

Fluorescent toxicity endpoints as promising tools to progress the fish embryo test towards practical screening applications

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Abbreviations

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AO	Acridine orange
AOP	Adverse outcome pathway
BLBP	brain lipid binding protein
cAMP	Cyclic adenosine monophosphate
<i>cdh5</i>	<i>VE-cadherin</i>
cDNA	complementary Deoxyribonucleic acid
CNS	Central nervous system
DA	Dorsal aorta
DIN	Deutsche Industrie Norm
DLAV	Dorsal longitudinal anastomic vessel
DMSO	Dimethyl sulfoxide
EC50	Effect concentration of 50%
EDA	Effect-directed analysis
EDC	Endocrine disruption chemical
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EPO	Erythropoietin
ETS	E-twenty six
FET	Fish Embryo Toxicity test
<i>fli1a</i>	<i>friend leukaemia integration1</i>
<i>flt4</i>	<i>fms-related tyrosin kinase 4</i>
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
HIF-1	Hypoxia-induced factor-1
hpf	Hours post fertilization
HTS	High-throughput screening
ISO	International Organisation for Standardization
ISV	Intersegmental vessel
ISVFA	Intersegmental vessel fluorescence assessment
MBFA	Morphological bright field assessment
mRNA	Messenger ribonucleic acid
OECD	Organization of Economic Co-operation and Development
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with triton
PCP	Personal care products
PCR	Polymerase chain reaction
PCV	Posterior cardinal vein
PFA	Paraformaldehyde

Abbreviations

Q-PCR	Quantitative polymerase chain reaction
RGC	Radial glial cell
RNA	Ribonucleic acid
ROI	Region of interest
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TRI	Trizol
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
YES	Yeast Estrogen Screen
zFET	Zebrafish embryo toxicity test

1. Introduction

1.1 Challenges of the current risk assessment

Manufactured chemicals have a wide dispersion in the environment and derive from many different human activities, like medical, industrial and agricultural application. The continuously increasing of chemical production and their release into the environment can pose a serious risk to environmental and human health. To ensure a high degree of protection of human health and environment current legislation in European and other industrial countries demand appropriate data on risk assessment of chemicals, pesticides, biocides and pharmaceuticals (Commission of the European Communities, 1994; CVMP/VICH 2000; EMEA/CHMP, 2006 and VICH, 2004). These data include information on the toxicity at various trophic levels and require testing on vertebrates. Solely in the context of the European Union Regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), a strong increase of animal tests is anticipated. On the contrary, next to ethical concerns, the enforcement of the renewed European cosmetic directive (EC/1223/2009) and the animal testing law (Directive, 2010/63/EU) arise the need of animal alternative testing methods. According to the cosmetic directive, animal test are not allowed anymore for testing cosmetic ingredients and products and to comply with the ethical principle of the 3 Rs (Reduction, Refinement and Replacement) of Russell and Burch (1959) alternatives are mandatory.

In vitro testing appears as an attractive alternative for toxicity testing, as it does not require direct animal use. The development and validation of such methods is the aim of the European Union Reference Laboratory for alternative methods (EURL) in Ispra, Italy. Currently, there are many different *in vitro* methods routinely used in toxicology testing, especially for mechanistic approaches, screenings and research, but their application for regulatory use is strongly limited. An *in vitro* system, which detects the mutagenic potential of chemicals and which is accepted and established provides the Ames test (OECD 471 test guideline). Based on auxotrophic mutants of bacteria strains, it finds application in pharmaceutical testing and chemical evaluation under REACH. Another example for an *in vitro* test, which is an applied and validated animal alternative test, is the steroidogenesis assay (OECD 456, in July 2011). The steroidogenesis assay with H295R cell line detects endocrine disrupting chemicals (EDCs) and therefore, replaces the rodent minced testis assay

(OECD 440) (Lilienblum, 2008). However, most of these tests are only suitable for screening possible toxicity of chemicals, not for definitive hazard identification. Moreover, no *in vitro* assay is able to reveal adverse apical effects (Brion et al, 2012). They cannot reflect the full range of chemically induced toxicity, as cell-based *in vitro* methods do not provide the complexity of animal models. Hence, it is in dispute, if *in vitro* tests alone are capable of providing accepted alternative methods. On the one hand, there is a need to reduce assays with higher vertebrates and on the other hand screening assays with *in vivo* properties are in demand.

Promising *in vivo* approaches with small-scale *in vitro* like attributes use embryos of zebrafish (*Danio rerio*) or Xenopus (*Xenopus spp.*). The Xenopus Embryonic Thyroid Signalling Assay (OECD TG231) for instance provides mechanistic information, as it measures interactions of chemicals with the thyroid receptor. Frog embryos represent like the embryos of the zebrafish, an intact organism, in which cell-cell interactions and intercellular signal transduction is preserved. The advantage of the zebrafish embryos is their closer (phylogenetic) relationship to mammals. They have a high number of conserved genes, which possess specialized functions also present in higher vertebrates (Embry et al., 2010). Some data indicate that zebrafish embryos are suitable models for predicting the toxicity and teratogenicity of chemicals in mammals including humans (Nagel, 2002; Selderslaghs et al., 2009; Strähle et al., 2012). Concurrently, fish embryos are not considered as protected animals according to the European Directive on experimental animal use (EU Directive 2010/63). Based on the assumption that embryos have only a limited or missing pain perception in comparison to adults (Belanger et al., 2010; Strähle et al., 2011), the zebrafish embryo is regarded by law as a non-animal until the onset of independent feeding. The zebrafish is a popular and well-established model in genetics and developmental biology. In addition, it is commonly used as a model for drug discovery (Parng et al., 2002; Zon and Peterson, 2005; Chakraborty et al., 2009) and in toxicology to study effects of chemicals (Carvan et al., 2000).

Furthermore, toxicological research is more and more interested in the identification of the mode and mechanisms of action of chemicals (National Research Council, 2007). Potential subtle and long-term effects may be indicated by toxicokinetics and toxicodynamics (Fent, 2003). The uptake, metabolism and excretion (toxicokinetics) are important parameters to assess bioavailability and bioconcentration of an environmental chemical in an organism.

Furthermore, any toxic effect of a chemical is preceded by an interaction at the molecular level (toxicodynamics). The toxic compounds may interact with membranes and receptors therein or bind to proteins, what e.g., influence DNA transcription. These interactions result in alterations in gene or protein expression, which may mediate the toxic effects on the morphological level. Thus, the impact of chemicals comprises all ecological levels, from the molecular to the ecosystem level (e.g., food chain) (Fig. 1). But the limited knowledge about biological systems has hindered effort to use mechanistic information as a basis of toxicity extrapolation and thus, risk assessment (OECD 184, 2013).

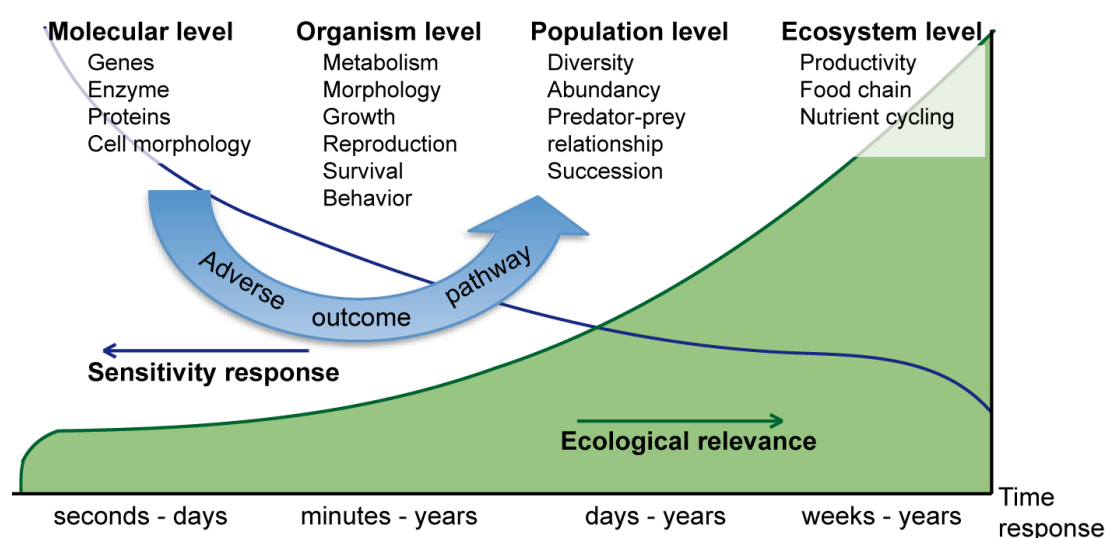


Fig. 1: Ecotoxicological impact on different biological levels.

