	**3**	**3**	**3**	**3**	**3**
	C	**2**	**2**	**3**	**3**	**2**	**3**	**3**
	D	**2**	**3**	**2**	**2**	**3**	**3**	**2**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

)a: Statistically significant higher testicular stage compared to solvent control; Step-down Jonckheere-Terpstra Test; $p<0.05$; two-sided)

Table 96: Study 7, ZEOGRT with Prochloraz: Median gonad maturation stages for F1 generation

Parameter		Mean measured concentration Prochloraz [µg/L]						
		Control	**Solvent control**	**3.36**	**10.8**	**33.0**	**112**	**331**
Maturation stages, male [1)]		Stage 0 (0/51) Stage 1 (5/51) Stage 2 (41/51) Stage 3 (5/51) Stage 4 (0/51)	Stage 0 (0/48) Stage 1 (0/48) Stage 2 (31/48) Stage 3 (17/48) Stage 4 (0/48)	Stage 0 (0/48) Stage 1 (6/48) Stage 2 (29/48) Stage 3 (13/48) Stage 4 (0/48)	Stage 0 (0/41) Stage 1 (0/41) Stage 2 (39/41) Stage 3 (2/41) Stage 4 (0/41)	Stage 0 (0/50) Stage 1 (0/50) Stage 2 (35/50) Stage 3 (15/50) Stage 4 (0/50)	Stage 0 (0/57) Stage 1 (0/57) Stage 2 (22/57) Stage 3 (35/57) Stage 4 (0/57)	Stage 0 (0/37) Stage 1 (0/37) Stage 2 (3/37) Stage 3 (34/37) Stage 4 (0/37)
Maturation stage, male, median value/ replicate	A	**2**	**2**	**1**	**2**	**2**	**2**	**3**
	B	**2**	**2**	**2**	**2**	**2**	**3**	**3**
	C	**2**	**2**	**2**	**2**	**2**	**2**	**3**
	D	**2**	**2**	**3**	**2**	**2**	**3**	**2**
Treatment found to be significantly different compared to solvent control.		-	-	-	-	-	-	**)[a]**
Maturation stages, female [2)]		Stage 0 (0/22) Stage 1 (0/22) Stage 2 (17/22) Stage 3 (5/22) Stage 4 (0/22) Stage 5 (0/22)	Stage 0 (0/26) Stage 1 (0/26) Stage 2 (21/26) Stage 3 (5/26) Stage 4 (0/26) Stage 5 (0/26)	Stage 0 (0/26) Stage 1 (0/26) Stage 2 (16/26) Stage 3 (10/26) Stage 4 (0/26) Stage 5 (0/26)	Stage 0 (0/23) Stage 1 (0/23) Stage 2 (14/23) Stage 3 (9/23) Stage 4 (0/23) Stage 5 (0/23)	Stage 0 (0/25) Stage 1 (0/25) Stage 2 (24/25) Stage 3 (1/25) Stage 4 (0/25) Stage 5 (0/25)	Stage 0 (0/8) Stage 1 (0/8) Stage 2 (6/8) Stage 3 (2/8) Stage 4 (0/8) Stage 5 (0/8)	-
Maturation stage, female, median value/ replicate	A	**3**	**3**	**2**	**2**	**2**	**3**	**-**
	B	**2**	**2**	**2**	**2**	**2**	**2**	**-**
	C	**2**	**2**	**3**	**2**	**2**	**2**	**-**
	D	**2**	**2**	**-**	**3**	**2**	**2**	**-**

1) Criteria for staging testes: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-spermatogenic; stage 3 = late spermatogenic; stage 4 = spent

2) Criteria for staging ovaries: stage 0 = undeveloped; stage 1 = early spermatogenic; stage 2 = mid-development; stage 3 = late development; stage 4 = late development/hydrated; stage 5 = post ovulatory

)a: Statistically significant higher testicular stage compared to solvent control; Step-down Jonckheere-Terpstra Test; $p<0.05$; two-sided)

3.2.3.7.1 Histopathology results, parental generation (F0)

In five fish (one fish from group 0 (ZF23), one fish from group 2 (ZF86), one fish from group 5 (ZF220) and two fish from group 4 (ZF254, ZF272) gonads were not detected after processing (including step sections). Eleven fish (listed as missing on dispatch list) and one fish (listed as not missing on dispatch list) were not delivered.

Control – group 0

Two female fish (2/19) had the ovary stage 1, twelve female fish (12/19) had the ovary stage 2 and five females (5/19) the ovary stage 3.

Two male fish (2/19) were diagnosed with the testis stage 1 and seventeen male fish (17/19) were diagnosed with the testis stage 2.

Additional diagnoses were not observed in the female fish of this group. In three of the male fish (3/19), there was a very slight presence of hyper-eosinophilic interstitial cells in the testis.

Solvent control – group 1

Five female fish (5/19) had the ovary stage 2, thirteen females (13/19) the ovary stage 3 and one female (1/19) the ovary stage 4.

Two female fish showed egg debris (one grade 1, one grade 2) and a granulomatous inflammation (one grade 1, one grade 2).

Four male fish (4/19) were diagnosed with the testis stage 1 and fifteen male fish (15/19) were diagnosed with grade 2. One male fish (1/19) showed very slight presence of hyper-eosinophilic interstitial cells in the testis.

3.36 µg Prochloraz/L (mean measured) – group 2

One female fish showed the ovary stage 0 (1/18). Seven female fish (7/18) had the ovary stage 2 and ten females (10/18) the ovary stage 3.

In the female fish with ovary stage 0 a lymphoma was apparent in various tissues.

Three male fish (3/18) were diagnosed with the testis stage 1, fourteen male fish (14/18) with stage 2, and one male fish (1/18) with testis stage 3. Two male fish (2/18) showed very slight presence of hyper-eosinophilic interstitial cells in the testis.

10.8 µg Prochloraz/L (mean measured) – group 3

Eight female fish (8/18) had the ovary stage 2 and ten females (10/18) the ovary stage 3. One female showed egg debris (stage 2) and a granulomatous inflammation (stage 3).

Five male fish were diagnosed with the testis stage 1 (5/20) and fifteen male fish were diagnosed with the testis stage 2 (15/20).

Four male fish (4/20) showed very slight presence of hyper-eosinophilic interstitial cells in the testis.

33.0 µg Prochloraz/L (mean measured) – group 4

Twelve female fish (12/20) had the ovary stage 2, and eight female fish (8/20) the ovary stage 3.

One female fish showed increased oocyte atresia (grade 2).

Three male fish (3/19) were diagnosed with the testis stage 1 and sixteen males (16/19) with the stage 3.

Seven male fish (7/19) showed hyper-eosinophilic interstitial cells in the testis (two with grade 1, one with grade 2, four with grade 3).

112 µg Prochloraz/L (mean measured) – group 5

Seven female fish (7/19) had the ovary stage 2 and twelve female fish (12/19) had the ovary stage 3.

One female fish showed a granulomatous inflammation (grade 1).

Thirteen male fish (13/19) were diagnosed with the testis stage 2 and six males (6/19) with the stage 3.

One male fish (1/19) showed very slight presence of hyper-eosinophilic interstitial cells in the testis.

331 µg Prochloraz/L (mean measured) – group 6

Seven female fish (7/17) were diagnosed with ovary stage 2 and ten females (10/17) had the ovary stage 3.

Three female fish showed increased oocyte atresia (two with grade 2, one with grade 3), egg debris (one with grade 1, two with grade 2), and a granulomatous inflammation (all grade 1).

Seven male fish had the testis stage 2 (7/19) and twelve male fish were diagnosed with testis stage 3 (12/19). One male fish showed testis-ova (grade 3) and one male showed increased testicular degeneration (grade 1).

3.2.3.7.2 Histopathology results, first filial generation (F1)

Control – group 0

Seventeen female fish (17/22) had the ovary stage 2 and five females (5/22) the ovary stage 3. One female fish showed increased oocyte atresia (grade 1).

Five male fish (5/51) were diagnosed with the testis stage 1, fourty-one male fish (41/51) were diagnosed with the testis stage 2, and five male fish (5/51) were diagnosed with the testis stage 3.

One male fish showed increased testicular degeneration, one male showed interstitial cell hypertrophy/hyperplasia, one male was diagnosed with increased proportion of spermatogonia, and two males showed asynchronous germ cell development (all grade 1). Five male fish (5/51) showed very slight presence of hyper-eosinophilic interstitial cells in the testis. In two male fish, lesions outside the testes (granulomatous inflammation in the body cavity / skeletal musculature) were observed.

Solvent control – group 1

Twenty-one female fish (21/26) had the ovary stage 2 and five females (5/26) the ovary stage 3. Two female fish showed egg debris (one with grade 1, one with grade 3) and granulomatous inflammation (both grade 1).

Thirty-one males (31/48) were diagnosed with the testis stage 2 and seventeen (17/48) with the testis stage 3.

One male fish showed testis-ova (grade 1). Two male fish (2/48) showed very slight presence of hyper-eosinophilic interstitial cells in the testis.

3.36 µg Prochloraz/L (mean measured) – group 2

Sixteen female fish (16/26) had the ovary stage 2 and ten females (10/26) the ovary stage 3.

Five females showed increased oocyte atresia (four grade 1, one grade 2). Two females showed egg debris (both grade 1) and four females showed granulomatous inflammation (three with grade 1, one with grade 2). One female showed increased post-ovulatory follicles (grade 1).

Six male fish (6/48) were diagnosed with the testis stage 1, twenty-nine male fish (29/48) were diagnosed with the testis stage 2, and thirteen male fish (13/48) with the stage 3. One male showed testis-ova (grade 1), five males had increased proportion of spermatogonia (all grade 1), and one male was diagnosed with asynchronous germ cell development (grade 1). Eight male fish (8/48) showed hyper-eosinophilic interstitial cells in the testis (six with grade 1, two with grade 2).

10.8 µg Prochloraz/L (mean measured) – group 3

Fourteen female fish (14/23) had the ovary stage 2 and nine females (9/23) the ovary stage 3.

One female fish showed increased oocyte atresia (grade 1).

Thirty-nine male fish (39/41) were diagnosed with the testis stage 2 and two males (2/41) with the testis stage 3. Fifteen male fish (15/41) showed hyper-eosinophilic interstitial cells in the testis (five with grade 1, seven with grade 2, three with grade 3).

33.0 µg Prochloraz/L (mean measured) – group 4

Twenty-four female fish (24/25) were diagnosed with the ovary stage 2 and one female fish (1/25) with the stage 3. Increased oocyte atresia was observed in 5 female fish (all grade 2). Six females showed egg debris (5 with grade 1, one with grade 3), five had a granulomatous inflammation (4 with grade 1, one with grade 2), and one female fish showed increased post-ovulatory follicles (grade 1).

Thirty-five male fish (35/50) were diagnosed with the testis stage 2 and fifteen males (15/50) with the stage 3.

Three male fish showed increased testicular degeneration, and one male fish showed increased proportion of spermatogonia (all grade 1). Thirty-two male fish (32/50) showed hyper-eosinophilic interstitial cells in the testis (fourteen with grade 1, fifteen with grade 2, three with grade 3).

112 µg Prochloraz/L (mean measured) – group 5

Six female fish had the ovary stage 2 (6/8) and two females were diagnosed with the ovary stage 3 (2/8). Three females showed increased oocyte atresia (two with grade 1, one with grade 3), egg debris (two with grade 1, one with grade 3), and granulomatous inflammation (one with grade 1, one with grade 2, one with grade 3).

Twenty-two male fish were diagnosed with testis stage 2 (22/57) and thirty-five males had testis stage 3 (35/57). Six males showed increased testicular degeneration (all grade 1), three males showed interstitial cell hypertrophy/hyperplasia (all grade 1), and there was increased proportion of spermatogonia in four males (all grade 1) and asynchronous germ cell development in one male (grade 1). Twenty male fish (20/57) showed hyper-eosinophilic interstitial cells in the testis (seven with grade 1, ten with grade 2, three with grade 3).

331 µg Prochloraz/L (mean measured) – group 6

No females were observed in this group.

Three male fish had the testis stage 2 (3/37) and thirty-four were diagnosed with testis stage 3 (34/37). Three males showed increased proportion of spermatogonia (all grade 1).

3.2.3.8 Summary/Conclusion

All results of the Study 7 (phase II) are summarized in Table 97.

Table 97: Study 7, ZEOGRT with Prochloraz: Summary of NOEC / LOEC determination during the study

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
F0-Reproduction	Fecundity (Egg number per day and female)	≥320 / >320	≥331 / >331
	Fertilization rate	≥320 / >320	≥331 / >331
F0-Growth	**Wet weight males)[a]**	**100 / 320**	**112 / 331**
	Wet weight females)[a]	**100 / 320**	**112 / 331**
	Total length males	≥320 / >320	≥331 / >331
	Total length females)[a]	**100 / 320**	**112 / 331**
F0-Sex ratio	Sex ratio (% males)	Right setting was confirmed	
	Sex ratio (% females)		
F0-Histopathology	Maturity stage ovary	≥320 / >320	≥331 / >331
	Maturity stage testis)[b]	**100 / 320**	**112 / 331**
	Histopathology (females)	No treatment-related differences in the occurrence of the investigated lesions.	
	Histopathology (males)	Dose-dependent increase of hypereosinophilic interstitial cells in the testis with a peak at 32 µg Prochloraz/L.	
F0-Biomarker	Vitellogenin males	≥320 / >320	≥331 / >331
	Vitellogenin females	≥320 / >320	≥331 / >331
F1-ELS	Hatch day 5 *	≥320 / >320	≥331 / >331
	Survival day 21 **)[c]	**100 / 320**	**112 / 331**
	Survival day 35 **)[c]	**100 / 320**	**112 / 331**
	Total length day 35)[d]	**100 / 320**	**112 / 331**
F1-Juveniles	Survival day 35 – day 63	≥320 / >320	≥331 / >331
	Total length day 63	≥320 / >320	≥331 / >331
F1-Reproduction***	Time to first spawning	≥100 / >100	≥112 / >112
	Fecundity (Egg number per day and female)	≥100 / >100	≥112 / >112

Generation/phase	Endpoint	NOEC / LOEC Nominal concentration Prochloraz [µg/L]	NOEC / LOEC Mean measured concentration Prochloraz [µg/L]
	Fertilization rate	≥100 / >100	≥112 / >112
F1-Termination***	Survival (test end)	≥320 / >320	≥331 / >331
	Total length males	≥320 / >320	≥331 / >331
	Total length females***	≥100 / >100	≥112 / >112
	Wet weight males	≥320 / >320	≥331 / >331
	Wet weight females***	≥100 / >100	≥112 / >112
	Sex ratio (% males))[e]	**100 / 320**	**112 / 331**
	Sex ratio (% females))[e]	**100 / 320**	**112 / 331**
F1-Biomarker***	Vitellogenin males	≥320 / >320	≥331 / >331
	Vitellogenin females	≥100 / >100	≥112 / >112
F1-Histopathology***	**Maturity stage testis)[b]**	**100 / 320**	**112 / 331**
	Maturity stage ovary	≥100 / >100	≥112 / >112
	Histopathology (males)	No treatment-related differences in the occurrence of the investigated lesions.	
	Histopathology (females)	Dose-dependent increase of hypereosinophilic interstitial cells in the testis with a peak at 32 µg Prochloraz/L.	
F2-Embryo****	Hatch day 4	≥100 / >100	≥112 / >112

)[a]: Significantly increased compared to solvent control.; Williams Multiple t-test; p<0.05; two-sided.