The challenge of risk assessment is to understand the linkage between the sensitive toxicity response on the molecular level (mechanistic information) and the individual fitness (organism level) to the more ecological relevant population and at least ecosystem level. The use of mechanistic information for ecological risk assessment is a prerequisite of the conceptual framework of Adverse Outcome Pathways (AOP). (Figure is modified from Fent, 2003)

There is a need for a more ecotoxicological understanding of a potential linkage between a molecular initiating event and an adverse outcome across all levels of biological organization (Ankley et al., 2010). And to implement a predictive strategy for risk assessment, a sentinel system is required, which is suitable to facilitate predictions of realistic pollutant stress responses from the molecular to the whole organism and population level. This can not be achieved by a single test system but rather by developing the Adverse Outcome Pathways (AOPs). The framework of AOPs is a conceptual construct, what collects and evaluates relevant chemical, biological and toxicological information to support the use of mode and/or mechanism of action as basis for the understanding of adverse effects (OECD 184, 2013). The AOPs portrays existing knowledge concerning the pathways of causal linkages between a

molecular initiating event (chemicals interaction with e.g., DNA binding, protein oxidation) and a final adverse effect at a biological level of organization that is relevant to a regulatory decision (Ankley et al., 2010). An adverse outcome can be found on the organism level as a change in morphology, physiology, growth, development, and reproduction or on the population level. In all cases it results in an impairment of functional capacity and a loss of compensation for additional stress or an increase in susceptibility to other influences (IPCS, 2004; Keller et al., 2012). To promote the AOP concept, more approaches are needed, which elucidate the molecular toxicity responses for the identification of mode of action of chemicals. Desirable are test systems, which combine the complexity of an intact organism with metabolic processes to increase the understanding of toxicity pathways.

1.2 The zebrafish embryo toxicity test

Fish are in direct contact with the aquatic environment, which is one of the main sink of pollutants. As an aquatic organism they are exposed to chemical substances during their whole life cycle and over generations. Their important position in the food chain as primary, secondary or tertiary consumer is why they became an important model in aquatic ecotoxicology. To date, the application of the zebrafish (*Danio rerio*) embryo toxicity test (zFET) in ecotoxicology has focused on acute toxicity assessments. Acute toxicity in fish embryos correlates very well with acute toxicity in adult fish (Nagel, 2002), which is why the zFET was suggested to replace the acute fish test (TG, 203, OECD, 1992). In Germany, it was already applied for routine wastewater effluent testing (Bundesgesetzblatt, 2005; DIN 15088, 2007) replacing the acute test on adult golden ide (*Leuciscus idus melanotus*) (DIN 38412-L31) since the 1st January 2005. With the adoption of the new OECD 236 test guideline (Fish Embryo Acute Toxicity Test, 2013) it is now also internationally accepted to determine the fish acute toxicity of chemicals on embryonic stages of zebrafish. Recently, the European Centre for Validation of Alternative Methods (ECVAM) published a protocol to promote the embryo toxicity test as alternative to the acute fish toxicity test. The protocol describes the application of the zFET as a refinement/replacement, reduction part of an integrated testing strategy and screening method for higher tier tests (ECVAM DB-ALM, 2013).

In the zFET according to the new OECD 236 the effects coagulation of the embryo, lack of somite formation, non-detachment of tail and lack of heartbeat are considered lethal and determine mortality, thus acute toxicity (OECD 236, 2013). Beyond acute toxicity assessments, there is plenty of evidence for the potential to also evaluate specific and subchronic toxicities like teratogenicity or neurotoxicity (Braunbeck, 2005; Parng et al., 2007;

Scholz et al., 2008; Muth-Köhne et al., 2012). The numerous available methods for zebrafish open up possibilities to increase the specificity and sensitivity and to improve the understanding of toxic effects. Accordingly, the definition of more refined, meaningful and quantifiable toxicological endpoints is required to broaden the applicability of the zFET (Frayse et al., 2006; Scholz et al., 2008). Valuable in this context is the gene specific promoter-driven expression of fluorescent marker proteins of transgenic fish lines, which allow tracing of gene expression spatiotemporally *in vivo*. This, however, requires a refined endpoint evaluation using detailed morphological and submorphological analysis methods, which are so far neither established nor standardized. Furthermore, the zFET in combination with so-called genomic approaches, which try to elucidate the molecular responses of the transcriptome, proteome or metabolome, has increasingly been proposed for the identification of modes of actions of chemicals (Scholz et al., 2008).

Nevertheless, the applicability of the zFET is not currently suitable for regulatory standard testing as well as for industrial use also in pharmaceutical product development. Despite numerous intentions to refine the application of the zFET according to DIN/ISO 15088 (replacement of the DIN/ISO 38415-6: 2003) or OECD 236 (OECD 236, 2013) for specific toxicity assessments (Bachmann, 2002; Hollert et al., 2003, Voelker et al., 2007), an important prerequisite for industrial use has not been established yet: for screening, also the zFET procedures will need adjustments to high or even middle throughput applications. Automation of processes can help to increase time and cost efficiency, which are important parameters for commercial test systems, which can be achieved by automation. Therefore, the bright field microscopic assessment method of the zFET poses a noticeable drawback as it is manually performed. However, a computer-based automated phenotypic assessment of embryonic effects by bright field microscopy is not invented so far. The current zFET is based on the assessment of characteristic morphological effects on the development of zebrafish embryos. Therefore, the orientation of the embryo significantly contributes to the quality of the assessment. Only an optimal orientation of the embryo enables the determination of all relevant morphological structures, which ensure a sufficient toxicological evaluation. A possible approach to solve this difficulty could be provided by computer-based 3 D-projections, where a virtualized rotation of the embryo can compensate the unfavorable position. Present 3 D-projections can only be appropriate achieved for fluorescence images. High-capacity computers for the procession of fluorescence image are already available on

the market in contrast for bright field images. Thus, automated image recognition and analysis could only be achieved for fluorescence techniques.

1.3 Fluorescence microscopy

Epifluorescence microscopy reveals only the objects of interest against otherwise black background. It requires the object of interest to be fluorescent what can be achieved by a wide range of different fluorophores. Fluorescence is the emission of light that occurs after the absorption of light of shorter wavelength. The difference between excitation and emission wavelength is called Stokes shift, and constitutes the specific property that makes fluorescence so powerful. By completely filtering out the exciting light without blocking the emitted fluorescence, it is possible to see only the objects, which are fluorescent. If the background has no autofluorescence, fluorescence microscopy enables visualization of even single fluorescent molecules (Lichtman and Conchello, 2005). The utilization of *in vivo* fluorophores has already become a beneficial tool for the detection of specific cell structures or tissues or even gene expression within the living organism.

An additional advantage of using fluorescent tools concerns image analysis. Fluorescence microscopy offers many possibilities of automated computer-based image analysis, which are superior to bright field imaging methods due to less complex image features. Fluorescence signal based images are easier to process than bright field images, which contain a large scale of different grey values or even color information. Moreover there is the possibility of fluorescent double stainings, where different structures can be assessed at the same time in the same organism, what favors time efficiency. Fluorescence microscopy applications have already been established over recent decades in medical research, but still stand at the beginning in environmental research.

1.4 Fluorescence imaging in zebrafish embryos

The GFP, an intrinsically fluorescent gene product, allows tagging of protein components in living organisms. This promotes for example *in vivo* investigations of developmental processes like morphogenesis, as well as the detection of disturbed developmental processes. Thus, adverse effects can be visualized and assessed *in vivo*.

Hence, fluorescence microscopy finds application in toxicology. Several studies already deployed transgenic zebrafish models for simple read-outs to analyze chemical exposure induced changes in expression of target genes (Mattingly et al., 2001; Blechinger et al., 2002;

Hill, 2003; Behra, 2004; Yu, 2005; Hernández and Allende, 2008; Chen et al., 2009). Hill et al. (2003), for instance, linked a dioxin-induced reduction of the brain capacity in zebrafish to a decreased expression of *neurogenin* and *sonic hedgehog*. They used transgenic zebrafish lines expressing GFP under the control of the promoters of these genes, which are two key regulatory genes in brain development. The *in vivo* reporter system of Mattingly et al. (2001) allowed the prediction of dioxin-induced toxicity. They used GFP-expression regulated by the promoter of the aryl hydrocarbon receptor (AhR), to show that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-exposed transgenic zebrafish embryos express *ahr* in the eye, nose and vertebrae (Mattingly et al., 2001). Tong et al. (2009) developed a transgenic zebrafish *Tg(cyp19a1b-GFP)* line that expresses GFP under the control of the *cyp19a1b* promoter. This line shows co-expression of GFP and endogenous aromatase B in radial glial cells, what enables screening of estrogenic activities of chemicals or mixtures in living zebrafish embryos (Tong et al., 2009; Brion et al., 2012). The fluorescence intensity of the GFP signal in the brain was determined as indicator for estrogenic active compounds. Furthermore, *Tg(cyp19a1b-GFP)* were applied in an effect-directed analysis (EDA) to assess the estrogenic activity of environmental sediment samples (Fetter et al., 2014).

The specific promotor-regulated expression of fluorophors can increase sensitivity and specificity for the detection of chemical impacts. Analog to the transgenic fish lines, fluorescent antibody stainings as well as *in vivo* stainings with vital-fluorophores can be used to visualize substance-induced alterations of tissues or even cells of the zebrafish embryo. In our laboratories, for example, we could demonstrate that whole mount immunostainings of motorneurons in zebrafish embryos enabled the classification of neurotoxic compounds (Muth-Köhne et al., 2012). Also, tissue-specific vital stainings have shown to be applicable for effect detection in zebrafish embryos. They are already suggested for drug screening applications (Parnig et al., 2002).

These examples illustrate the good potential of zebrafish embryos as a test organism for environmental and human risk assessment, as well as a model organism in medical and pharmaceutical research and development. In contrast to cell-based systems, their characteristic attributes facilitate a direct effect detection in a living organism in real-time. Zebrafish embryos are small, transparent and develop *ex utero* and provide all characteristic properties of vertebrates. At 48 hours post fertilization (hpf), the somatogenesis, as well as the development of the most organs and extremities are already completed in zebrafish (Kimmel et al., 1995). Consequently, complex biological processes based on cell-cell interactions and

signal cascades can be assessed during the whole embryonic development. Even if metabolic processes are considered in cell-based systems, they cannot provide information on systemic effects. The possibility to assess the fluorescent signal in living organism provides the basis for an automated image-based phenotypic analysis. Moreover, under laboratory conditions several thousand zebrafish eggs are easily available per day, which can be used for a high number of parallel experimental treatments (Scholz et al., 2008). The time-, cost- and material efficiency of the zFET makes it attractive also for high-throughput applications.

1.5 Objectives and experimental approach

The overarching objective of this thesis was to apply fluorescence microscopy methods to the fish embryo toxicity test (FET) with the aim to increase the sensitivity and explanatory power of the test. The FET is an animal alternative test widely applied in ecotoxicology. Thus far, the main application of the FET with zebrafish (*Danio rerio*) as test species (zFET) focused on the assessment of the acute toxicity of chemicals, where only lethal morphological effects are accounted for. However, an application of the zFET beyond acute toxicity is evident and desirable, but necessitates the establishment of more refined and quantifiable toxicological endpoints. I therefore intended to assist its development to an alternative and supplementary test method for fish toxicity by improving and accelerating the microscopic assessment through fluorescence image analysis. A valuable tool in this context is the use of gene expression-dependent fluorescent markers and stains that can even be measured *in vivo*.

My task was to compare the fluorescence with standard bright field for the microscopic evaluation of the zFET. Particular consideration was given to the practical issues and the optimization and automation of image analysis, as these aspects play an important role in high-throughput applications. Consequently, I elaborated the benefits of using fluorescence applications as convenient tools to identify and specify toxicity *in vivo* and to increase the sensitivity of the zFET. Transgenic zebrafish lines were chosen which enable the assessment of the vascular and neuronal development. Furthermore I tried to visualize additional structures of the embryos by different staining methods. The whole mount immunostaining of the muscle morphology and the vital staining of apoptotic cells appeared as the most promising stainings to study further potential chemical-induced effects. The developed fluorescence endpoints were scrutinized in a proof-of-principle with chemicals, which have an occurrence in the environment, to verify their feasibility for toxicity testing. After testing of a bright range of chemicals (pharmaceutical and insecticides), triclosan, genistein and cartap were selected as model compounds.

The identification of molecular initiating events of toxicity pathways is key for early effect detection. Thus, gene expression analysis was chosen to validate the morphological effects, which were detected via fluorescence microscopy.

For the first time, two different phenotypic assessment methods, the microscopic bright field assessment of the conventional zFET with different fluorescence-based endpoint methods, were directly compared, in terms of their potential to increase the sensitivity and value of the zebrafish embryo test system.

An overview of the objectives and the experimental approach of this thesis are illustrated in figure 2.