)[b]: Statistically significant higher testicular stage compared to solvent control; Step-down Jonckheere-Terpstra Test; p<0.05; two-sided).

)[c]: Significantly higher mortality compared to solvent control.; Step-down Rao-Scott-Cochran-Armitage Test Procedure; p<0.05; one-sided greater.

)[d]: Significantly reduced compared to solvent control; Williams Multiple t-test; p<0.05; one-sided smaller.

)[e]: Significant increase (males), respective decrease (females) compared to solvent control; Multiple Sequentially-rejective Welsh-t-test After Bonferroni-Holm; p<0.05; two-sided.

*: Value slightly below the defined validity criterion of 80 % (refers to solvent control).

**: Value slightly below the defined validity criterion of 75 % (refers to both controls).

***: Only male fish present at 320 µg Prochloraz/L in F1 generation.

****: Only male fish present at 320 µg Prochloraz/L in F1 generation. Subsequently, no eggs for starting second filial generation could be produced at this treatment level.

4 Conclusion and Discussion

4.1 Testing strategy

4.1.1 Validity criteria

Within the four ZEOGRT studies performed in Phase I, there was a broad compliance with the defined acceptance criteria. The exception was the parameter sex ratio in the F1 generation of the study with tamoxifen-citrate (study 1). For controls a mean ratio of 25.0 and 75.0% was calculated for males and females, respectively.

During the study with dienogest (study 3), there was a timely limited deviation from the recommended range of water temperature. Over a period between day 2 and 10 after test start, single temperatures exceeded the recommended maximum level of 27.5 °C. A maximum of 28.0 °C was not exceeded. High water temperatures were measured at all treatment levels including controls. From the results obtained from the Fish studies conducted in phase I, it can be postulated that the ZEOGRT provides an appropriate setup to detect the relevant apical endpoints. For the test substances applied the test showed sufficient sensitivity. Moreover, the defined validity criteria were all met for both studies conducted confirming the robustness of the protocol applied. The comparison with the validity criteria is shown in Table 98.

Table 98: Comparison with validity criteria defined for ZEOGRT, Validation phase I

Validity criterion	Study 1: TMX		Study 2:PRO		Study 3: DNG		Study 4: DEXA	
	value	met	value	met	value	met	value	met
Test conditions (all replicates)								
Dissolved oxygen content: > 60%	74 - 107 %	yes	80 - 116 %	yes	83 - 107 %	yes	88 - 115 °C	yes
Water temperature: 26.0 +/- 2.0 °C	25.8 - 27.3 °C	yes	26.4 - 27.8 °C	yes	26.0 – 28.0 °C	yes	25.6 - 27.2 °C	yes
F0 generation (control replicates only)								
Reproduction: replicate mean of ≥ 10 eggs per female and day	55 ± 9 [eggs / female and day]	yes	54 ± 6 [eggs / female and day]	yes	64 ± 11 [eggs / female and day]	yes	75 ± 9 [eggs / female and day]	yes
≥ 80 % fertility	84.4 ± 4.5 [%]	yes	84.9 ± 3.5 [%]	yes	90.7 ± 3.4 [%]	yes	91.1 ± 2.5 [%]	yes
F1 generation (control replicates only)								
Early life stage: ≥ 80 % hatching success	91.7 ± 6.0 [%]	yes	100 ± 0.0 [%]	yes	100 ± 0.0 [%]	yes	98.6 ± 2.8 [%]	yes
≥ 75 % Post-hatch survival	86.0 9.6 [%]	yes	84.7 ± 4.8 [%]	yes	92.4 ± 4.7 [%]	yes	94.4 ± 9.6 [%]	yes

Validity criterion	Study 1: TMX		Study 2:PRO		Study 3: DNG		Study 4: DEXA	
	value	met	value	met	value	met	value	met
Reproduction: replicate mean of ≥ 10 eggs per female and day	31 ± 10 [eggs / female and day]	yes	21 ± 6 [eggs / female and day]	yes	31 ± 9 [eggs / female and day]	yes	21 ± 3 [eggs / female and day]	yes
≥ 80 % fertility	80.2 ± 7.3 [%]	yes	86.5 ± 9.5 [%]	yes	86.4 ± 3.6 [%]	yes	89.9 ± 2.5 [%]	yes
Survival ≥ 90% (juveniles and adults)	100 ± 0.0 [%]	yes	98.8 ± 2.5 [%]	yes	98.8 ± 2.5 [%]	yes	100 ± 0.0 [%]	yes
Sex ratio 30 to 70% (males or females)	23.8 ± 10.3 [% males]	no	43.6 ± 17.9 [% males]	yes	60.9 ± 10.7 [% males]	yes	43.3 ± 7.6 [% males]	yes
F2 generation (control replicates only)								
Early life stage: ≥ 80 % hatching success	88.8 ± 8.5 [%]	yes	85.0 ± 14.1 [%]	yes	83.8 ± 11.1 [%]	yes	100 ± 0.0 [%]	yes
The analytical measurement of the test concentrations is compulsory.	A weekly measurement of at least two tank replicates was conducted.	yes	A weekly measurement of at least two tank replicates was conducted.	yes	A weekly measurement of at least two tank replicates was conducted.	yes	A weekly measurement of at least two tank replicates was conducted.	yes

Abbreviations:
PRO: Prochloraz
TMX: Tamoxifen-Citrate
DNG: Dienogest
DEXA: Dexamethasone

The acceptance criteria were also assessed for the three studies performed in the external labs (phase II). For one study (study 7), there were discrepancies regarding the water parameters applied. For single measurements, the water temperatures were outside the range of 26.0 ± 2.0 °C. Moreover, low oxygen concentrations were measured in single cases. In the same study, the F1 post hatch survival of at least 75 % was not met. Moreover, the minimum hatching rate for F2 was not achieved. For study 6, a repeat of F1 preparation was reported, which was necessary to finally meet the acceptance criterion. To summarize, although there were single outliers from the defined criteria, the laboratories were able to transfer the test protocol including sufficient biological performance of the test animals applied.

Table 99: Comparison with validity criteria defined for ZEOGRT: Validation phase II

Validity criterion	Study 5: TMX		Study 6: PRO		Study 7: PRO (based on DWC)	
	value	met	value	met	value	met
Test conditions (all replicates)						
Dissolved oxygen content: > 60%	72 - 110 %	yes	72 - 104 %	yes	27 - 106 %	no
Water temperature: 26.0 +/- 2.0 °C	25.0 – 27.1 °C	yes	24.7 – 27.2 °C	yes	23.6 – 27.9 °C	no
F0 generation (control replicates only)						
Reproduction replicate mean of ≥ 10 eggs per female and day	33 ± 13 [eggs / female and day]	yes	47 ± 13 [eggs / female and day]	yes	49 ± 4 [eggs / female and day]	yes
≥ 80 % fertility	94.7 ± 0.5 [%]	yes	96.9 ± 0.8 [%]	yes	83.3 ± 2.4 [%]	yes
F1 generation (control replicates only)						
Early life stage: ≥ 80 % hatching success	100 ± 0.0 [%]	yes	100 ± 0.0 [%]	yes	88.2 ± 10.7 [%]	yes
≥ 75 % Post-hatch survival	88.9 ± 8.3 [%]	yes	87.5 ± 8.0 [%]	yes	74.0 ± 15.6 [%]	no
Reproduction replicate mean of ≥ 10 eggs per female and day	56 ± 17 [eggs / female and day]	yes	33 ± 11 [eggs / female and day]	yes	17 ± 5 [eggs / female and day]	yes
≥ 80 % fertility	91.4 ± 4.1 [%]	yes	88.7 ± 6.9 [%]	yes	86.0 ± 3.5 [%]	yes
Survival ≥ 90% (juveniles and adults)	95.0 ± 4.1 [%]	yes	92.5 ± 6.5 [%]	yes	97.5 ± 2.9 [%]	yes
Sex ratio 30 to 70% (males or females)	62.8 ± 15.0 [% males]	yes	30.1 ± 12.1 [% males]	yes	69.3 ± 18.4 [% males]	yes
F2 generation (control replicates only)						
Early life stage: ≥ 80 % hatching success	100 ± 0.0 [%]	yes	100 ± 0.0 [%]	yes	78.8 ± 14.4 [%]	no

Validity criterion	Study 5: TMX		Study 6: PRO		Study 7: PRO (based on DWC)	
	value	met	value	met	value	met
The analytical measurement of the test concentrations is compulsory.	A regular measurement was conducted.	**yes**	A regular measurement was conducted.	**yes**	A regular measurement was conducted.	**yes**

Abbreviations:
DWC: Dilution water control
PRO: Prochloraz
TMX: Tamoxifen-Citrate

4.2 Summary of available control data

4.2.1 Validation phase I: Studies at Fraunhofer IME

To derive information about the variability of intra-laboratory data the mean control values and standard deviations for each endpoint were calculated and are summarized in Table 100.

The comparison of the available control data derived from the phase I studies revealed findings, discussed as follows. The obtained fecundity data showed low variability between the studies for both F0 and F1 generation, with a relative standard deviation (RSD) of 15.7 and 21.0%, respectively. In this context, it was evident, that the mean total egg numbers per female per day were lower in F1 compared to F0, which is a consequence of the younger age of the fish.

The F0 spawning groups are prepared using adult fish taken from a lab fish culture. To be sure, that the required egg numbers are finally achieved there is much emphasis in the setting of appropriate groups and thus to use the best spawners but also to have low variability between the spawning groups. The fish will also arise from different conditions as finally applied in the test (e.g. culture tanks with lower density). The final setting of 5 males and 5 females represents an optimized setting to achieve high egg numbers. Typically, the fish already have an age of >6 months, which means a higher grade of maturation (compared to F1).

In contrast, the F1 fully originates out of the test setting (=offspring from F0) including shorter time for development. The recording of reproduction success directly starts after the fish reached maturity (estimated around day 60). Although able to reproduce and to fertilise the eggs, the fish are “just” adult and the ovaries are not at their capacity limit.

Currently, there is no validity criterion for zebrafish fecundity defined in e.g. OECD TG 229, however, for the choice of groups showing appropriate spawning performance the document states: “… but it is relatively common to observe average spawns of >10 eggs/female/day for each species”. This criterion was considered to assess the reproductive performance in the ZEOGRT studies for both F0 and F1.

The mean fertility rates obtained were characterized by low standard deviations and were similar for F0 and F1, with RSDs of 4.2 and 4.7%, respectively.

The recording for the “Time to first spawning” has to be initiated once the fish achieved sufficient maturity to allow the reproductive output. For two phase I studies, it was found that the groups were already able to spawn from the first day spawning trays were placed in the test vessels. Thus, for that studies it was not possible to define the date of spawning start. Thus, the final protocol should consider an earlier start of reproduction recording.

The sex ratio represents an important parameter for the assessment of endocrine acting chemicals. Within the ZEOGRT studies performed, the sex ratio showed the highest control variability of all population relevant parameters recorded. From those studies that met the F1 acceptance criterion, sex ratios for male fish were between 43.3 and 60.9 %. The overall variability may be triggered by different factors related to e.g., test conditions or gene distribution in the fish groups. To identify these influencing factors, further investigations will have to be conducted. This includes e.g. different water temperature settings, feeding amounts but also the assessment of varying group densities.

The recorded growth parameters for adult fish of both F0 and F1, i.e. total length and wet weight, showed clear sex-specific differences and thus should be evaluated separately. Adequate growth performance can also be used to estimate on sufficient maturation. For the phase I studies, the mean total lengths were 3.8 and 4.0 cm for F0 males and females, respectively. The

mean wet weights were 0.474 and 0.701 g for F0 males and females, respectively. The fish of F1 were generally smaller, but the sex-specific differences of the growth parameters could be identified. The mean total lengths were 3.7 and 3.6 cm for F1 males and females, respectively. The mean wet weights were 0.423 and 0.517 g for F1 males and females, respectively

The mean VTG concentration in females were very similar between the control groups, and also between the two generations applied. The mean VTG concentrations in male fish was found to be in the expected low range, however, single outliers induced uncertainty of the data sets, also impacting the statistical evaluation and thus the robust detection of effect concentrations with respect to VTG decrease. By calculating geometric mean concentrations of VTG for the single replicates, the bias induced by the outliers should be reduced.

A summary of the mean control data derived from the phase I of the validation are presented in Table 100.