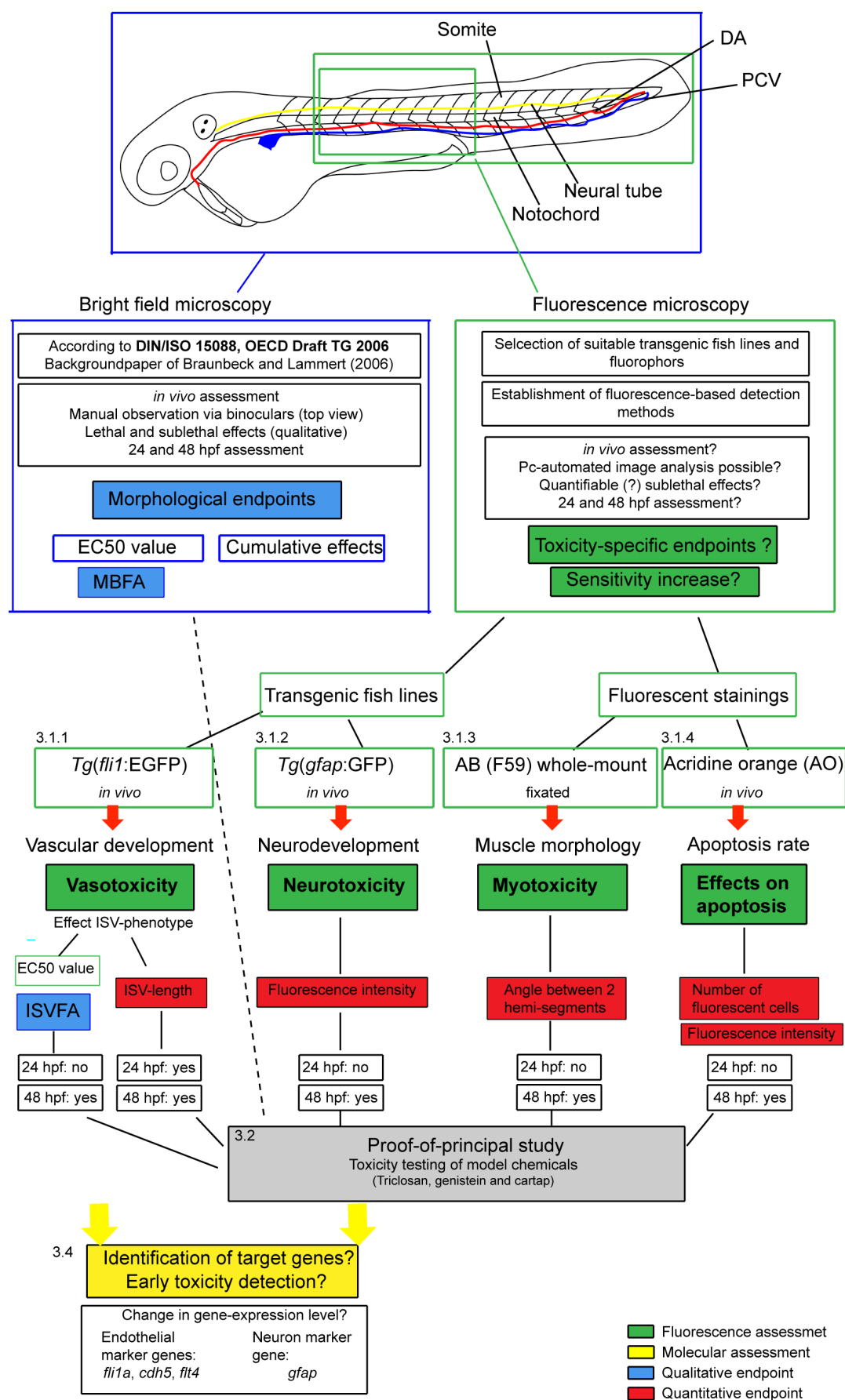


Fig. 2: Work flow and objectives of this PhD thesis.

Numbers refers to the chapters in which the assessment methods are described. DA: dorsal aorta, PCV: posterior cardinal vein ISV: intersegmental blood vessel.

2. Materials and Methods

All used materials and the corresponding manufactures (incl. product number) as well as the equipment used are described in the following chapters.

2.1 Fish husbandry

The test organism zebrafish (*Danio rerio*, Hamilton-Buchanan) belongs to the family of the *Cyprinidae* (carp family) within the class of the *Osteichthyes* (bony fish). It is originally found in the branches and tributaries of the rivers in India and South Asia. Males can be distinguished from females by their smaller appearance and an orange or reddish tint to the silvery bands along their body. Due to the large number of egg production, females can also be recognized by their often swollen bellies. They have a very short reproduction circle as they reach maturity within three month. One female can spawn about 100 eggs per day, which are fertilized by sperm release of the male into the water (Kimmel et al., 1995). Under good laboratory conditions several thousand eggs can easily be produced daily. The transparent and non-adherent chorions of zebrafish eggs enable an easy observation and manipulation. After fertilization embryos develop fully within 48 hpf and hatch between 48 hpf and 72 hpf, depending on the water temperature (Kimmel et al., 1995). The optimal temperature for zebrafish keeping and raising is $26 \pm 0.5^{\circ}\text{C}$ (Braunbeck et al., 2005).

Transgenic *Tg(fli1:EGFP)^{y1}* zebrafish (Westerfield, 1995) were obtained as eggs from the zebrafish facility GIGA (Groupe Interdisciplinaire de Génomprotéomique Appliquée), Liège (Belgium). Transgenic zebrafish *Tg(gfap:GFP)* eggs were kindly provided from the Tübingen Zebrafish stock collection (Max-Planck-Institute for Developmental Biology, Tübingen, Germany). Wild type zebrafish were originally obtained from the West Aquarium GmbH (Bad Lauterberg, Germany) and maintained for several generations and years in the laboratories of the Fraunhofer IME. All zebrafish stocks were raised and kept according to high standards of general zebrafish keeping and husbandry (Brand et al., 2002; Lawrence, 2007). Transgenic fish were handled according to German regulations of genetically modified organism (GMO, GVO). Fish were maintained in 100 – 200 l glass aquaria at $27 \pm 1^{\circ}\text{C}$ at a 14:10 h light : dark photoperiod. For obtaining eggs, metal lattice-covered glass spawning trays were inserted into the tanks for 1 h at the beginning of the light period.

All experiments were solely performed on embryos before the start of external feeding. All tests were terminated at 48 ± 1.5 hpf (hours post fertilization) and can be regarded as non-

animal tests according to the legislation and regulations of the German federal state of North Rhine Westphalia and the EU Directive 2010/63/EU. Meanwhile, the German law harmonized the EU Directive 2010/63/EU and allows testing on fish embryos until the onset of independent feeding, which occurs around day 5 post fertilization.

2.2 Control and model chemicals

For a proof-of-principle study the newly established fluorescence endpoints should be validated using suitable model chemicals in comparison to reference (control) chemicals. Firstly, for proofing the validity of this test paradigm, three chemicals were selected as control substances: the small molecule SU4312, ethanol and the pharmaceutical pentoxifylline.

SU4312 is a known antiangiogenic compound, for which vascular impairment is confirmed (Molina et al., 2007; Tran et al., 2007; Vogt et al., 2009). It was selected as a positive control for the ISV-phenotype and length assessment. Vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) are the major regulators of angiogenic signaling by promoting endothelial cell differentiation, survival and migration (Ferrara and Alitalo, 1999; Zachary and Gliki, 2001; Chan et al., 2002). SU4312 specifically inhibits the VEGFR and thus, prevents the ISV outgrowth (Kendall et al., 1999; Molina et al., 2007). Therefore, it was suggested as a suitable positive control for the ISV-phenotype and length assessment and in addition as negative control for the other fluorescence endpoints. I chose pentoxifylline, a synthetic dimethylxanthine drug used to treat circulatory disorders (Oshihma et al., 1981; Mueller, 1985) as a negative control for the vasotoxicity assessment. It has haematological effects and its main mechanism of action is the inhibition of the erythrocyte phosphodiesterase, thereby increasing the intracellular concentration of cAMP (Bessler et al., 1986; Semmler et al., 1993). An antiangiogenic potential of pentoxifylline has so far not been described in the literature. Ethanol served as positive control for the evaluation of the neuro-, myotoxic and apoptotic potential. Ethanol is known to act as a neurotoxin, which additionally causes axial (teratogenic) defects as well as an increase of the apoptosis rate (Muth-Köhne et al., 2012).

In addition, triclosan, genistein and cartap were selected as model compounds. Triclosan is a commonly used antibacterial agent in personal care products (PCPs), textiles and furniture. Triclosan is effective against microbial growth through inhibition of the enoyl reductase in the fatty acid synthesis of bacteria and fungi (Russell, 2004; Dann and Hontela, 2010).

Moreover, toxicological studies showed triclosan to act as an endocrine disruptor in fish (Foran et al., 2000; Ishibashi et al., 2004) and frogs (Veldhoen et al., 2006). Additionally, Oliveira et al. (2009) suggested a vasotoxic potential of triclosan and proposed further investigations on the mode of action, as no detailed information are available so far (Oliveira et al., 2009). Genistein, a naturally occurring phytohormone present in e.g., soy products has been subject of numerous studies because of its known estrogenic activity. Sassi-Messai et al. (2009) discovered apoptotic effects in zebrafish embryos, which were estrogen-receptor independent and instead caused by inhibition of tyrosine-specific tyrosine kinases of the epidermal growth factor (EGF) receptor and of transthyretin amyloidosis (Green et al., 2005; Sassi-Messai et al., 2009). Additionally, Kalin et al. (2009) showed antiangiogenic activity of genistein in frogs, where it induced a severe ISV-phenotype. Cartap is an insecticide that targets the nicotinic acetylcholine receptor at the neuromuscular synapse (Nagata et al., 1997). It is known to act as a neurotoxin (Kwak et al., 2000; Zhou et al., 2009; Muth-Köhne et al., 2012) but no vasotoxic potential has been described as yet. Although, Zhou et al. (2009) described an aberrant ISV-phenotype in cartap-exposed zebrafish embryos.

2.2.1 Exposure concentrations

Zebrafish embryos were exposed from 1 – 2 h post fertilization (hpf) on 48 h to different concentrations of the following test substances: ethanol (9065.1, ROTIPURAN®, purity $\geq$ 99.8 %, p.a., Carl Roth, Karlsruhe, Germany), SU4312 (S8567, Sigma Aldrich, Munich, Germany), pentoxifylline (P1784), triclosan (Irgasan: 72779, Sigma Aldrich), genistein (G6649, Sigma Aldrich) and cartap (cartap hydrochloride: 45995, Sigma Aldrich).

According to initial range finding experiments and the calculated effect thresholds derived from the corresponding concentration-response relationships, I defined the following nominal test concentrations: ethanol (v/v): 0.5%, 0.71%, 1%, 1.41%, 2%; SU4312: 0.38, 0.61, 0.91, 1.44, 2.23, 3.48 μ M; pentoxifylline: 179.66, 303.83, 593.22, 1019.44 μ M; triclosan: 1.38, 1.73, 1.93, 2.18, 2.45, 2.76 μ M; genistein: 7.4, 8.81, 10.47, 12.43, 14.80 μ M; cartap: 0.42, 0.55, 0.67, 0.84 μ M.

2.3 Fish Embryo Toxicity Test

The Fish Embryo Toxicity Tests were conducted following the draft OECD Guideline for the testing of chemicals (OECD 2006) with modifications: 96-well plates were used instead of

24-well plates and the lethal as well as sub-lethal morphological effects assessed according to the descriptions of Nagel (2002) and Braunbeck and Lammer (2006).

For the test solution preparation, ethanol, pentoxifylline and cartap were dissolved directly in ISO water (prepared according to OECD guideline 203, Annex 2), whereas SU4312 was dissolved in dimethyl sulfoxide (DMSO, D8418, Sigma Aldrich), and triclosan and genistein at first in a solvent carrier. The stock solution of SU4312 in DMSO was prepared at a concentration of 5 g/L (w/v) SU4312 in 100% (v/v) DMSO. Test solutions were prepared by dilution of the corresponding volume of DMSO stock solution in ISO water and the addition of 100% (v/v) DMSO in order to ensure equal amounts of the carrier DMSO in all treatments. A DMSO concentration of 0.03% (v/v) was the minimum to achieve complete dissolution of SU4312. Stock solutions for ethanol, pentoxifylline, triclosan, genistein, and cartap had concentrations of 0.1 g/L, 1.0 g/L, 0.5 g/L, 1.0 g/L, and 0.1 g/L (w/v), respectively. The triclosan and genistein stock solutions were dissolved in 100% acetone (ROTIPURAN®, p.a., Carl Roth, Karlsruhe, Germany) and further dilutions were prepared in a final volume of 1 ml acetone each, with the dilution factors of 1.26 and 1.19, respectively. These stock solutions were used to coat the inner surface of 100 ml-Schott bottles. As solvent control, one bottle was coated with 1 ml of acetone only. After coating and complete evaporation of acetone, 100 ml of ISO water was added to the bottle to dissolve the coated chemicals. The solutions were stirred for a minimum of 2 h or until the test substance was completely dissolved and evenly mixed. If necessary, the solutions were sonicated for 20 min. To ensure oxygen saturation of $\geq 90\%$ and provide sufficient agitation, all test solutions were strongly aerated overnight prior to test start. In parallel, the U-bottom 96-well microtiter test plates (Greiner Bio-One, Frickenhausen, Germany) were saturated with the corresponding test chemical by incubating the wells with 200 μ l of the test solutions over night at 27°C. The solutions were then renewed prior to the test start. For the final tests, one healthy and fertilized egg was transferred to each well and exposed for 48 h to the corresponding test solution.

The embryos were microscopically assessed for lethal and sublethal developmental morphological effects after 24 and 48 h of exposure via bright field. Although the actual assessments took place between 1 and 2 h after 24 and 48 hpf, the denotation of the assessment time points were simplified to 24 hpf and 48 hpf to reflect the exposure time. This nomenclature is used throughout the text, all data charts and legends. The morphological effects were assessed and recorded following the description of the OECD draft guideline for the zFET of 2006 (OECD Draft TG 2006) and the background paper of Braunbeck and

Lammer (2006). Coagulation, no detachment of tail, no somites, no heartbeat and no differentiation were recorded as lethal effects in embryos at 48 hpf. The listing of all effects in embryos 24 and 48 hpf is provided in the Annex (8. 1). The data from the morphological bright field assessment are displayed as 1) effective concentration values (EC50 value), which derived from the concentration-response curves based on probit analysis using ToxRat® Professional (version 2.10, ToxRat Solutions GmbH, Alsdorf, Germany) and 2) cumulative effects of all embryos in percentage. The results of the effective concentration curves for the morphological bright field assessment are named as MBFA for a better discrimination from comparison with the qualitative fluorescence endpoint within the *Tg(fli1:EGFP)^{y1}* analysis (see 3.1.1). The presented zFET results were conducted on embryos of the *Tg(fli1:EGFP)^{y1}* line.

2.3.1 Test design and validity

For the test, wells of U-bottom 96-well plates were filled with 200 µl of test solution. One egg was transferred in one well, respectively. 2 – 3 replicates on individual plates for each treatment and control were prepared, where one replicate was defined as 12 embryos on a single plate. All tests were repeated independently for 3 times, resulting in three biological replicates with 2 – 3 technical replicates respectively, which were pooled for calculation.

To ensure the quality of the zFET results, I applied following validity criteria according the OECD draft (OECD Draft TG 2006): 1) the oxygen saturation should be at least 90% at the test start, 2) the temperature has to be kept at $26 \pm 1.0^\circ\text{C}$ throughout the test, 3) pH of the controls should range between 6.8 and 8.0 and the pH of the treatments between 6.5 and 8.5 and 4) the survival rate in the controls should exceed 90%. If one or more criteria could not be met, a test was considered invalid and had to be repeated.

2.4 Transgenic zebrafish lines *Tg(fli1:EGFP)^{y1}* and *Tg(gfap:GFP)*

The transgenic zebrafish line *Tg(fli1:EGFP)^{y1}*, generated by Lawson and Weinstein (2002), expresses enhanced GFP (EGFP) under the control of the *fli1* promoter, which belongs to the E-twenty six (ETS) transcription factor family. *Fli1* is crucial for vascular development (Liu et al., 2008). It is expressed in the very early haematopoietic precursors within the posterior lateral mesoderm from the three-somite stage and later in the endothelial precursors, which

develop into the blood vessels of the head and axial- and intersegmental blood vessels (Fig. 3 A+B).