Table 100: ZEOGRT validation studies, phase I: Comparison of control data, mean values ± SD

Endpoint	Unit	Study 1	Study 2	Study 3	Study 4	Mean	SD	RSD [%]
F0 generation								
Egg number per day and female	[n]	**55** (±9)	**54** (±6)	**64** (±11)	**75** (±9)	**62**	**10**	**15.7**
Fertilization rate	[%]	**84.4** (±4.5)	**84.9** (±3.5)	**90.7** (±3.4)	**91.1** (±2.5)	**87.8**	**3.6**	**4.1**
Total length, males	[cm]	**3.8** (±0.1)	**3.7** (± 0.0)	**3.9** (± 0.1)	**3.8** (±0.1)	**3.8**	**0.1**	**2.1**
Total length, females	[cm]	**4.0** (±0.1)	**4.0** (±0.1)	**3.9** (±0.2)	**3.9** (±0.1)	**4.0**	**0.1**	**1.5**
Wet weight, males	[g]	**0.451** (±0.049)	**0.461** (±0.013)	**0.503** (±0.019)	**0.482** (±0.036)	**0.474**	**0.023**	**4.9**
Wet weight, females	[g]	**0.688** (±0.061)	**0.700** (±0.064)	**0.711** (±0.055)	**0.704** (±0.054)	**0.701**	**0.010**	**1.4**
F1 generation Early life stages								
Hatching success	[%]	**91.7** (±6.0)	**100** (±0)	**100** (±0)	**98.6** (±2.8)	**97.6**	**4.0**	**4.1**
Post-hatch survival rate, day 21 pf	[%]	**88.9** (±8.5)	**88.2** (±4.2)	**93.1** (±3.6)	**97.2** (±4.8)	**91.9**	**4.2**	**4.5**
Post-hatch survival rate, day 35 pf	[%]	**86.0** (±9.6)	**84.7** (±4.8)	**92.4** (±4.7)	**94.4** (±9.6)	**89.4**	**4.7**	**5.3**
Total length, day 35 pf	[cm]	**1.8** (±0.1)	**1.8** (±0.1)	**1.9** (±0.0)	**1.8** (±0.0)	**1.8**	**0.0**	**2.8**

Endpoint	Unit	Study 1	Study 2	Study 3	Study 4	Mean	SD	RSD [%]
F1 generation, Juveniles								
Survival, day 63 pf	[%]	**100** (±0)	**98.8** (±2.5)	**98.8** (±2.5)	**100** (±0)	**99.4**	**0.7**	**0.7**
Total length, day 63 pf	[cm]	**3.0** (±0.1)	**3.0** (±0.1)	**3.1** (±0.1)	**3.0** (±0.0)	**3.0**	**0.1**	**1.7**
F1 generation Reproduction								
Time to first spawn	[days]	**68 - 78**	**≤56**	**≤64 - 65**	**58 - 61**	**-**	**-**	
Egg number per day and female	[n]	**31** (±10)	**22** (±6)	**31** (±9)	**21** (±3)	**26**	**6**	**21.0**
Fertilization rate	[%]	**80.2** (±7.3)	**86.5** (±9.5)	**86.4** (±3.6)	**89.9** (±2.5)	**85.8**	**4.0**	**4.7**
F1 generation, Adult phase								
Survival	[%]	**100** (±0)	**98.8** (±2.5)	**100** (±0)	**100** (±0)	**99.7**	**0.6**	**0.6**
Total length, males	[cm]	**3.9** (±0.1)	**3.4** (±0.1)	**3.7** (±0.1)	**3.8** (±0.1)	**3.7**	**0.2**	**5.8**
Total length, females	[cm]	**3.8** (±0.1)	**3.4** (±0.1)	**3.6** (±0.1)	**3.7** (±0.1)	**3.6**	**0.2**	**4.7**
Wet weight, males	[g]	**0.474** (±0.035)	**0.356** (±0.028)	**0.436** (±0.027)	**0.425** (±0.017)	**0.423**	**0.049**	**11.6**
Wet weight, females	[g]	**0.580** (±0.040)	**0.447** (±0.046)	**0.512** (±0.063)	**0.528** (±0.027)	**0.517**	**0.055**	**10.6**
Sex ratio, %males	[%]	**23.8** (±10.3)	**43.6** (±17.9)	**60.9** (±10.7)	**43.3** (±7.6)	**42.9**	**15.2**	**35.3**
Sex ratio, %females	[%]	**73.8** (±14.4)	**56.4** (±17.9)	**39.1** (±10.7)	**56.7** (±7.6)	**56.5**	**14.2**	**25.1**
Sex ratio, %undiff	[%]	**2.5** (±5.0)	**0.0** (±0.0)	**0.0** (±0.0)	**0.0** (±0.0)	**0.6**	**1.3**	**200**
F2 generation Early life stage								
Hatching success	[%]	**88.8** (±8.5)	**85.0** (±14.1)	**83.8** (±11.1)	**100** (±0)	**89.4**	**7.4**	**8.3**

Endpoint	Unit	Study 1	Study 2	Study 3	Study 4	Mean	SD	RSD [%]
Biomarker								
F0, VTG males	[ng/ mL]	**5.98E+01** (± 1.17E+01)	**1.41E+02** (± 1.04E+02)	**6.20E+01** (± 2.15E+01)	**7.54E+01** (± 1.85E+01)	**8.45E+01**	**3.83E+01**	**45.3**
F0, VTG females	[ng/ mL]	**1.51E+07** (± 3.19E+06)	**4.07E+07** (± 9.12E+06)	**1.64E+07** (± 2.58E+06)	**4.89E+07** (± 2.77E+07)	**3.03E+07**	**1.71E+07**	**56.5**
F1, VTG males	[ng/ mL]	**3.03E+02** (± 2.72E+02)	**4.21E+01** (± 4.98E+01)	**1.78E+02** (± 7.58E+01)	**2.80E+01** (± 0.71E+01)	**1.38E+02**	**1.29E+02**	**93.8**
F1, VTG females	[ng/ mL]	**1.46E+07** (± 1.83E+06)	**1.23E+07** (± 2.19E+06)	**1.81E+07** (± 1.11E+07)	**2.76E+07** (± 1.78E+07)	**1.82E+07**	**6.74E+06**	**37.1**

4.2.2 Validation phase II: Studies at external laboratories

In a second step, the control performance for the phase II studies is presented. It has to be noted that the laboratories involved in the validation exercise had established own zebrafish strains. Therefore, different growth performance could be observed. The calculation of overall mean values and standard deviations is of no further value, as it would be impeded by the strain-specific differences. The mean values (± SD) derived from the phase I controls were added to the summarizing Table (see below) to allow for comparison.

Study 7, conducted with Prochloraz, required the application of a solvent control. The data obtained are also presented to allow at least a limited assessment of the impact of solvent use.

Some findings are highlighted in the following. Basically, it can be stressed that all studies expressed a very good performance regarding the reproduction parameters. Notably, all studies were able to identify the "time to first spawning" within the same period of testing. The acceptance criterion for sufficient fecundity of both F0 and F1, i.e. at least 10 eggs per female and day, was met in each case. As also recorded and discussed for the phase I studies, the egg numbers for F0 were usually higher as for F1. The exception was Study 5, where F1 egg numbers were finally higher. The reasons for this difference were already explained in chapter 4.2.1.

Also, the fertilization rates were at a high level, without major differences for F0 and F1. Moreover, obviously no difference was induced by solvent use. As described for the phase I data set, the fertilization rates were characterized by a very low variability under control conditions.

As already mentioned, fish strain-specific differences in the growth performance could be observed. For example, the F0 adults displayed mean female wet weights of 1.191 and 1.078 g in study 5 and 6, respectively. The mean male wet weights were calculated to be 0.894 and 0.813 g for study 5 and 6, respectively. Thus, sex-specific difference could also be determined in these studies. F1 controls wet weights were detected in the same ranges.

The mean sex ratios (expressed as % males) were all found to be in line with the given acceptance criteria. However, high variability of control sex ratio was identified and ranged between 30.1 % (study 6) and 69.3 % (study 7, dilution water control). However, the standard deviations within the control replicates were within an acceptable range between 12.1 % (study 6) and 23.1% (study 7, solvent control). Notably, no difference was seen between the mean sex ratio of dilution water and of solvent control (both study 7).

The vitellogenin concentrations found were all characterized by high variation for both females and males. The occurrence of single outliers resulted partly in large standard deviations within the same order of magnitude as the obtained mean values. Moreover, the mean VTG concentrations detected for males in the studies 5 and 6 already displayed high background concentrations, higher than the male VTG concentrations measured in the phase I dataset. However, in any case, a clear sex-specific difference of the mean values was obtained for males and females. Moreover, comparable mean VTG concentrations were detected for both F0 and F1.

Available results for the controls of validation phase II studies are presented in Table 101.

Table 101: ZEOGRT validation studies, phase II: Comparison of control data, mean value ± SD

Endpoint	Unit	Study 5	Study 6	Study 7 DWC	Study 7 Solvent control	Mean phase I	SD phase I
F0 generation							
Egg number per day and female	[n]	**33** (± 13)	**47** (± 13)	**49** (± 4)	**58** (± 16)	**62**	10
Fertilization rate	[%]	**94.7** (± 0.5)	**96.9** (± 0.8)	**83.3** (± 2.4)	**82.7** (+/- 3.3)	**87.8**	3.6
Total length, males	[cm]	**4.8** (± 0.1)	**4.7** (± 0.1)	**3.9** (± 0.1)	**3.9** (± 0.1)	**3.8**	0.1
Total length, females	[cm]	**5.0** (± 0.1)	**4.9** (± 0.1)	**4.1** (± 0.1)	**4.2** (± 0.1)	**4.0**	0.1
Wet weight, males	[g]	**0.894** (± 0.066)	**0.813** (± 0.020)	**0.624** (± 0.039)	**0.602** (± 0.022)	**0.474**	0.023
Wet weight, females	[g]	**1.191** (± 0.045)	**1.078** (± 0.138)	**0.807** (± 0.033)	**0.840** (± 0.052)	**0.701**	0.010
F1 generation Early life stages							
Hatching success	[%]	**100** (± 0.0)	**100** (± 0.0)	**88.2** (± 10.7)	**77.1** (± 9.7)	**97.6**	4.0
Post-hatch survival rate, day 21 pf	[%]	**90.3** (± 9.8)	**87.5** (± 8.0)	**78.4** (± 17.7)	**74.7** (± 13.0)	**91.9**	4.2
Post-hatch survival rate, day 35 pf	[%]	**88.9** (± 8.3)	**87.5** (± 8.0)	**74.0** (± 15.6)	**73.9** (± 14.4)	**89.4**	4.7
Total length, day 35 pf	[cm]	**1.4** (± 0.0)	**1.5** (± 0.1)	**1.0** (± 0.1)	**1.1** (± 0.1)	**1.8**	0.0
F1 generation Juveniles							
Survival, day 63 pf	[%]	**100** (± 0)	**100** (± 0)	**100** (± 0)	**100** (± 0)	**99.4**	0.7
Total length, day 63 pf	[cm]	**3.7** (± 0.1)	**3.8** (± 0.1)	**2.5** (± 0.0)	**2.4** (± 0.1)	**3.0**	0.1
F1 generation Reproduction							
Time to first spawn	[days]	**70 – 73**	**65 – 79**	**71 – 76**	**71 – 76**	-	-
Egg number per day and female	[n]	**56** (± 17)	**33** (± 11)	**17** (± 5)	**26** (± 13)	**26**	6
Fertilisation rate	[%]	**91.4** (± 4.1)	**88.7** (± 6.9)	**86.0** (± 3.5)	**83.2** (± 5.1)	**85.8**	4.0

Endpoint	Unit	Study 5	Study 6	Study 7 DWC	Study 7 Solvent control	Mean phase I	SD phase I
F1 generation Adult phase							
Survival	[%]	**95.0** (± 4.1)	**92.5** (± 6.5)	**97.5** (± 2.9)	**98.8** (± 2.5)	**99.7**	0.6
Total length, males	[cm]	**4.7** (± 0.2)	**4.6** (± 0.2)	**3.6** (± 0.1)	**3.6** (± 0.1)	**3.7**	0.2
Total length, females	[cm]	**4.8** (± 0.1)	**4.6** (± 0.1)	**3.7** (± 0.2)	**3.6** (± 0.2)	**3.6**	0.2
Wet weight, males	[g]	**0.886** (± 0.090)	**0.796** (± 0.104)	**0.414** (± 0.026)	**0.434** (± 0.014)	**0.423**	0.049
Wet weight, females	[g]	**1.086** (± 0.107)	**0.922** (± 0.084)	**0.554** (± 0.086)	**0.481** (± 0.056)	**0.517**	0.055
Sex ratio, %males	[%]	**62.8** (± 15.0)	**30.1** (± 12.1)	**69.3** (± 18.4)	**66.0** (± 23.1)	**42.9**	15.2
Sex ratio, %females	[%]	**33.3** (± 13.4)	**68.6** (± 13.0)	**30.7** (± 18.4)	**34.0** (± 23.1)	**56.5**	14.2
Sex ratio, %undiff	[%]	**3.9** (± 7.9)	**1.3** (± 2.6)	**0.0** (± 0.0)	**0.0** (± 0.0)	**0.6**	1.3
F2 generation Early life stages							
Hatching success	[%]	**100.0** (± 0.0)	**100.0** (± 0.0)	**78.8** (± 14.4)	**98.8** (± 2.5)	**89.4**	7.4
Biomarker							
F0, VTG males	[ng/mL]	**7.76E+04** (± 1.03E+05)	**6.33E+03** (± 7.24E+03)	**9.63E+02** (± 3.09E+02)	**1.10E+03** (± 5.69E+02)	**8.45E+01**	3.83E+01
F0, VTG females	[ng/mL]	**6.79E+07** (± 4.60E+07)	**3.40E+07** (± 1.84E+07)	**2.49E+06** (± 2.41E+06)	**2.74E+06** (± 2.78E+06)	**3.03E+07**	1.71E+07
F1, VTG males	[ng/mL]	**5.21E+03** (± 1.66E+03)	**5.45E+04** (± 7.18E+04)	**9.24E+01** (± 6.55E+01)	**2.05E+02** (± 1.62E+02)	**1.38E+02**	1.29E+02
F1, VTG females	[ng/mL]	**4.46E+07** (± 9.1E+06)	**2.82E+07** (± 2.75E+07)	**3.16E+05** (± 1.69E+05)	**8.23E+05** (± 6.74E+05)	**1.82E+07**	6.74E+06

SD: Standard deviation

DWC: Dilution water control.

4.2.3 Comparison with available industry studies

To extend the validation data base, especially to allow an evaluation of the protocol specific variables, additional control data sets were identified from contracted ZEOGRT studies. In total, three studies were considered, i.e. presented as studies 8, 9 and 10. Notably, all three studies were conducted with the same strain of zebrafish, i.e. the "West Aquarium"-strain as described in chapter 2.1.1. The data were transferred from the available study reports. Although following the given ZEOGRT protocol, the data presentation can differ due to the underlying procedures and methods. The units are presented as originally recorded, see Table 99 for details.

The control data obtained from studies 9 and 10 are quite close to the mean control values distilled from validation phase I. This refers e.g. to the reproduction endpoints egg numbers and fertilization rates for both F0 and F1. Also, the growth parameters in terms of length and weight measurements for the different life stages showed good compliance. The biological performance regarding hatching and life-stage specific survival was in line with the defined acceptance criteria.

The comparison of VTG concentrations was impeded due to differences in the data sets. Within study 8, VTG concentrations were measured from body homogenates, while VTG levels out of studies 9 and 10 originated from blood samples. However, in study 9, the VTG results were related to total protein and VTG in study 10 was only related to the blood volume. Nevertheless, it is transparent that there are sex-specific differences in the mean value of several orders of magnitude, fulfilling a basic requirement for VTG evaluation.

The control parameters for study 8, however, are characterized by lower growth performance. For example, both length and weight of the F0 do not show sex-specific differences. Compared with the phase I data, especially the female size seems retarded. As a secondary effect, the egg numbers were also lower compared to the other studies. This observation was also made for the F1 with the same range of sizes and reproduction output. However, the fertilization success did not show any limitations.

All data from the additional studies is presented in Table 102.

Table 102: ZEOGRT validation studies: Comparison of control data, mean value ± SD

Endpoint	Study 8	Study 9	Study 10	Mean ± SD phase I
F0 generation				
Egg number per day and female	**21** (+/- 29.9 %)	**69** (+/- 10)	**55** (+/- 7)	**62** (+/- 10)
Fertilization rate	**92.2 %**	**93.3** (+/- 2.6)	**92.3** (+/- 1.3)	**87.7 %** (+/- 3.6)
Total length, males	**3.7 cm**	**38** mm (+/- 1)	**38** mm (+/- 1)	**3.8 cm** (+/- 0.1)
Total length, females	**3.5 cm**	**39** mm (+/- 1)	**40** mm (+/- 2)	**4.0 cm** (+/- 0.1)
Wet weight, males	**443** mg	**0.463 g** (+/- 0.037)	**0.447 g** (+/- 0.018)	**0.474 g** (+/- 0.023)
Wet weight, females	**415** mg	**0.615 g** (+/- 0.063)	**0.639 g** (+/- 0.067)	**0.701 g** (+/- 0.010)

Endpoint	Study 8	Study 9	Study 10	Mean ± SD phase I
Sex ratio, %males	-	-	**51.4 %** (+/- 2.8)	-
Sex ratio, %females	-	-	**48.6 %** (+/- 2.8)	-
F1 generation Early life stages				
Hatching success	**99 %**	**98.6 %** (+/- 2.8)	**100 %** (+/- 0.0)	**97.6 %** (+/- 4.0)
Post-hatch survival rate, day 21 pf	-	**89.4 %** (+/- 9.2)	**86.8 %** (+/- 11.6)	**91.9 %** (+/- 4.2)
Post-hatch survival rate, day 35 pf	**83 %**	**88.7 %** (+/- 8.2)	**81.9 %** (+/- 13.1)	**89.4 %** (+/- 4.7)
Total length, day 35 pf	**1.7 cm** (Std. length) (+/- 3.2 %)	**17 mm** (+/- 1)	**18 mm** (+/- 1)	**1.8 cm** (+/- 0.0)
F1 generation Juveniles				
Survival, day 63 pf	**100 %**	**100 %** (+/- 0)	**100 %** (+/- 0)	**99.4 %** (+/- 0.7)
Total length, day 63 pf	**2.7 cm** (Std. length) (+/- 1.4 %)	**30 mm** (+/- 1)	**30 mm** (+/- 1)	**3.0 cm** (+/- 0.1)
F1 generation Reproduction				
Time to first spawn	-	**day 62 - 69**	**day 58 - 60**	-
Egg number per day and female	**14** (+/- 12.2 %)	**22** (+/- 7)	**31** (+/- 13)	**26** (+/- 6)
Fertilization rate	**91.4 %**	**94.5 %** (+/- 1.1)	**91.3 %** (+/- 2.0)	**85.8 %** (+/- 4.0)
F1 generation Adult phase				
Survival	**100 %**	-	-	**99.7 %** (+/- 0.6)
Total length, males	**3.7 cm** (+/- 2.4 %)	**38 mm** (+/- 1)	**34 mm** (+/- 2)	**3.7 cm** (+/- 0.2)
Total length, females	**3.6 cm** (+/- 0.5 %)	**38 mm** (+/- 0)	**35 mm** (+/- 2)	**3.6 cm** (+/- 0.2)
Wet weight, males	**403** mg	**0.434 g** (+/- 0.044)	**0.355 g** (+/- 0.045)	**0.423 g** (+/- 0.049)
Wet weight, females	**449** mg	**0.524 g** (+/- 0.052)	**0.422 g** (+/- 0.067)	**0.517 g** (+/- 0.055)

Endpoint	Study 8	Study 9	Study 10	Mean ± SD phase I
Sex ratio, %males	**40.7 %**	**31.3 %** (+/- 11.1)	**50.5 %** (+/- 19.2)	**42.9 %** (+/- 15.2)
Sex ratio, %females	-	**68.8 %** (+/- 11.1)	**49.5 %** (+/- 19.2)	**56.5 %** (+/- 14.2)
Sex ratio, %undiff.	-	-	-	**0.6 %** (+/- 1.3)
F2 generation Early life stage				
Hatching success	-	**95.0 %** (+/- 7.1)	-	**89.4 %** (+/- 7.4)
Biomarker				
F0, VTG males	-	**0.60 ng/µg** (+/- 0.36)	**48.3 ng/mL** (+/- 11.3)	**8.45E+01 ng/mL** (+/-3.83E+01)
F0, VTG females	-	**1752.4 ng/µg** (+/- 173.5)	**9.07E+05 ng/mL** (+/- 3.00E+05)	**3.03E+07 ng/mL** (+/-1.71E+07)
F1, VTG males	**14.2 ng/mg** 1)	**0.57 ng/µg** (+/- 0.25)	**38.5 ng/mL** (+/- 19.9)	**1.38E+02 ng/mL** (+/-1.29E+02)
F1, VTG females	**33475 ng/mg** 1)	**1870.1 ng/µg** (+/- 1160.8)	**8.61E+05 ng/mL** (+/- 6.02E+05)	**1.82E+07 ng/mL** (+/-6.74E+06)

Remark: 1) VTG was measured from tissue homogenates, median values.
Std. length: Standard length.
Study 8 presented Standard deviation as [%].
Additional remark: The data were transferred from the available study reports. Although following the given ZEOGRT protocol, the data presentation can differ due to the underlying procedures and methods. The units are presented as originally recorded.

4.3 Comparison of effect concentrations (NOEC/LOEC)

4.3.1 Studies with Tamoxifen-Citrate (TMX)

The available data allows the comparison of two studies conducted with Tamoxifen-citrate in the same range of test concentrations. For both studies, a stable application of the test chemical was confirmed. For **F0 generation** a sensitive effect on reproduction was observed in the study conducted in phase I of the validation. However, no effect on F0 reproduction was observed in the corresponding study of phase II.

During the **F1 generation**, both studies showed corresponding effects on the early life stage performance. One study displayed already a reduced hatching success, but for both studies, the post hatch survival rate was found to be reduced at the same concentration range. In study 1 (phase I), an effect on survival was also present in the juvenile phase.

Furthermore, egg numbers were significantly reduced in both studies. However, a concentration dependent delay for the start of first spawning as well as a reduced fertilization capacity was only found in one of the two studies.

The growth performance of the adult fish was characterized differently in both studies. While remaining females expressed enhanced growth in study 1, the males within study 5 (phase II) were retarded in growth. However, both studies clearly identified a sex ratio shift towards an increased number of male fish in the exposed groups. These observations were concentration dependent, and a 100% shift towards males was detected. In the further course, the hatching success in the **F2 generation** was found to be reduced in study 1, but no effect was found in the corresponding study 5.

Corresponding results were identified regarding the measurement of VTG. For both studies, the VTG concentrations for F0 females were significantly reduced. For F1, the decrease of female VTG was confirmed in the study 1.

The histopathological analysis of fish gonads was only meaningful for study 1, which identified a concentration dependent increase of maturity stages of both males and female gonads.

All effect concentrations are summarized in Table 103.

Table 103: ZEOGRT validation studies: Comparison of nominal effect concentrations (NOEC/LOEC): ZEOGRT with Tamoxifen-Citrate (TMX)

Endpoint	Study 1 (phase I) [µg/L]	Remark	Study 5 (phase II) [µg/L]	Remark
	NOEC/LOEC		**NOEC/LOEC**	
F0 generation				
Egg number per day and female	**2.0 / 6.3**	decrease	≥26.3 / >26.3	
Fertilization rate	≥20 / >20	-	≥26.3 / >26.3	
Total length, males	≥20 / >20	-	≥26.3 / >26.3	
Total length, females	≥20 / >20	-	**8.3 / 26.3**	increase
Wet weight, males	≥20 / >20	-	≥26.3 / >26.3	
Wet weight, females	≥20 / >20	-	≥26.3 / >26.3	

Endpoint	Study 1 (phase I) [µg/L]	Remark	Study 5 (phase II) [µg/L]	Remark
	NOEC/LOEC		**NOEC/LOEC**	
Sex ratio, %males	≥20 / >20	Confirmation of correct group setting	≥26.3 / >26.3	Confirmation of correct group setting
Sex ratio, %females	≥20 / >20	Confirmation of correct group setting	≥26.3 / >26.3	Confirmation of correct group setting
F1 generation, Early life stages				
Hatching success	≥20 / >20	-	**8.3 / 26.3**	decrease
Post-hatch survival rate, day 21 pf	**0.2 / 0.63**	decrease	**2.92 / 8.3**	decrease
Post-hatch survival rate, day 35 pf	**2.0 / 6.3**	decrease (no larvae survived at 20 µg/L)	**2.92 / 8.3**	decrease (no larvae survived at 26.3 µg/L)
Total length, day 35 pf	**0.63 / 2.0**	decrease	≥8.3 / >8.3	no larvae survived at 26.3 µg/L
F1 generation Juveniles				
Survival, day 63 pf	**2.0 / 6.3**	decrease (no fish survived at 20 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L
Total length, day 63 pf	≥ 6.3 / >6.3	no larvae survived at 20 µg/L	≥8.3 / >8.3	no fish survived at 26.3 µg/L
F1 generation Reproduction				
Time to first spawn	≥ 6.3 / >6.3	no fish survived at 20 µg/L; only males at 6.3 µg/L	**0.29 /0.92**	increase (delay) (no fish survived at 26.3 µg/L)
Egg number per day and female	**0.20 / 0.63**	decrease (no fish survived at 20 µg/L; only males at 6.3 µg/L)	**2.92 / 8.3**	decrease (no fish survived at 26.3 µg/L)
Fertilization rate	≥2.0 / >2.0	only male fish at 6.3 µg/L	**2.92 / 8.3**	decrease (no fish survived at 26.3 µg/L)

Endpoint	Study 1 (phase I) [µg/L]	Remark	Study 5 (phase II) [µg/L]	Remark
	NOEC/LOEC		**NOEC/LOEC**	
F1 generation Adult phase				
Survival	≥6.3 / >6.3	no fish survived at 20 µg/L	≥8.3 / >8.3	no fish survived at 26.3 µg/L
Total length, males	≥6.3 / >6.3	no fish survived at 20 µg/L	**<0.29 / ≤0.29**	decrease (no fish survived at 26.3 µg/L)
Total length, females	**0.20 / 0.63**	increase (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L
Wet weight, males	≥6.3 / >6.3	no fish survived at 20 µg/L	**<0.29 / ≤0.29**	decrease (no fish survived at 26.3 µg/L)
Wet weight, females	**0.20 / 0.63**	increase (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L
Sex ratio, %males	**0.63 / 2.0**	increase (only male fish at 6.3 µg/L)	**2.92 / 8.3**	increase (no fish survived at 26.3 µg/L)
F2 generation Early life stages				
Hatching success	**0.20 / 0.63**	decrease (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L
Biomarker				
F0, VTG males	≥20 / >20		≥26.3 / >26.3	-
F0, VTG females	**2.0 / 6.3**	decrease	**0.29 / 0.92**	decrease
F1, VTG males	≥6.3 / >6.3	no fish survived at 20 µg/L	≥8.3 / >8.3	no fish survived at 26.3 µg/L
F1, VTG females	**0.20 / 0.63**	decrease (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L

Endpoint	Study 1 (phase I) [µg/L]	Remark	Study 5 (phase II) [µg/L]	Remark
	NOEC/LOEC		**NOEC/LOEC**	
Histopathology				
F0, maturity stages, male testis	**2.0 / 6.3**	increase (towards stage 3)	≥26.3 / >26.3	only trend for increase
F0, maturity stages, female ovaries	**2.0 / 6.3**	increase (towards stage 3)	≥26.3 / >26.3	-
F1, maturity stages, male testis	**0.20 / 0.63**	increase (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L
F1, maturity stages, female ovaries	**0.63 / 2.0**	Increase (no fish survived at 20 µg/L; only males at 6.3 µg/L)	≥8.3 / >8.3	no fish survived at 26.3 µg/L

4.3.2 Studies with Prochloraz (PRO)

During the validation project, three studies were conducted with Prochloraz in the same range of test concentrations. For all studies, a stable application of the test chemical was confirmed. For **F0 generation,** a sensitive effect on reproduction was only observed in one study conducted in phase II of the validation. However, no effect on F0 reproduction was observed in the corresponding studies of phase I (study 2) and phase II (study 7). Notably, both studies conducted in phase II revealed increasing growth performance for the adult F0 fish.

The early life stages of the **F1 generation** were found to be affected in all three studies. In one study, the hatching rate was already reduced, but most prominent was a decreased post hatch survival. Although the effect was identified in each study, the related effect concentrations differed. Two of the studies displayed an additional growth retardation of the fish larvae, in terms of a reduced length. The juvenile stages were not impacted in any of the studies.

No effect on F1 reproduction was recorded in any of the studies. Furthermore, with exception of an increased male length in study 2, no growth effects on the adult fish occurred. The most prominent effect derived consistently in all studies was a significant shift in sex ratio towards an increased number of male fish. At the top concentration, 100 % male fish were identified in all three studies.

The final evaluation of the **F2 generation** revealed no significant effect on hatching in all available studies.

The measurement of VTG revealed a significant decrease of VTG concentrations for both F0 and F1 for two of the studies. In the third study, no effect was detected. Interestingly, in that study increased male gonad maturity was identified, while in the other studies no effects on the fish gonads were found.

All effect concentrations are summarized in Table 104.

Table 104: ZEOGRT validation studies: Comparison of effect concentrations (NOEC/LOEC): ZEOGRT with Prochloraz (PRO)

Endpoint	Study 2 (phase I) [µg/L]	Remark	Study 6 (phase II) [µg/L]	Remark	Study 7 (phase II) [µg/L]	Remark
F0 generation						
Egg number per day and female	≥320 / >320	-	**32 / 100**	decrease	≥320 / >320	
Fertilization rate	≥320 / >320	-	**100 / 320**	decrease	≥320 / >320	
Total length, males	≥320 / >320	-	**100 / 320**	increase	≥320 / >320	
Total length, females	≥320 / >320	-	**100 / 320**	increase	**100 / 320**	increase
Wet weight, males	≥320 / >320	-	**100 / 320**	increase	**100 / 320**	increase
Wet weight, females	≥320 / >320	-	**100 / 320**	increase	**100 / 320**	increase

Endpoint	Study 2 (phase I) [µg/L]	Remark	Study 6 (phase II) [µg/L]	Remark	Study 7 (phase II) [µg/L]	Remark
F1 generation Early life stages						
Hatching success	≥320 / >320	-	**32 / 100**	decrease	≥320 / >320	
Post-hatch survival rate, day 21 pf	**<3.2 / ≤3.2**	decrease	**32 / 100**	decrease	**100 / 320**	decrease
Post-hatch survival rate, day 35 pf	**<3.2 / ≤3.2**	decrease	**32 / 100**	decrease	**100 / 320**	decrease
Total length, day 35 pf	**100 / 320**	decrease	≥320 / >320		**100 / 320**	decrease
F1 generation Juveniles						
Survival, day 63 pf	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
Total length, day 63 pf	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
F1 generation Reproduction						
Time to first spawn	≥100 / >100	(spawning start was unintentio-nally missed)	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)
Egg number per day and female	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)
Fertilization rate	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)
F1 generation Adult phase						
Survival	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
Total length, males	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
Total length, females	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)

Endpoint	Study 2 (phase I) [µg/L]	Remark	Study 6 (phase II) [µg/L]	Remark	Study 7 (phase II) [µg/L]	Remark
Wet weight, males	**3.2 / 10**	increase	≥320 / >320	-	≥320 / >320	-
Wet weight, females	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)	≥100 / >100	(no females at the top concentrati on)
Sex ratio, %males	**100 / 320**	increase (100% males at the top concen-tration)	**3.2 / 10**	increase (100% males at the top concen-tration)	**100 / 320**	increase (100% males at the top concen-tration)
F2 generation Early life stages						
Hatching success	≥100 / >100	-	≥100 / >100	-	≥100 / >100	-
Biomarker						
F0, VTG males	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
F0, VTG females	**100 / 320**	decrease	**32 / 100**	decrease	≥320 / >320	-
F1, VTG males	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
F1, VTG females	**32 / 100**	decrease (no females at the top concen-tration)	**32 / 100**	decrease (no females at the top concen-tration)	≥100 / >100	(no females at the top concen-tration)
Histopathology						
F0, maturity stages, male testis	≥320 / >320	-	≥320 / >320	-	**100 / 320**	increase
F0, maturity stages, female ovaries	≥320 / >320	-	≥320 / >320	-	≥320 / >320	-
F1, maturity stages, male testis	≥320 / >320	-	≥320 / >320	-	**100 / 320**	Increase
F1, maturity stages, female ovaries	≥320 / >320	-	≥100 / >100	-	≥320 / >320	

4.4 Comparison with scientific literature

4.4.1 Effects of Tamoxifen-Citrate in long term fish studies

Tamoxifen was designed to act as a selective estrogen receptor modulator. Results from the literature show that it can act as an agonist or an antagonist, depending on the fish species exposed.