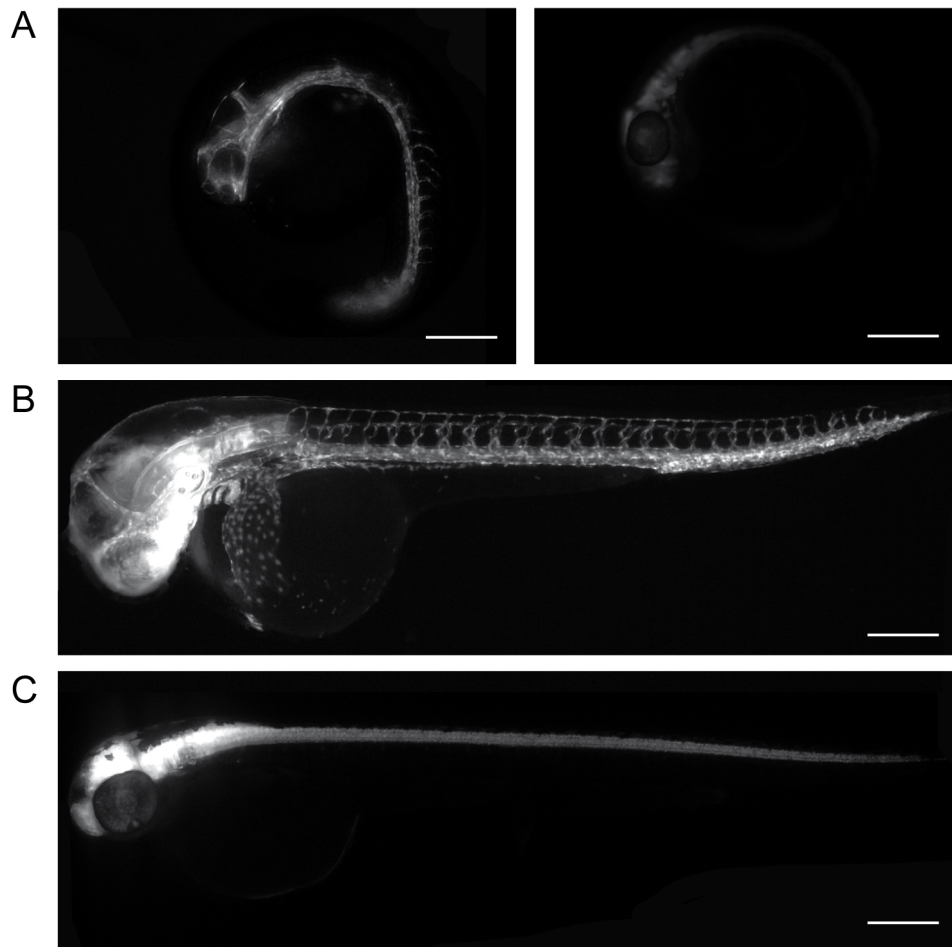


Fig. 3: Fluorescence images of zebrafish embryos 24 and 48 hpf of *Tg(fli1:EGFP)^{y1}* and *Tg(gfap:GFP)*. (A) Zebrafish embryos 24 hpf of *Tg(fli1:EGFP)^{y1}* (left) and *Tg(gfap:GFP)* (right) in their chorions. (B) Dechorionated zebrafish embryo 48 hpf of *Tg(fli1:EGFP)^{y1}*. GFP expression is located in the vasculature of the embryo. (C) Dechorionated zebrafish embryo 48 hpf of *Tg(gfap:GFP)*, which expresses GFP in the central nervous system (CNS). Scale bar: 250 μ m.

The EGFP-signal persists throughout all stages of development and can also be detected in the microvasculature of the fins of the adult fish (Lawson, 2002). The expression is strongest in the trunk and tail and can be found in the walls of the major vessels, e.g., dorsal aorta (DA), axial vein and intersegmental vessels (ISVs) (Thompson et al., 1998; Brown et al., 2000). The EGFP-signal of *Tg(fli1:EGFP)^{y1}* embryos corresponds to the expression pattern of the endogenous Fli1 protein (Lawson, 2002). Therefore, *Tg(fli1:EGFP)^{y1}* embryos facilitate the *in vivo* observation of both present and forming blood vessels and thus, of blood vessel development.

The development of the intersegmental blood vessels (ISVs) was chosen as a parameter to determine chemical-induced vascular impairment. ISVs sprout out from the dorsal aorta (DA)

and run between each pair of somites. Somites are the primary segments of the embryonic development of vertebrates, which develop from the mesoderm. The ISVs ventrally follow the chevron shape of the somite and more dorsally they cross the somite borders to terminally form the dorsal longitudinal anastomic vessel (DLAV) (Isogai, 2003) (Fig. 4). They arrange in a regular array and show a characteristic S-like pattern (Serbedzija et al., 1999; Childs et al., 2002; Isogai, 2003). The ISV development is time dependent and well described in Ellertsdóttir et al. (2010). It is suggested that chemical-induced ISV morphology aberrations can be used as an indication of vasotoxicity.

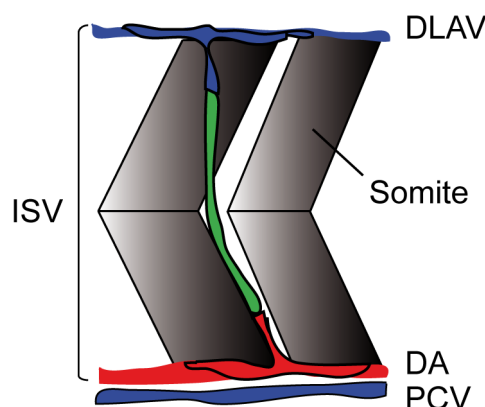


Fig. 4: Detailed sketch of the intersegmental vessel (ISV) of zebrafish embryos 48 hpf.

The ISV is composed of 3 types of endothelial cells (black framed): inverted T-shape cell (red) sprouts out from the DA, the connecting cell (green) courses between the somites ventral and dorsally it crosses the somite borders and the DLAV connecting T-shaped cell (blue). DA, dorsal aorta; DLAV, dorsal longitudinal anastomic vessel; PCV, posterior cardinal vein; ISV, intersegmental vessel. (Figure is modified from Childs et al., 2002)

The embryos of the transgenic zebrafish line *Tg(gfap:GFP)* co-express GFP with the gene of the glial fibrillary acidic protein (GFAP), which occurs mainly in the central nervous system (CNS) (Fig. 3 A+C). It is primarily expressed in the radial glial cells and astrocytes (Bernardos and Raymond, 2006). Next to brain lipid-binding protein (BLBP), GFAP is a common radial glial cell (RGC) marker in fish and mammals. RGCs differentiate from neuroepithelial cells and serve as a guiding scaffold for newborn neurons to migrate to their final destination. They also serve as progenitors of neuronal precursors, astrocytes and oligodendrocytes (Xing et al., 2014) but only the RGCs and astrocytes are GFAP-positive (Fig. 5). The RGCs represent the major cell type in the neural tube and persist throughout the entire life of zebrafish. Therefore, I chose the fluorescence intensity of the neural tube as a parameter to determine chemically induced neurodevelopmental impairment. Abnormalities in RGC development, function and differentiation as well as cellular interaction (between

RGCs and RGCs and neurons), can cause developmental brain disorders (reviewed in Xing et al., 2014).

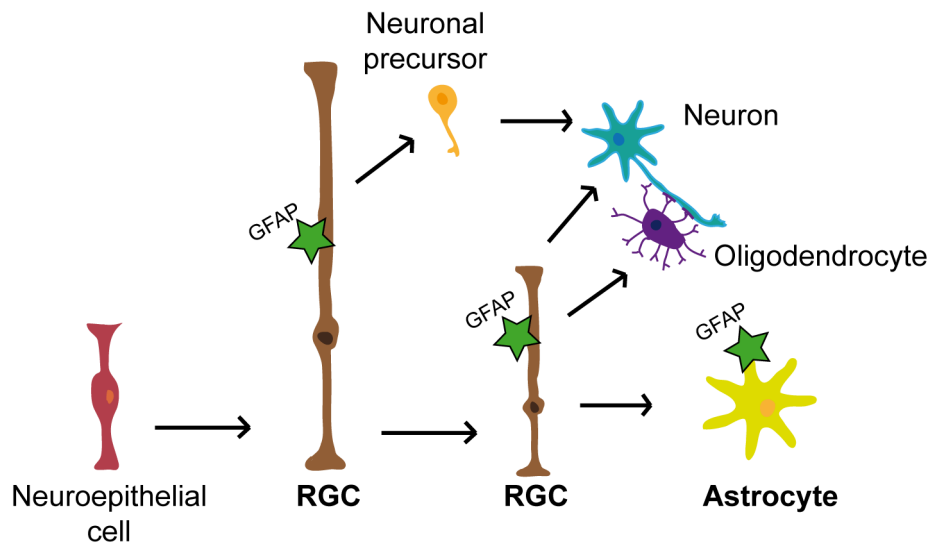


Fig. 5: Differentiation of GFAP-positive radial glial cells (RGCs) during CNS development.