Williams et al (2007) investigated the effects of tamoxifen-citrate to fathead minnows (*Pimephales promelas*) in a Partial Life Cycle Test (PLC) at measured concentrations of 0.11, 0.41, 1.65, 5.97, 18.2 µg tamoxifen-citrate/L and a Full Life Cycle Test (FLCT) at measured concentrations of 0.01, 0.07, 0.61, 4.08 µg/L. An effect on reproduction in terms of reduced egg numbers was detected at 18.2 µg/L (NOEC: 5.97 µg/L). The growth parameters, especially in the early stages of life, were also significantly reduced in the PLC (NOEC: 1.65 µg/L). In both studies, fertilization rates and sex ratios were not assessed.

In the FLCT, the effect on growth was unclear. The measurement of vitellogenin in whole- body homogenates revealed a dose-dependent reduction (NOEC: 0.11 µg/L), but the analysis of blood plasma samples could not confirm this finding (Williams et al., 2007).

In a study with Medaka of Sun et al. (2007), animals in the early stages and the reproductive phase were exposed to tamoxifen. There was a delay in hatch and a reduced hatching rate at 125 und 625 µg/L (NOEC: 25 µg/L). The fertilization rate and egg counts were significantly reduced in the highest test concentration (NOEC: 125 µg/L). The measurement of vitellogenin showed a significant increase in males from the first test concentration (NOEC: < 1 ng/L). In females, VTG concentrations were significantly reduced (NOEC: 5 µg/L). In contrast to the Fathead minnow study, an anti-estrogenic pattern of effects was clearly visible (Sun et al., 2007). Of the parental groups, eggs were collected and kept for hatching. An effect on hatching success was only observed at 625 µg/L.

The analysis of the genetic sex of the embryos revealed an increase of male fish already at the low-test levels (NOEC: 5 µg/L) (Sun et al., 2007).

In a zebrafish PLC with tamoxifen (nominal: 3.2; 10; 32; 100; 320 µg/L), van der Ven et al. (2007) identified the hatching rate and growth of the F1 generation as the most sensitive endpoints with population relevance (NOEC: < 32 µg/L). The histopathological examination of the gonads showed an increase in Leydig cells in male gonads. This endpoint was less sensitive by a concentration level factor than the above endpoints (NOEC: 32 µg/L).

In studies published in Knacker et al. (2010), the effect of tamoxifen citrate on zebrafish was investigated in a two-generation test. The sex ratio of the F1 generation was detected to be the most sensitive endpoint with population relevance. A prominent shift towards an increased number of males was observed with a NOEC of 0.22 µg/L (LOEC: 0.77 µg/L) which is below the effect thresholds for the reproduction parameters. The fertilization rate of the parental animals was less sensitive (NOEC 4.0 µg/L). Vitellogenin (VTG) reacted in the same range of concentration as the sex ratio, an increase of VTG concentrations in males of the F1 generation was statistically significant at 0.77 µg/L.

The VTG concentration in females of the parental generation proved to be more sensitive than the fertilization rate (NOEC: 1.2 µg/L). A decrease of VTG concentration was also observed for the F1 generation, but this was considered as a trend.

Hatching success and early life stage survival was significantly reduced at 11 µg/L (NOEC: 2.7 µg/L).

In the presented ZEOGRT data from study 1 (phase I), most of the findings reported in Knacker et al. (2010) could be confirmed at the same level of sensitivity.

Reproduction in terms of egg numbers was found to be significantly reduced at 0.63 µg/L for both the parental and the filial generation (NOEC: 0.20 µg/L). The corresponding study of phase II displayed a decreased F1 fertilization rate (NOEC: 2.92 µg/L) and thus highly comparable to the results of the published study.

The early life and juvenile stages recorded in the ZEOGRT were equally sensitive to the reproduction endpoints (NOEC: 1.9 µg/L for post hatch survival) or more sensitive (NOEC: 0.59 µg/L for growth after 35 days pf).

The publication of Flynn et al. (2017) summarized results from nine available Medaka EOGRT studies. In a study with tamoxifen-citrate at mean measured concentrations of 1.32, 2.51, 5.08, 10.17 and 20.44 µg/L, hatch and fertilization success were identified to be sensitive, with LOEC of 2.51 and 10.17 µg/L, respectively. An increased expression of male specific secondary sex characteristics was observed at the same effect concentration (LOEC: 10.17 µg tamoxifen-citrate/L). Fecundity as well as the growth parameters were significantly declined at 20.44 µg/L. The vitellogenin concentration in females were significantly reduced already at 1.32 µg/L.

Compared to the ZEOGRT results from the present study it seems, that medaka showed similar sensitivity towards the exposure to tamoxifen-citrate. Although fecundity of zebrafish was reduced already at 6.5 µg/L (LOEC, vs. 20.44 µg/L in the MEOGRT), fertilization did not respond in the zebrafish test (phase I test), but was significantly reduced in the MEOGRT at 10.17 µg/L.

However, as not assessed in the MEOGRT, a comparison of the effect level on sex ratio is not possible. This was the most sensitive endpoint with population relevance in the zebrafish test. The increased expression of male phenotypic secondary sex characteristics (SSCs) in the medaka test at 10.17 µg/L underlines at least a hint to the anti-estrogenic potential of the substance.

In both studies vitellogenin responded very sensitive and was significantly reduced at <1.32 µg/L and 0.59 µg/L in MEOGRT and ZEOGRT, respectively.

4.4.2 Effects of Prochloraz in long term fish studies

A Fish Sexual Development Test (FSDT) with prochloraz (performed bei Kinnberg et al., 2007) identified the sex ratio as the most sensitive population-relevant endpoint in zebrafish. A shift towards a complete male group was observed at 202 µg/L (NOEC: 64 µg/L). An increase in VTG concentrations in males has already been observed at 16 and 64 µg/L of prochloraz and was thus more sensitive (NOEC: <16 µg/L). In contrast, the exposure to 202 µg/L resulted in a decrease in VTG in both male and female animals.

In another FSDT with prochloraz, which was conducted with both, zebrafish and fathead minnows at three test concentrations (32, 100 and 320 µg/L, nominal), a significantly reduced number of females was found in 100 µg/L (zebrafish) and in 320 µg/L (fathead minnow) (Thorpe et al., 2011). A decrease in VTG concentrations in the blood plasma was observed for both fish species. In the case of zebrafish, the decrease was significant at 320 µg/L, and for the fathead minnows a concentration level below (LOEC: 100 µg/L; NOEC: 32 µg/L). The exposed zebrafish also showed an increased incidence of ovotestis structures at 100 µg/L (NOEC: 32 µg/L). The exposed fathead minnows showed an increased proportion of undifferentiated gonads at 320 µg/L (NOEC: 100 µg/L).

In fish sexual development tests performed by Holbech et al. (2012) effects of prochloraz on both zebrafish and fathead minnows were assessed. A shift towards an increased number of male fish was found at 134 µg/L and 293 µg/L for zebrafish and fathead minnow, respectively.

The measurement of VTG concentrations revealed a decline in females at 110 µg/L and 68 µg/L for zebrafish and fathead minnow, respectively. In conclusion, both species were identified to have similar sensitivity regarding the assessed parameters.

In a MEOGRT with prochloraz presented by Flynn et al. (2017), hatch, early life stage survival and fecundity were affected at the same level of sensitivity (LOEC: 25 µg/L). The growth parameters length and weight as well as the expression of SSC and a decrease of vitellogenin concentrations of females were by two concentration steps more sensitive (LOEC: 9.2 µg/L). No information on sex ratio impacts was presented.

In the present ZEOGRT studies, the shift in the sex ratios of the exposed zebrafish groups towards males was again the most prominent endpoint with population relevance. However, the studies expressed different sensitivities. For studies 2 and 7, a LOEC of 320 µg/L (nominal concentration) was found, while in study 6 a significant difference was already detected at a LOEC of 10 µg/L (nominal concentration). This was the only study detecting an impact on reproduction, i.e. reduced egg numbers (LOEC: 100 µg/L) and fertilisation rates (LOEC: 320 µg/L) for the parental fish (F0). A decrease of female VTG levels was induced at the same effect level (LOEC: 100 µg/L, nominal concentration). Finally, the comparison of MEOGRT and ZEOGRT data revealed a higher sensitivity of the medaka test. However, important information on the sex ratio shift is missing.

5 List of references

All genetics (2017): Report on fish strain identification by DNA barcoding. Project ID C08P01. February 06, 2017. AllGenetics Biology, SL; Coruna Spain

Armitage, P (1955). "Tests for Linear Trends in Proportions and Frequencies". Biometrics. 11 (3). International Biometric Society: 375–386.

Colborn, Theo, Frederick S. Vom Saal and Ana M. Soto. "Developmental effects of endocrine-disrupting chemicals in wildlife and humans." Environmental health perspectives 101.5 (1993): 378-384.

Dunnett, C. W. (1955). "A multiple comparison procedure for comparing several treatments with a control." Journal of the American Statistical Association 50(272): 1096-1121.

Dunnett, C. W. (1964). "New tables for multiple comparisons with a control." Biometrics 20(3): 482-491.

EC (2000). "European Commission, Directorate General Health and Consumer Protection: SANCO/3029/99 rev.4 (11/07/2000), Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414.".

EEC (2010). "European Union. 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. Official Journal of the European Union L276/33."

Flynn K, Lothenbach D, Whiteman F, Hammermeister D, Touart LW, Swintek J, Tatarazako N, Onishi Y, Iguchi T, Johnson R. 2017. Summary of the development the US Environmental Protection Agency's Medaka Extended One Generation Reproduction Test (MEOGRT) using data from 9 multigenerational medaka tests. Environ Toxicol Chem. 36(12):3387–3403.

Holbech, H., L. Andersen, G. I. Petersen, B. Korsgaard, K. L. Pedersen and P. Bjerregaard (2001). "Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*)." Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 130(1): 119-131.

Holbech H, Kinnberg KL, Brande-Lavridsen N, Bjerregaard P, Petersen GI, Norrgren L, Orn S, Braunbeck T, Baumann L, Bomke C, et al. 2012. Comparison of zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) as test species in the Fish Sexual Development Test (FSDT). Comp Biochem Physiol C Toxicol Pharmacol. 155(2):407–15.

Jonckheere, A. (1954). "A test of significance for the relation between m rankings and k ranked categories." British Journal of Statistical Psychology.

Kinnberg K, Holbech H, Petersen GI, Bjerregaard P. 2007. Effects of the fungicide prochloraz on the sexual development of zebrafish (*Danio rerio*). Comp Biochem Physiol C Toxicol Pharmacol. 145:165–170.

Knacker, T., Boettcher, M., Frische, T., Rufli, H., Stolzenberg, H.C., Teigeler, M., Zok, S., Braunbeck, T., Schaefers, C., 2010. Environmental effect assessment for sexual endocrine - disrupting chemicals: Fish testing strategy. Integr. Environ. Assess. Manag. 6, 653–662.

Länge, R., T. H. Hutchinson, C. P. Croudace, F. Siegmund, H. Schweinfurth, P. Hampe, G. H. Panter and J. P. Sumpter (2001). "Effects of the synthetic estrogen 17α-ethinylestradiol on the life-cycle of the fathead minnow (Pimephales promelas)." Environmental Toxicology and Chemistry 20(6): 1216-1227.

Maack, G. and H. Segner (2003). "Morphological development of the gonads in zebrafish." Journal of fish biology 62(4): 895-906.

Nagel, R. (1986). "Untersuchungen zur Eiproduktion beim Zebrabärbling (Brachydanio rerio, Ham.-Buch.)." Journal of Applied Ichthyology 2(4): 173-181.

Nagel, R. (1998). Der vollständige Life Cycle Test (Complete Life Cycle Test, CLC Test) mit dem Zebrabärbling (Danio rerio, vormals Brachydanio rerio), Entwurf. UBA-Texte 58/98, 166-175.

OECD (2006). "Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application)." Environmental Health and Safety Publications Series on Testing and Assessment No.54, OECD, Paris.

OECD (2008). "Detailed review paper on fish life-cycle tests. OECD Environmental Health and Safety Publications Series on Testing and Assessment No. 95 (ENV/JM/MONO (2008) 22). Paris, France."

OECD (2009). "Test No. 230: 21-day Fish Assay: A Short-Term Screening for Oestrogenic and Androgenic Activity, and Aromatase Inhibition." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2010). "Guidance document on the diagnosis of endocrine-related histopathology in fish gonads. Series on testing and Assessment No. 123." ENV/JM/MONO(2010)14.

OECD (2011). "Test No. 234: Fish Sexual Development Test." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2012). "Test No. 229: Fish Short Term Reproduction Assay." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2013). "Test No. 210: Fish, Early-life Stage Toxicity Test." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2013). "Test No. 236: Fish Embryo Acute Toxicity (FET) Test." OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

OECD (2023). “Test No. 240: Medaka Extended One Generation Reproduction Test (MEOGRT)”. OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Rao, J.N.K. and A.J. Scott (1992). "A simple method for the analysis of clustered binary data." Biometrics 48, 577-585.

Rao, J.N.K. and A.J. Scott (1999). "A simple method for analyzing overdispersion in clustered Poisson data." Statistics in Medicine 18, 1373-1385.

Sumpter, John P. "Feminized responses in fish to environmental estrogens." Toxicology letters 82 (1995): 737-742.

Sun, L., Zha, J., Spear, P.A., Wang, Z., 2007. Tamoxifen effects on the early life stages and reproduction of Japanese medaka (*Oryzias latipes*). Environ. Toxicol. Pharmacol. 24, 23–29.