Radial glial cells (RGCs) differentiate from neuroepithelial cells, which serve as progenitors for the differentiation into neuronal precursor, astrocytes, oligodendrocytes and also into further RGCs. The GFAP-positive RGCs and astrocytes are indicated with green stars. In these cells the *Tg(gfap:GFP)* zebrafish embryos express GFP. (Figure is modified from Xing et al., 2014)

The fluorescence signal of *Tg(gfap:GFP)* can be detected in the brain but also weakly in the retina, and most prominently in the spinal cord of 24 and 48 hour post fertilization (hpf) embryos (Bernardos and Raymond, 2006). The GFAP expression in zebrafish brain is reported to begin at the 12-somite stage and persists in the CNS glia of adult zebrafish (Bernardos and Raymond, 2006).

The transgenic embryos were treated and exposed to chemicals according to previous description (2. 3). The fluorescence microscope settings for imaging were excitation at 475 nm and emission at 509 nm for both.

2. 5 Whole-mount immunostaining

To analyze muscle morphology and thus the myotomal development, whole-mount immunostainings were performed with a specific primary antibody and a fluorescent secondary antibody. The primary antibody was F59, a mouse monoclonal antibody (IgG1, kappa light chain), which specifically binds to myosin heavy chain and adaxial muscle cells in zebrafish. Due to the binding of a fluorescent secondary antibody to the Fc kappa fragment of the primary antibody, the muscle structures became visible by fluorescence microscopy. The

secondary antibody used was DyLightTM 549-conjugated AffiniPure Goat Anti-Mouse IgG, specific for Fc kappa fragment (Jackson ImmunoResearch, Europe, code: 115-505-008). F59 was obtained from the Developmental Hybridoma Bank, University of Iowa, USA.

The visualization of the muscle fibers enabled the assessment of the angle between hemi-segments, as well as of the fiber morphology of zebrafish embryos (Fig. 9). The fluorescence microscope settings for analyzing were excitation at 512 nm and emission at 615 nm. Thus, the staining method could be conducted on both transgenic lines without losing fluorescence signal information as well on wild type embryos.

For the whole-mount antibody staining the control and treated embryos were manually dechorionated after 48 h of substance exposure and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) for 4 h at room temperature (RT). Then they were rinsed three times for 10 min with PBS/0.1% (v/v) Triton X-100 (PBST) and stored at 4°C. I performed whole-mount immunostainings as described in a book chapter written by Westerfield (2000), with the following modifications: Briefly, fixed embryos were permeabilized in ice-cold acetone for 7 min at -20°C, rinsed with distilled water for 5 min and washed three times for 10 min with PBST. To prevent nonspecific binding, the embryos were incubated in blocking solution (PBST/2% (v/v) normal goat serum) for 4 h at room temperature with agitation, and then incubated with the primary antibody (5 µg/ml in blocking solution) over night at 4°C. The embryos were washed five times for 10 min with PBST and subsequently incubated with the appropriate secondary antibody (1:500 dilution) for 4 h at room temperature. They were washed another five times for 10 min with PBST and mounted on glass slides in methylcellulose for imaging.

2.6 Vital staining with acridine orange

As apoptosis is an important cellular process in embryonic development (Abrams et al., 1993) it was the intention to evaluate the potential of the chemicals to influence the normal apoptosis rate. Cole and Ross (2001) have described the normal patterns of apoptosis in various structures of the developing zebrafish embryos. The dynamics of apoptosis in zebrafish embryos follow a time course and mainly occur during neurulation and somitogenesis (Cole, 2001). Quantification of the level of apoptosis in zebrafish embryos is commonly performed by staining with the vital dye acridine orange (AO) (Parnig et al., 2002; Tucker and Lardelli, 2007).

For staining live zebrafish embryos, control and treated embryos 48 hpf of the wild type were incubated in 2 µg/ml acridine orange (Sigma-Aldrich) for 30 min after manual dechoriation. After three washing steps of 10 min each in ISO water, the embryos were anesthetized in phosphate-buffered benzocaine (4-aminobenzoic acid ethyl ester, E1501, Sigma-Aldrich; 2 mM Na₂HPO₄) and mounted in 3% (w/v) methylcellulose for imaging. The fluorescent cells could be detected by an excitation at 502 nm and an emission maximum at 525 nm.

2.7 Fluorescence imaging

For the microscopic assessment, embryos were mounted laterally on a glass slide in 3% (w/v) methylcellulose and covered with a cover slip for imaging. 5 – 8 embryos per treatment of each assessment method were mounted on one slide, respectively, to assume optimal lateral position of at least 3 embryos per treatment for a convenient assessment. To maximize the statistical value I tried to analyze more embryos than 3, but due to practicability and time effort the number varies between 3 – 5 embryos per treatment and assessment method. Thus, the minimal number of embryos assessed was three for each treatment within each assessment. For live assessment, embryos were anesthetized in phosphate-buffered benzocaine (0.5 mM 4-aminobenzoic acid ethyl ester, E1501, Sigma–Aldrich; 2mM Na₂HPO₄). All embryos were analyzed on a motorized, inverse bright field microscope with integrated fluorescence application (Leica System AF6000) and photographed under a 5x and 10x objective; depending on the examined structure (whole embryos for GFAP expression in RGCs and apoptosis measurements, and detailed regions for the assessment of the vascular and myotomal structures, respectively). For imaging two different fluorescence filter cubes had to be used: for the GFP and AO imaging a filter cube with excitation filter: 480/40 nm, dichromatic mirror: 505 nm and suppression filter: 527/30 nm and for the DyLight imaging a filter cube with excitation filter: 540/40 nm, dichromatic mirror: 565 nm and suppression filter: 610/75 nm.

Embryos of one assessment method were photographed using always the same microscopic settings (exposure, gain and intensity), respectively. Microscopic z-stack images were acquired and processed using the Leica LAS-AF software (Leica Application Suite, Advanced Fluorescence, version 2.2.0, Wetzlar, Germany), and analyzed using the free software ImageJ (National Institutes of Health). All focus areas of the individual images of each z-stack were integrated into a full-focused image (extended depth of field) using the ImageJ plugin “Stack

focuser". The full-focused images were subsequently analyzed according to the region of interest (ROI, see chapter 3. 1) and the fluorescence endpoint assessed.

All images were acquired and processed as black and white images (8 bit). For illustration, full-focused images were manually colored and edited, like cut to size, color processed, contrast control, etc. with Photoshop Adobe® CS4 (version 11.0.2). All shown images display a lateral view of the embryos with anterior towards the left and dorsal up. For the final evaluation of the fluorescence assessment methods, 3 independent experiments were performed, respectively.

The development of the fluorescence endpoint analysis methods and the image analysis was a major task of this thesis and is therefore described in the results section (chapter 3. 1).

2. 8 Real-time quantitative PCR

The question was raised whether the quantification of target gene expression could further increase the sensitivity of the zFET. With gene expression analysis, it was also envisaged to validate the morphological effects, which were detected via fluorescence microscopy. In particular the linkage between the phenotypic effects, which were detected in the transgenic lines, alterations in gene expression levels were aimed to be established.