Takahashi, H. (1977). "Juvenile hermaphroditism in the zebrafish, *Brachydanio rerio*." Bulletin of the Faculty of Fisheries Hokkaido University 28(2): 57-65.

Teigeler, M., K. Franzen, E. Eilebrecht, D. Schaudien and C. Schäfers (2024). “New-Generation Steroid Hormones, Tailored Assessment Strategies for Environmental Protection.” Project No. (FKZ) 3717 67 405 0. Texte | 57/2024. German Environment Agency.

Terpstra, T. J. (1952). "The asymptotic normality and consistency of Kendall’s test against trend, when ties are present in one ranking." Indagationes Mathematicae 14(3): 327-333.

Thorpe KL, Marca Pereira ML, Schiffer H, Burkhardt-Holm P, Weber K, Wheeler JR. 2011. Mode of sexual differentiation and its influence on the relative sensitivity of the fathead minnow and zebrafish in the fish sexual development test. Aquat Toxicol. 105:412–420.

van der Ven, L.T.M., van den Brandhof, E.-J., Vos, J.H., Wester, P.W., 2007. Effects of the estrogen agonist 17beta-estradiol and antagonist tamoxifen in a partial life-cycle assay with zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 26, 92–99.

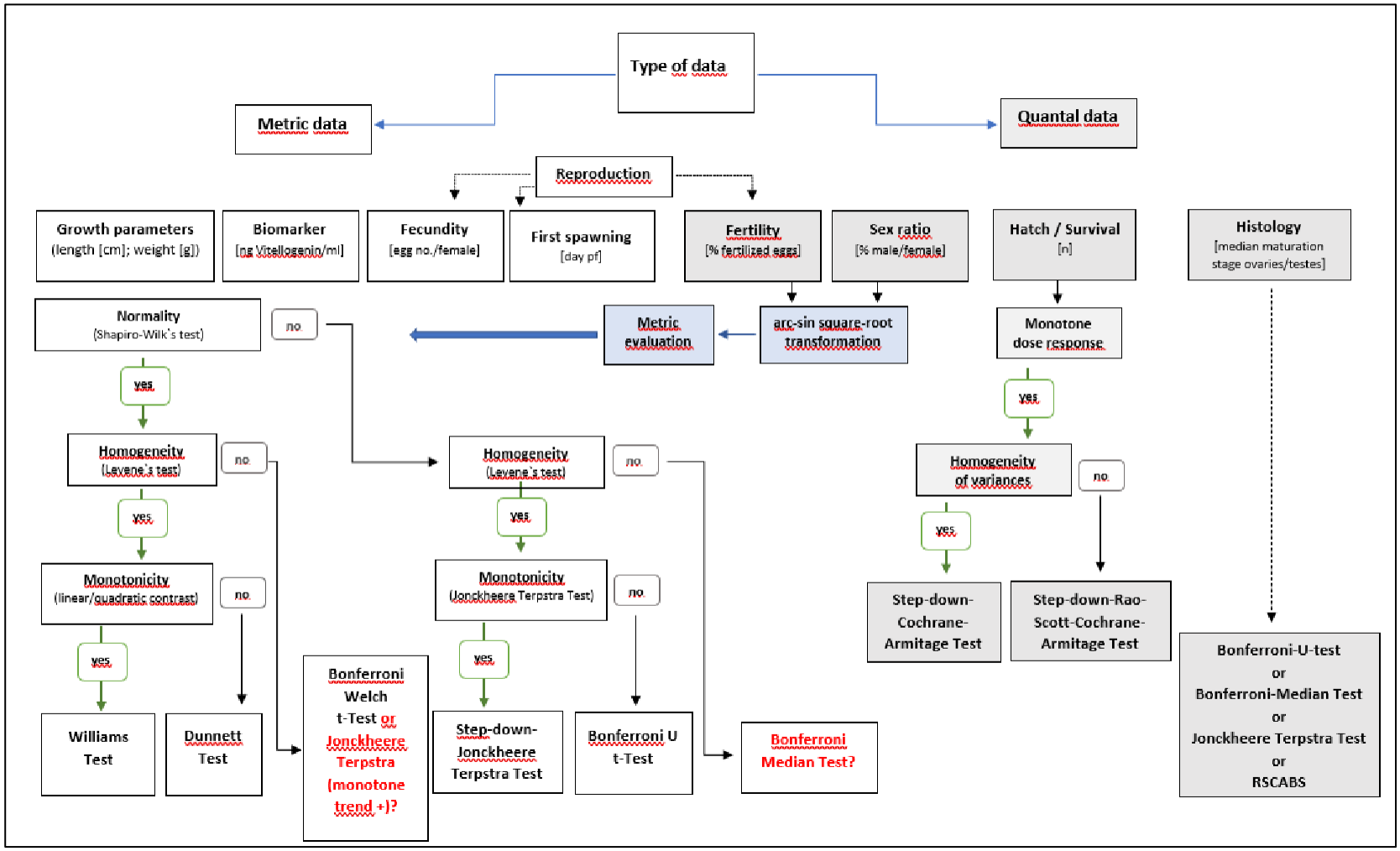
Williams, D. (1971). "A test for differences between treatment means when several dose levels are compared with a zero-dose control." Biometrics: 103-117.

Williams, D. (1972). "The comparison of several dose levels with a zero-dose control." Biometrics: 519-531.

Williams, T.D., Caunter, J.E., Lillicrap, A.D., Hutchinson, T.H., Gillings, E.G., Duffell, S., 2007. Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (*Pimephales promelas*). Environ. Toxicol. Chem. 26, 695 – 707.

6 Annex

6.1 Statistical flow chart



Source: Own illustration, Fraunhofer IME

6.2 Draft Test Guideline

DRAFT Standard operating procedure

Zebrafish extended one generation reproduction test (ZEOGRT)

Author

Dr. Matthias Teigeler

Fraunhofer Institute for
Molecular Biology and Applied Ecology (IME)
Auf dem Aberg 1
57392 Schmallenberg
Germany

November XX, 2023

Version 2.1

Contents page

1 Introduction

Aims

1. This SOP describes a test procedure to assess the effects of prolonged exposure to chemicals on one generation of zebrafish including adult parental fish, a first filial generation from embryonal to adult stage and finally of the embryonal and early larval stage of a second filial generation.

2. The data obtained from this study can be used to assess the endocrine potential of test substances in the sense of Level 5 of the OECD Conceptual framework to detect endocrine active substances [1].

3. Biological endpoints to be assessed are hatch, survival and growth of different life stages, sex ratio, time to first spawning, egg numbers, fertilization rates. To address the potential link to endocrine disrupting mechanisms, additional evaluation should be included, e.g. the measurement of biomarkers (i.e. vitellogenin) and histopathology of fish gonads and further target organs, if relevant.

Principle of the test

4. The objective of this study is the assessment of the effects of continuous exposure to the test item on different life stages and life performances of zebrafish (*Danio rerio*) during a life cycle. The primary focus is on assessment of the impacts on population relevant endpoints. The results obtained can be either used for environmental risk and hazard assessment, with main focus on endocrine acting substances.
This list of relevant life stages includes the spawning period of the parental generation (F_0), early life stages, juvenile growth, sexual maturation, and reproduction of the first filial generation (F_1-generation), and the early embryonal stage of the second filial generation (F_2-generation). Endpoints determined are hatching success, mortalities during different life stages, and juvenile growth (F_1-generation, only). The sex ratio of the exposed fish groups is determined by macroscopic inspection of gonads and confirmed by histopathological analysis (F_1- generation) of the fish.
Spawning performance (fecundity) and fertilization rates are recorded for adult fish of F_0- and F_1-generation and the time to first spawning for the F_1-generation.

5. To derive mechanistic data for further interpretation of observed effects the study includes the measurement of biomarkers and a histopathological examination of exposed fish.
Blood plasma samples of the adult fish of F_0- and F_1-generation are taken and measured for vitellogenin (VTG) concentration. Furthermore, a histopathological examination of the fish gonads of the adult fish of F_0- and F_1- generation is performed.

From the results obtained, the threshold concentrations of the chemical is determined, i.e. the concentration with significant observed sublethal or lethal toxic effects ("LOEC") and the highest concentration tested without any significant effects ("NOEC").

6. The test protocol is open to include methodical approaches to assess the underlying mode of action, e.g. -omic methodologies.
The test protocol is considered to identify the full range of EATS modalities, i.e. estrogen, androgen, thyroid and steroidogenic compounds.

2 Validity of the test

7. For a test to be valid, the following conditions must be fulfilled in controls (water and solvent) over the test period:

- There should be greater than 80% fertility and hatchability of eggs and embryos, respectively, from the control animals.
- Post hatch survival of fish larvae, fry and juveniles in the controls should be greater than or equal to 75% during the early life stage phase.
- Sex ratio (% males or % females) in the controls should preferably between 30-70%.
- Dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test.
- The mean water temperature over the entire duration of the study should be 26 ± 1.5 °C. Brief excursions from the mean by individual aquaria should not be more than ± 1.5 °C.
- There should be more than 90% survival of juvenile and adult control animals in all test phases over the duration of the chemical exposure.
- The control fish in each replicate in the two spawning phases should spawn regularly.
- An analytical measurement of the test concentrations is compulsory.

8. If a deviation from the test validity criteria is observed, the consequences should be considered in relation to the reliability of the test results and these deviations and considerations should be included in the test report.

3 Description of the method

Apparatus

9. Normal laboratory equipment should be available and especially the following:

- oxygen and pH meters;
- equipment for determination of water hardness and alkalinity;
- adequate apparatus for temperature control and preferably continuous monitoring;
- tanks made of chemically inert material and of a suitable capacity in relation to the recommended loading and stocking density (i.e. 1 g fish/L);
- suitably accurate balance (i.e. accurate to ± 0.5 mg).

Test vessel

10. The test vessels should be glass aquaria serving at least approx. 25 L of test solution. Each replicate group is kept in an individual test vessel. Full glass aquaria are clearly preferred, however, if bonded vessels are used care should be taken that high-quality silicone adhesive is used. The choice of test vessel size should at least ensure a robust observation and recording of reproduction success, i.e. the use of appropriate spawning trays.
To prepare the filial generations, each test vessel should be equipped with two fry cages. These can be e.g. being analytical sieves of stainless steel with a diameter of 10 cm and a brim height of 4.5 cm. The sieve should have a net at the bottom with preferably a mesh width of approximately 400 µm. The fry chamber should be designed to ensure a reliable observation of egg survival, hatching success and larval swim-up.
Any fry-keeping system allowing a robust observation of hatching success and survival and secondly a stable test item exposure can be used.

11. Glass, stainless steel, or other chemically inert material should be used for construction of the test system that has not been contaminated during previous tests. The use of plastic material should be avoided as far as possible. However, if plastic material is used leaching of plasticizers or other compounds has to be avoided.

Test medium

12. The water used in this study should be of constant quality and should be in compliance with the water quality criteria listed in the Annex. For example, tap water can be used after charcoal filtering to achieve dechlorination. Water used in the test should be the same as used for culturing of fish stock. Measurement of metals should be performed in regular intervals, e.g. every three months. Chemical characteristics of acceptable dilution water are presented in Annex 4.

13. The following water chemistry data should be recorded on a regular base: pH, conductivity [µS/cm], dissolved oxygen content [% air saturation value or mg O2/L], total residual chlorine [mg/L], content of nitrate [mg/L], nitrite [mg/L], ammonium [mg/L], phosphate [mg/L], calcium [mmol/L], magnesium [mmol/L], total hardness [mmol/L], alkalinity [mmol/L], DOC content [mg/L].

Water temperature and light regime

14. Water temperature can be maintained e.g. by placing the vessels into a temperature controlled water bath set to 26 °C ± 2 °C. The light regime should be 12 hours light / 12 hours dark (light intensity of approximately 1000 lux is appropriate, measured 5 cm above the water surface in the middle of the test vessel).

Aeration

15. The oxygen concentration of the test solution should not be lower than 60% of air saturation value. If sufficient exchange of test media is applied via flow through system, no additional aeration of test vessels needs to be applied. Test vessels need to be aerated, if the oxygen concentration falls below 70%.
Special emphasis should be related on the water condition in the fry cage (see above for details). Additional aeration in those cages can improve water quality for the sensitive stages.

Flow through system

16. To apply the test chemical, appropriate application solutions should be prepared. The application solutions serving the flow through device are prepared by dilution of primary stock solution. The stock solution should preferably be prepared by mixing the test chemical in dilution water by mechanical means, e.g. stirring and/or ultra-sonification. Other methods, e.g. saturator columns or passive dosing, can be used to achieve a suitable stock solution.
All efforts should be made to avoid solvents or carriers. However, if a solvent carrier is used, maximum solvent concentrations should not exceed 100 µL/L. It is recommended to keep the solvent amount as low as possible, i.e. ≤ 20 µL/L [21].

17. If difficult substances should be tested, the recommendations of Guidance Document 23 should be taken into consideration [11].

18. The application solutions should be delivered by a suitable pump system. The flow rate of the dosing system should be calibrated before the initiation of the exposure. During the test, flow rates and consumption of application solutions should be checked at regular intervals. Prior to initiation of the exposure period, proper function of the dosing system should be ensured. In the pre-test phase the behavior of the test item under test conditions should be evaluated and stability of test concentrations should be confirmed. During the equilibration phase, the test item concentrations should be analysed at regular intervals, e.g. at least once per week. This analysis can be limited to one replicate vessel per treatment step if the test vessels are served by the same dosing device.
At test start, all test vessels should be measured to confirm equal distribution of test media to all replicates. During the test, the concentrations of the test chemical should be determined at least once per week.
It is recommended that all results are based on mean measured concentrations. However, if concentration of the test chemical in solution has been satisfactorily maintained within +/- 20% of the nominal values throughout the test, then the results can either be based on nominal or mean measured values.

Test organism

19. Zebrafish (*Danio rerio*) is recommended by the OECD TG 210 (ref. [6]), TG 229 (ref.[8]) and TG 234 (ref.[9]) as suitable test species to cover fish early life stages, reproduction and sexual development. It is also one of the four test species which is recommended by the OECD fish drafting group for fish life cycle studies (ref. [4])).
20. Test fish should be selected from a single laboratory stock, which has been acclimated for at least two weeks prior to the test under conditions of water quality and illumination similar to those used in the test. Fish age should not exceed 1 year. Fish should be fed once a day with a commercially available flake food. Additionally, brine shrimp nauplii should be fed once a day.

21. During the acclimation phase, mortalities in the culture fish should be recorded and the following criteria applied following a 48-h settling-down period:

- Mortalities of greater than 10% of the culture population in seven days preceding transfer to the test system: reject the entire batch;
- Mortalities of between 5% and 10% of the population in the seven days preceding transfer to the test system: acclimation for seven additional days to the 2 week acclimation period; if more than 5% mortality during the second seven days, reject the entire batch;
- Mortalities of less than 5% of the population in the seven days preceding transfer to the test system: accept the batch.

22. Fish should not receive treatment for disease in the two-week acclimation period preceding the test and during the exposure period, and disease treatment should be completely avoided, if possible. Fish with clinical signs of disease should not be used in the study. A record of observations and any prophylactic and therapeutic disease treatments during the culture period preceding the test should be maintained. A regular screen on lab- and species-specific pathogens is recommended.

23. The test should be started with sexually dimorphic adult fish. It should be ensured that all fish groups are actively spawning throughout a conditioning period of at least two weeks prior to exposure start.

4 Test design

Test concentrations and number of replicates

24. The setup of five test concentrations is recommended for this test. A negative control (dilution water) is tested in parallel. Controls and treatments are prepared in four replicates each.

If the use of solvent is necessary to achieve adequate dosing of test item, a solvent control has to be applied.
A spacing factor of ≤ 3.2 can be considered.

25. All sources of information should be considered when selecting the range of test concentrations to be applied. The results from other fish tests should be considered, e.g. OECD 210, 229, 230, 234.

Additionally, the following aspects should be considered:

- The highest test concentration should not exceed water solubility.
- The highest test concentration should not exceed 10 mg/L.
- The highest test concentration should not exceed 1/10 of the LC_{50} value (96 h).

Annex 5 can provide further help in setting a suitable concentration series.

5 Test procedure

Initiation of the test

26. At test start, 5 male and 5 female fish are allocated to each replicate. Prior to test start, the fish groups are held under test conditions for at least 14 days to record spawning success during the pre-treatment phase. Females and males are taken from one batch of the same age. If single fish from the main spawning groups have to be replaced, they are taken from the same batch.
The spawning groups are composed by randomized distribution of males and females.
When fish in all test vessels achieve daily spawning of at least 10 eggs per female and fertilization rates equal to or above 80%, the exposure phase can be started. One spawning group each is placed in each test vessel. The fish groups are randomly distributed to the test vessels.

Conditions of exposure

27. A complete summary of test parameters and conditions are shown in Annex 3.

28. During the test, dissolved oxygen, pH and water temperature should be measured in regular intervals in all test vessels of treatment groups and controls. Water temperature should be measured at least at each working day. Additionally, water temperature should be measured continuously in e.g. two control vessels.
Oxygen and pH should be measured at least twice a week in each test vessel.

Duration of exposure

29. The test exposes parental fish from F_0-generation during a spawning period of three weeks. In week 4, fertilized eggs are kept to prepare a F_1- generation. The F_1 fish are monitored over 5 weeks during their early life stage phase, followed by a juvenile growth phase for further 4 weeks. After reaching sexual maturity, start of reproduction and spawning success are observed and recorded over a period of least 10 weeks. An F_2 generation is prepared by keeping eggs of F_1 parental animals, and are exposed until hatch.
The total duration of the test is 20 weeks, until hatch of F_2 animals.

30. Deviations from the time schedule (as shown in Annex 2) may occur, especially by retardation of the development of fish. As a result of retardation of the development, it may be necessary to prolong the observations of the reproduction phase of these animals.

A time schedule of the in life phase is shown in Annex 2.

Feeding regime, juvenile and adult life stages

31. Animals are fed once daily *ad libitum* with commercially available flake food and brine shrimp nauplii (*Artemia salina*). Both food components should be provided not at once, but divided by a time period of at least 4 hours.
During the in life phase feed portions should be estimated and documented for each life phase as precisely as possible.

Analytical determination and measurements

32. Methods for analytical measurements of the test chemical in aqueous media should be available and validated before the start of the test period.

33. Prior to the proposed test start, the flow through system should be equilibrated and samples from all test vessels should be taken for chemical analysis.
During the test period, samples should be taken at test start and at least once per week afterwards. If the stability of the chemical in the test water allows it, rotating between the replicates of each treatment group is possible.
For sampling it is advisable to take retain samples. The retain samples should be stored to enable additional analyses on request. Additional samples should be taken if technical irregularities, e.g. malfunction of the dosing system occurs. Stability of the test chemical in the retain samples should be ensured by suitable pre-testing.

34. Beside chemical analysis, the flow rates of dilution water and application solutions should be checked daily, if possible, e.g. by inspection of consumption in respective tanks.

Observation and biological endpoints

Parental generation (F0)

35. After start of exposure, spawned eggs are collected daily from each test vessel for at least 20 days. Total egg numbers per replicate as well as the number of fertilized eggs are determined. Eggs should be collected daily without interruption as this may cause variability in the data set.

36. At the end of this phase, fertilized eggs are collected from each fish tank to prepare the F_1 generation.
To avoid a genetic drift and bottleneck respectively and to prevent that eggs used for the preparation for next generation originates from too few females, eggs from two successive days should be pooled per replicate and then systematically redistributed to the fry chamber placed in the test tank.

37. After successful start of the F_1-generation, based on fry post hatch survival recorded after three weeks post fertilization, the fish of the P-generation are terminated.

38. All fish are anaesthetised using appropriate anaesthetic. A blood sample is taken from each fish, e.g. via heart puncture (see Annex 7 for a detailed description). After successful blood sampling, the fish are killed humanly with a dorsal cut.
The phenotypic sex of each fish is determined by macroscopic inspection of fish gonads.
Individual total lengths and weights are measured.
After determination of length and weight, the fish are transferred to appropriate fixative to allow a histopathological analysis of fish tissue.

39. The phenotypic sex of each fish is confirmed by histopathological evaluation of fish gonads.

First Filial generation (F_1)

40. At test start, 36 (2 x 18) fertilized eggs are placed randomised in the suitable fry cages fixed under the water surface of each test vessel. Each aquarium is equipped with two fry cages. 144 eggs are used for each test concentration and the control.

41. Hatch of zebrafish fry usually starts at day 3 post fertilisation (pf) and is finished after 6 to 7 days pf. After approximately day 6 pf, fish fry are fed daily *ad libitum* with breeding food. Approximately from day 14 pf onwards, brine shrimp nauplii (*Artemia salina*) are added *ad libitum* to the daily food. From approximately day 21 pf onwards, breeding food is exchanged by ground flake food (*ad libitum*).

42. After 21 days pf, survival is determined by e.g photographic counting. After 21 days pf, the fish from the two fry chambers are pooled and released into the test vessels. After 35 days pf, length of each individual fish is measured and the fish are reduced randomly to 20 individuals, in order to create identical conditions for reproduction in each test vessel. The surplus fish are humanly killed and stored appropriately to enable further analysis, e.g. on physiological parameter. After 63 days pf, the length of individual fish is again measured. From day 63 pf onwards, spawning trays are introduced into the aquaria. Spawned eggs are collected daily, counted and the number of fertilized eggs is determined.

Second Filial generation (F_2)

43. For the start of the Filial 2 (F_2) -generation, 20 fertilized eggs spawned by the adult fish of each replicate are placed randomized in a fry cage and are fixed under the water surface of each test vessel. Hatching success and time to hatch is observed daily and is finished after 96 h. After 96 h, all fry are terminated. Complete hatch should be observed in the controls.

Test termination

44. Fish not further used in the study and all fish not being subject to blood collection and tissue sampling at the end of the study are euthanized after over-dosage with an appropriate anaesthetic.

45. At termination of the adult fish groups, the fish are prepared as follows: the adult fish of the P-and F_1-generation are anaesthetised using appropriate procedures. A blood sample is taken from each fish afterwards e.g. via heart puncture or other suitable methods to finally measure biomarkers, e.g. vitellogenin.
The procedure of blood sampling via heart puncture is described in Annex 7.
The preparation of head and tail homogenates is described in Annex 8.

46. Individual total length and individual wet weight (blotted dry) are measured for each fish. After successful blood collection, the fish are killed with a dorsal cut. The fish are sexed by inspection of the gonads and the whole body is placed into individual containers containing a fixative to allow a histopathological analysis of fish tissue. The sex ratio is calculated. The number of females per groups should be determined and used to calculate the number of total and fertilised eggs per female and day.

6 Observed effect criteria

47. Observations on fish of all life stages are made daily. Dead eggs, larvae, fry; dead juvenile and adult fish are recorded and removed immediately.

48. Hatching rates of the F_1- and F_2- generation are estimated by daily counting of non-hatched eggs between day 2 and 90 % hatch. Any abnormal behaviour is recorded.

49. Between hatch and day 35 of the F_1-generation, fry/juvenile fish are counted at least on day 21 and day 35 and the survival rates are estimated. The data can be confirmed by counting the fish on day 35. Digital photography is a suitable method for reliable detection of fish numbers.
50. Lengths of the F_1-generation fish are measured by digital photography after 35 and 63 days. For the F_1- generation, lengths of fish at day 35 and day 63 are measured e.g. using digital photography and appropriate evaluation software.
For this time interval, a growth rate based on length can be calculated for both generations. The time of first spawning, identified as first day at which eggs are found in the spawning trays, are recorded for the F_1-generation.

51. The reproductive endpoints like egg number and fertilisation rate are observed for 20 daily counts.
To collect the eggs, the use of spawning trays is recommended. A suitable method is described in Annex 6.

52. Regular spawning of the control groups should be achieved, before the counting period of eggs is started.
The proposed criteria for starting the quantitative evaluation can be as follows:
- Determination of the start of regular spawning with a daily egg production of minimum 15 eggs,
- Fertilisation rate of ≥ 80% for controls.
Both parameters should be found on three consecutive days.

7 Data evaluation

53. Statistical analysis of the data should preferably follow procedures described in the OECD guidance document [12].

54. For each endpoint, the NOEC and LOEC should be determined.
For NOEC / LOEC-determination, quantal data are arcsine-transformed prior to analysis. No Observed Effect Concentrations (NOEC) are calculated, using ANOVA, followed by appropriate post hoc tests, e.g. Dunnett's ([17]; [18]), or Williams test ([19]; [20]) or respective non-parametric approaches (e.g. Jonckheere-Terpsta test ([21]; [22]).
Details of all statistical analyses should be reported, including exact p-values for all statistical comparisons. Prior to use of parametric procedures, results of tests of normality and homogeneity of variance should be provided. Failure to confirm assumptions of normality and homogeneity of variance results in the use of a suitable non-parametric test for the data

involved. Results of all tests for normality and homogeneity of variance should be reported along with the results of the parametric or non-parametric tests.

55. If the test results show a concentration-response relationship, with e.g. a maximum effect size of at least 50 % compared to control, the data may be analysed by suitable regression models to determine the ECx-values including the 95 % confidence interval.

8 Further endpoints

56. Depending on available information on the mode of action of the test chemical to be assessed, the test procedure can be extended by further endpoints.

57. If available data suggest a mode of action linked to the thyroidal axes, measurement of blood parameters, e.g. T3 or T4 and/or a focused histopathology on thyroid follicle or glands can be included.
Moreover, it is possible to record the swim-bladder inflation for the early life stages. Respective protocols for the assessment of the different parameters are currently under validation at the OECD level.

9 References

[1] OECD (2012b). Guidance Document on Standardized Test Guidelines for Evaluating Endocrine Disrupters. Environment, Health and Safety Publications, Series on Testing and Assessment (No. 150.), Organisation for Economic Cooperation and Development, Paris.

[2] Organisation for Economic Co-operation and Development, ENV/JM/MONO(2008)22. Joint Meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology. Series on testing and assessment. Number 95, Detailed review paper on fish life cycle tests.

[3] U.S. Environmental Protection Agency (28 May, 2013). Validation of the Medaka Multigeneration Test: Integrated summary Report

[4] US Environmental Protection Agency (April 1996). Ecological Effects Test Guidelines. OPPTS 850.1500. Fish life cycle toxicity.

[5] Organisation for Economic Co-operation and Development, ENV/JM/MONO(2011)23/REV1. Joint Meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology. Validation report (Phase 2) for the fish sexual development test for the detection of endocrine active substances (updated 2012)

[6] OECD (2013): Fish early-life stage toxicity test. OECD Guidelines for the testing of chemicals (No. 210). Organisation for Economics Cooperation and Development, Paris

[7] OECD (2000): Fish, Juvenile Growth Test. OECD Guidelines for the testing of chemicals (No. 215). Organisation for Economics Cooperation and Development, Paris

[8] OECD (2012): Fish Short Term Reproduction Assay. OECD Guidelines for the testing of chemicals (No. 229). Organisation for Economics Cooperation and Development, Paris

[9] OECD (2011): Fish Sexual Development Test. OECD Guidelines for the testing of chemicals (No. 234). Organisation for Economics Cooperation and Development, Paris

[10] OECD (2015): Medaka Extended One Generation Reproduction Test (MEOGRT). OECD Guidelines for the testing of chemicals (No. 240). Organisation for Economics Cooperation and Development, Paris

[11] OECD. (2000). Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. OECD Environment, Health and Safety Publications, Series on Testing and Assessment (No. 23.), Organisation for Economic Cooperation and Development, Paris.

[12] OECD (2006). Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. Series on Testing and Assessement (No. 54), Organisation for Economic Cooperation and Development, Paris.

[13] Dunnett C.W. (1955). A multiple comparisons procedure for comparing several treatments with a control. Journal of American Statistical Association, 50, 1096-1121

[14] Dunnett C.W. (1964). New tables for multiple comparisons with a control. Biometrics, 20, 482-491

[15] Williams D.A. (1971). A test differences between treatment means when several dose levels are compared with a zero dose control, Biometrics 27, 103-117

[16] Williams D.A. (1972). The comparison of several dose levels with a zero dose control, Biometrics 28, 519-531

[17] Williams D.A. (1975). The Analysis of Binary Responses from Toxicological Experiments Involving Reproduction and Teratology. Biometrics 31, 949-952

[18] Williams D.A. (1977). Some inference procedures for monotonically ordered normal means, Biometrika 64, 9-14

[19] Jonckheere A.R. (1954). A distributon-free k-sample test against ordered alternatives, Biometrika 41, 133

[20] Terpstra, T. J. (1952): The asymptotic normality and consistency of Kendall's test against trend, when ties are present in one ranking. In: Indagationes Mathematicae. 14, 327-33.

[21] Hutchinson TH., Shillabeer N., Winter MJ and Pickford DB. (2006). Acute and Chronic Effects of Carrier Solvents in Aquatic Organisms: A Critical Review. Review. Aquatic Toxicology 76: 69–92.

10 Annex

○ Annex 1: Definitions/Abbrevations

11-kT	11-keto testosterone
ANOVA	Analysis of Variance
BSA	Bovine serum albumin
ELISA	Enzyme linked immunosorbent assay
ELS	Early life stage
F_0-generation	Parental generation
F_1-generation	Filial generation 1
F_2-generation	Filial generation 2
FLCT	Fish life cycle test
FSDT	Fish sexual development test
IME	(Fraunhofer) Institute for Molecular Biology and Applied Ecology
LOEC	Lowest observed effect concentration
MEOGRT	Medaka extended one generation reproduction test
MMT	Medaka Multigeneration test
NIES	National Institute for Environmental Studies, Japan
NOEC	No observed effect concentration
PBS	Phospahte-buffered saline
SOP	Standard operation procedure
SPSF	Standard Project Submission Form
SSC	Secondary sex characteristics
UBA	Umweltbundesamt (German Environment Agency)
OECD	Organisation for Economic Co-operation and Development
pf	post fertilisation
P-generation	Parental generation
US-EPA	US Environmental Protection Agency
VMG-ECO	Validation Management Group, Ecotoxicology
VTG	Vitellogenin
ZEOGRT	Zebrafish extended one generation reproduction test

○ Annex 2: Draft Test protocol

Time of exposure	Fish age	Phase	Course	Endpoints*
0 d	approx. 15 weeks	Reproduction F_0 generation	Start with spawning groups 5 male/5 female fish	**Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility)**
21 d	0 d	Early life stage F_1-generation	Start with 36 fertilized eggs per vessel (2x18 eggs in stainless steel fry cages)	**Time to hatch Hatching success**
24 d	3 d		Begin of hatch (hatch completion between 4 to 6 dpf)	
27 d	6 d		Feeding with breeding food *ad libitum*	
			Swimming up	
5 w	14 d		Feeding with *Artemia salina* (Lifefood)	
6 w	21 d		Photographic determination of survival; Transfer to main aquaria	**Post-hatch survival**
6 w	21 d	F_0-generation	Termination	**Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) Vitellogenin content in females and males**
8 w	35 d	Early life stage F_1-generation	Photographic determination of length and survival Random reduction to 20 individuals	**Post-hatch survival Length**
12 w	63 d	Juvenile growth F_1-generation	Photographic determination of length and survival	**Survival Length *growth rate***
13 - 18 w	70 - 105 d	Reproduction	Introduction of spawning trays Daily evaluation of egg numbers and fertilization rates	**Time to first spawning**
15 - 20 w	84 d - 119 d	F_1-generation		**Total egg no/ day/ female (Fecundity) Fertilization rate (Fertility)**
20 w	0 d	F_2-generation	Start with 20 fertilized eggs per vessel (20 eggs in stainless steel fry cages)	**Time to hatch Hatching success**
	96 h		Hatch	
20 - 22 w	119 - 133 d	Test termination F_1-generation	End of F_1-generation	**Length and weight at test termination Sex ratio Gonad histopathology (e.g. maturation stage; endocrine-related histopathology) Vitellogenin content in females and males**

* Only the most relevant endpoints for endocrine effects were listed. Furthermore, behavioural abnormalities (e.g. orientation in the water body, food consumption, increased / decreased motility) could be observed during the whole time course of the study.

○ Annex 3: Draft test conditions/Validity criteria

Test species	Zebrafish (*Danio rerio*)
Test type	Flow through
Mean water temperature	26 +/- 1.5 °C
Photoperiod	12 h light, 12 h dark
Water volume per test vessel	20 – 25 L
Volume exchanges of test solutions	minimum of 5 daily
Age of test organisms at start of exposure	adult fish, spawners (F_0-generation), approx. 3 to 6 months
Loading rate per replicate	F_0: 10 fish (5 males, 5 females) F_1: initiated with 36 eggs, reduced to 20 fish for juvenile growth and reproductive phase (randomised) F_2: 20 eggs (hatch only)
Loading rate per treatment	F_0: 40 fish F_1: initiated with 144 eggs, reduced to 80 fish for juvenile growth and reproductive phase (randomised) F_2: 80 eggs (hatch only)
Number of treatments	5
Number of replicates per treatment	4
Feeding regime	Fry food (dry), live food (nauplii of *Artemia salina*), flake food
aeration	None unless oxygen concentration falls below 60 % saturation
Dilution water	suitable water, considering guideline specifications
Test substance exposure duration	20-22 weeks
Biological endpoints	F_0: Reproduction (Fecundity and Fertility) F_1: Hatching success Survival, growth (Early life stage, juveniles and adults) Reproduction (Time to first spawning, Fecundity and Fertility) Sex ratio Vitellogenin, Gonad histopathology Optional: e.g. Measurement of further sex steroids, thyroid hormones Histopathological examination of further organs F_2: Hatching success
Test acceptability criteria for controls/ validity criteria	Dissolved oxygen ≥ 60 % Mean water temperature: 26 ± 1.5 °C F_0: Successful reproduction in controls: at least 10 eggs per female and day, 80 % fertility F_1: Post hatch survival (larvae), controls: ≥ 75 % F_0/F_1:Survival of juveniles and adults, controls: ≥ 90 % F_1: Sex ratio in controls preferably between 30 % to 70 %

○ **Annex 4: Some chemical characteristics of an acceptable dilution water (taken from OECD TG 240 [10])**

Substance	Limit Concentrations
Particulate matter	5 mg/L
Total organic carbon	2 mg/L
Unionised ammonia	1 µg/L
Residual chlorine	10 µg/L
Total organophosphorus pesticides	50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	50 ng/L
Total organic chlorine	25 ng/L
Aluminium	1 µg/L
Arsenic	1 µg/L
Chromium	1 µg/L
Cobalt	1 µg/L
Copper	1 µg/L
Iron	1 µg/L
Lead	1 µg/L
Nickel	1 µg/L
Zinc	1 µg/L
Cadmium	100 ng/L
Mercury	100 ng/L
Silver	100 ng/L

Remark: Deviations of the used test water from these characteristics (e.g. use of natural or tap water without further re-constitution) should be addressed and classified in the report.

○ **Annex 5: Logarithmic series of test concentrations suitable for toxicity test (taken from OECD TG 215 [7])**

Column (Number of concentrations between 100 and 10 or between 10 and 1)*						
1	**2**	**3**	**4**	**5**	**6**	**7**
100	100	100	100	100	100	100
32	46	56	63	68	72	75
10	22	32	40	46	52	56
3.2	10	18	25	32	37	42
1.0	4.6	10	16	22	27	32
	2.2	5.6	10	15	19	24
	1.0	3.2	6.3	10	14	18
		1.8	4.0	6.8	10	13
		1.0	2.5	4.6	7.2	10
			1.6	3.2	5.2	7.5
			1.0	2.2	3.7	5.6
				1.5	2.7	4.2
				1.0	1.9	3.2
					1.4	2.4
					1.0	1.8
						1.3
						1.0

* A series of three (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x+1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/L or µg/L). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainly on the toxicity level.

○ Annex 6: Spawning substrate for egg collection (example)

All glass instrument dish, for example 28 x 15 x 6 cm (l x w x d), covered with a removable stainless-steel wire lattice (mesh width 2mm). The lattice should cover the opening of the instrument dish at a level below the brim.

On the lattice, spawning substrate should be fixed. It should provide structure for the fish to move into and through it. For example, glass pearls fixed on stainless steel filament are suitable.
When using glass materials, it should be ensured that the fish are neither injured nor cramped during their vigorous actions.
Other material might be suitable. However, artificial plants of plastic material are often of bad quality and may leach out.

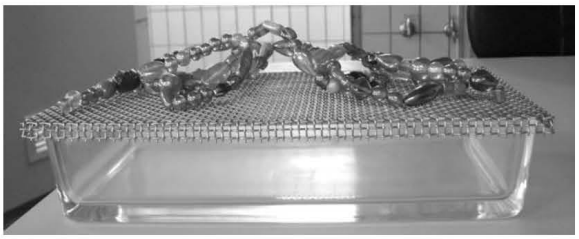


Figure 1: Spawning tray to collect zebrafish eggs, side view

Figure 2: Spawning tray placed in test vessel, top view

The distance between the tray and the glass panes of the test vessel should be at least 3 cm to ensure that the spawning is not performed outside the tray (see Figure 2). The eggs spawned onto the tray fall through the lattice and can be sampled 45-60 min after the start of illumination. The transparent eggs are non-adhesive and can easily be counted by using transversal light. Fertilisation status can be determined by observing cleavage stages (> four cell stage) using a binocular.

Please note, that the tray should be placed not directly on the bottom of the test vessel but e.g. on a cover plate (glass material, diameter of 10 cm) to prevent that fish get crushed to death below the tray due to handling mistakes.

When using five females per vessel, egg numbers up to 20 at a day can be regarded as low, up to 100 as medium and more than 100 as high numbers. The spawning tray should be removed, the eggs collected and the spawning tray re-introduced in the test vessel, either as late as possible in the evening or very early in the morning. The time until re-introduction should not exceed one hour since otherwise the cue of the spawning substrate may induce individual mating and spawning at an unusual time. If a situation needs a later introduction of the spawning tray, this should be done at least 9 hours after start of the illumination. At this late time of the day, spawning is not induced any longer.

○ Annex 7: Blood sampling by cardiac puncture

Heart puncture is performed with 1 mL syringes, which are equipped with a fixed cannula (0.3x12mm). The syringes were previously filled with 200 µL of a blood collection buffer. This buffer is composed as follows:

Approach for 100 mL buffer:
100.000 units Heparin (Ammonium salt from intestinal mucosa)
50 mg Aprotinin
Both components solve in 100 mL PBS
Then filter the buffer sterile (0.2 µm)

At the beginning of the blood sampling, the fish is anesthetized in a narcotization solution. Immediately after the active swimming movement of the fish has exposed, the animal is taken out of the solution and fixed with a paper towel in the back position. The position of the heart is determined by the visible heart beat and a translucent dark coloration of the organ.
If the position of the heart is determined, the cannula is pierced into the heart perpendicular to the belly of the fish, and gently moved up and down with a gentle pull. The success of the blood sampling is indicated by a deep red coloration of the sampling buffer.

After blood sampling, a complete mixing of blood and buffer is ensured by gentle shaking of the syringe. The blood / buffer mixture is transferred to a reaction vessel (500 µL) and stored on ice until centrifugation.
After the blood has been taken, the fish are killed by severing the neck part.
The reaction vessels with the blood samples are centrifuged in a cooling centrifuge for 30 minutes, at 4 °C and about 5000 revolutions per minute.

The blood plasma is then pipetted off and transferred into new reaction vessels and stored at -80°C until use.

- **Annex 8: VTG measurement, homogenisation of head and tail**

The fish are anaesthetized and euthanized. Head and tail are cut of the fish. It is to be noted that all dissection instruments and the cutting board should be rinsed and cleaned properly (e. g. with 96% ethanol) between handling of each single fish to prevent "VTG pollution" from females or induced males to un-induced males.
The weight of the pooled head and tail from each fish is determined. After being weighed, the parts are placed in appropriate tubes. It is important that the tubes should be numbered properly so that the head and tail from the fish can be related to their respective body-section used for gonad histology. Ice-cold homogenization buffer (12 mL 50mM Tris HCL pH 7.4 + 120 µL 1% Protease inhibitor cocktail) is added. With the pistils a homogeneous mass is to be produced. The samples are placed on ice until centrifugation at 4°C at 5000 g for 30 min. The supernatant should transferred into at least two tubes by dipping the tip of the pipette below the fat layer on the surface and carefully sucking up the supernatant without fat- or pellet fractions. Until use the tubes are stored at -80°C.

(taken from OECD 234)

- **Annex 9: Measurement of Vitellogenin content**

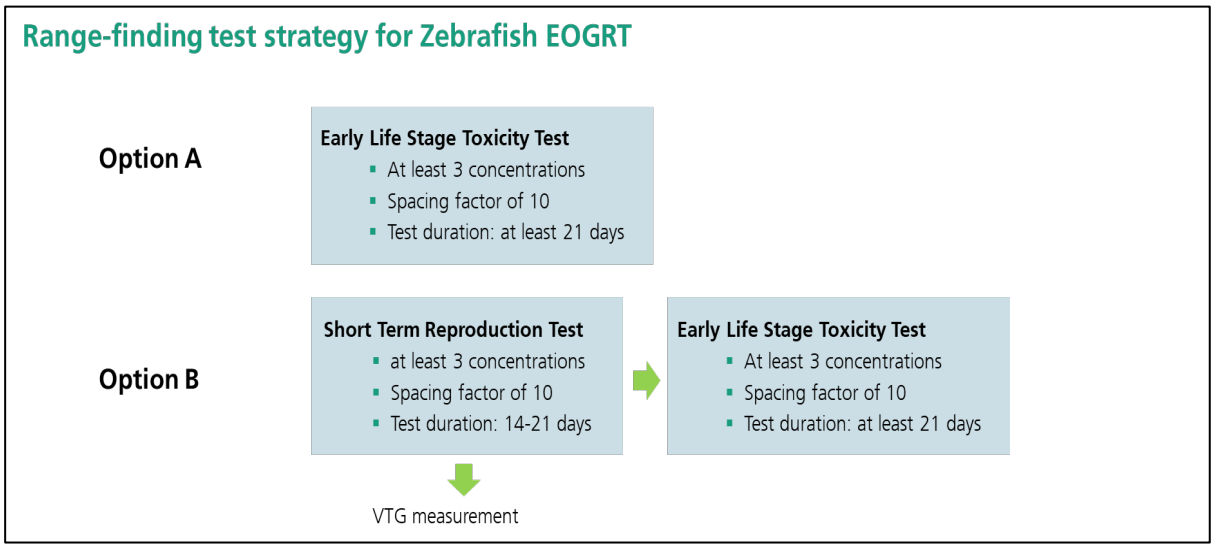
  [Lab-specific procedure, to be included]

- **Annex 10: Preparation of tissue sections for sex determination**

  [Lab-specific procedure, to be included]

6.3 Proposal for a pre-test strategy

Figure 2: Flowchart to illustrate an optional pre-test strategy to identify ZEOGRT test concentrations



Source: Own illustration, Fraunhofer IME

An appropriate concentration setting must ensure a clear identification of effects primary induced by endocrine acting property in contrast to toxic effects. A suitable pre-test strategy should allow a robust estimate of those concentrations. Two options for a pre-test strategy were proposed and discussed. The final choice and test design also depends on the amount of available data on acute and long-term fish toxicity of the chemical to be tested.

Option A represents a reduced fish early life stage toxicity test, basically following the requirements of OECD Test Guideline (TG) 210. However, the setup has only three test concentrations and two replicates per treatment and control. The spacing factor is broader to span a wide range of concentrations. The test records hatch of embryos, survival of larvae and allow the measurement of growth parameters, i.e. length at the end of the test. The test duration is considered with 21 days.

Option B combines two test designs and starts with a reproduction phase with adult fish. The setting of spawning groups can follow OECD TG 229, i.e. 5 males and 5 females per test vessel. The groups are exposed for at least 14 days and the reproduction success is recorded by the daily number of eggs. The assessment of fertilisation rates can be included. At the end of the parental phase, fertilized eggs are collected to prepare a second fish generation. This is also kept for 21 days to record the biological performance of the early life stages, e.g. embryos and larvae. The basic composition of this prolonged range finding test is also with three concentrations and a limited number of replicates. To record further information on the endocrine potential of the test chemical, the test can be extended by further evaluation e.g. by the measurement of vitellogenin concentrations of the adult fish.