Total RNA was extracted from control and treated embryos 48 hpf using an optimized Trizol-method: Total RNA was isolated from frozen, pooled embryos by homogenization in TRI-Reagent (T9424, Sigma-Aldrich) using motor-driven plastic pistils. After centrifugation, RNA extraction with chloroform and ethanol and purification using RNeasy Mini Spin Columns (QIAGEN, Hilden, Germany) were performed according to manufactures' protocols. RNA was then stored at -80°C until further analysis. RNA concentration was measured using a Nanotrop 1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany). All further processed RNA samples had RNA integrity at 260/280 absorbance between 1.8 and 2.2. These RNA samples were then diluted in RNase-free, DEPC-treated water and DNase I (Sigma-Aldrich, AMP-D1) digested according to manufacturer's instructions. I used 50 ng of total RNA for cDNA synthesis with Superscript III (Invitrogen, Carlsbad, USA). Real time quantitative PCR using SYBRGreen (BioRad®, Hercules, CA, USA; SYBR®GreenER qPCR SuperMix, Invitrogen) was performed on a BioRad iQ5 cycler (94°C for 3 min, 35 cycles: 94°C for 45 sec, 59°C for 30 sec, 72°C for 30 sec; 72°C for 10 min) using primers specific to

following gene transcripts: *fli1a* (*friend leukaemia integration1*) forward primer 5'-ggtgaacgtcaagcgagagt-3'; reverse primer 5'-atggcccagtctaaccactg-3', *cdh5* (*VE-cadherin*) forward 5'-tgcgtatgaggaaacacgac-3'; reverse 5'-cagctttgcaaggacaaca-3', *flt4* (*fms-related tyrosin kinase 4*) forward 5'-caggatgagagcaccagaca-3'; reverse 5'-agcttgtagagaagccatcgt-3' and *gfap* (*glial fibrillary acid protein*) forward 5'-gccgagctgaaccagctgaggg-3'; reverse 5'-tgccgcagggcagtcctcgtc-3'.

The specific primers were designed using the web-based NCBI PrimerBlast tool (www.ncbi.nlm.nih.gov/tools/primer-blast). I used the housekeeping genes *18s* and *rpl8* as internal standards and a negative control without cDNA template was run with every assay to assess overall specificity. Q-PCR cycling conditions were 95°C for 8 min, for amplification 40 cycles of 95°C for 15 s, 59°C for 30 s, 60°C for 30 s, followed by 95°C for 1 min, 55°C for 1 min and 55°C for 10 s. Relative expression ratio was calculated according to the $2^{-\Delta\Delta Ct}$ method (Paffl, 2004). RT-QPCR analysis was done with samples of three independent experiments.

2.9 Statistics

All experiments were independently repeated three times and means were calculated for comparison. The statistical analyses were performed with GraphPad Prism (version 4.0b). For normally distributed data (D'Agotino and Pearson omnibus normality test), one-way ANOVA and Dunnett's post-hoc test for multiple comparisons were performed. For non-normally distributed data, the Students t-test and Man-Whitney test were performed. Significant differences to the controls and the corresponding significant levels are indicated by asterisks (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

3. Results

Different fluorescence markers were used to visualize the vascular system, the neural tube and the muscle morphology as well as apoptotic cells in the zebrafish embryo, which were otherwise not visible in conventional bright field microscopy. Analysis methods for these morphological structures were developed and quantifiable fluorescence endpoints were defined. This will be described in detail in the following sub-chapter (chapter 3. 1). Subsequently in sub-chapter 3. 2, the applicability of these fluorescence endpoints and their potential for detecting and evaluating chemical-induced effects were investigated in a proof-of-principle study. The comparison of all bright field and fluorescence assessment endpoints is then presented in sub-chapter 3. 3. Finally, the results of the gene expression analysis are shown (3.4).

3.1 Establishment of fluorescence-based toxicity assessment methods and test endpoints

For the detection of vascular, neuronal and myotomal effects as well as change in the apoptosis rate fluorescent endpoints were assessed on the basis of image analysis. For each endpoint, a suitable morphological region within the embryo had to be defined to make the analysis most meaningful. Another important aspect that was taken into account when defining the analysis regions (= region of interest (ROI)) was the potential automation of the image analysis. The fish embryos were mounted laterally for easy analysis. The analysis was confined to the trunk section of the embryos including tail (Fig. 6). The region of the yolk sac was excluded from the analysis due to high auto-fluorescence. Endpoint analysis was conducted using full-focused images, which were assessed depending on the ROI and the corresponding fluorescence endpoint, as described as follows.

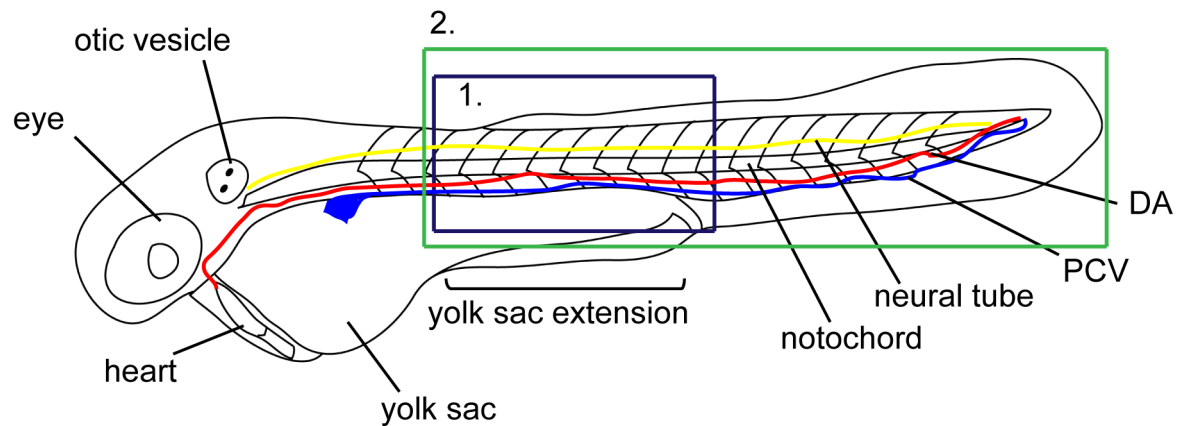


Fig. 6: Sketch of a zebrafish embryo 48 hpf, showing the regions of interest (ROIs) for the image analysis of the fluorescence microscopy.

For image analysis of the fluorescence endpoints, the regions of interest (ROI) were confined to the trunk and tail of the zebrafish embryo. Frame 1 (dark blue frame) indicates the ROI of the endpoints ISV-defects and ISV-length and angle between two hemi-segments. Frame 2 (green frame) indicates the ROI of the endpoints fluorescence intensity in the neural tube and the number of fluorescent cells). DA, dorsal aorta; PCV, posterior cardinal vein.

3.1.1 Vasotoxicity endpoint

To assess toxic effects on ISV development, eight blood vessels in the region above the yolk sac extension were evaluated for defects in each individual embryo of the *Tg(fli1:EGFP)^{y1}* line. Defects of the ISVs were classified according to the following criteria: truncated, looped, missing, irregularly branched. The individual defects were recorded when present (yes and no answer). Concentration-response relationships were established and EC50 values derived. For simplification, this qualitative effect evaluation was defined as ISV fluorescence assessment, ISVFA.

In a second step of the analysis, the lengths of the ISVs were determined. To account for biological interindividual size variation, the individual ISV-length measurements were normalized by calculating the ratio between the vessel length and the trunk width (dorso ventral axis), thereby obtaining the relative ISV-length (Fig. 7 C). To measure the vessel length, the tracing tool of the ImageJ Plugin *NeuronJ* was used (violet line). The trunk width in the area of each ISV measured was determined with the segmented line tool (blue line; Fig. 7 B). An average relative ISV-length across all control embryos at 24 hpf was determined 45% of the torso width and 80% at 48 hpf. Since the ISV-length is smaller than the trunk width, the calculated relative ISV-lengths remained <100% for the 48 hpf control embryos even though the ISVs had reached the maximum length. I therefore decided to set the relative ISV-length of untreated embryos at 48 hpf to 100%, and all other ISV-lengths were set in

relation to the control. The image section provided by the microscope enabled a convenient observation of eight ISVs independent of the lateral embryo orientation (horizontal or vertical). Furthermore, the measurement of more than eight vessels resulted in statistically imprecision, as even in control embryos the ISVs become shorter with the progression of the tail region. Therefore, eight vessels/embryo were analyzed of 3 – 5 individual embryos per treatment to ensure significance validity.

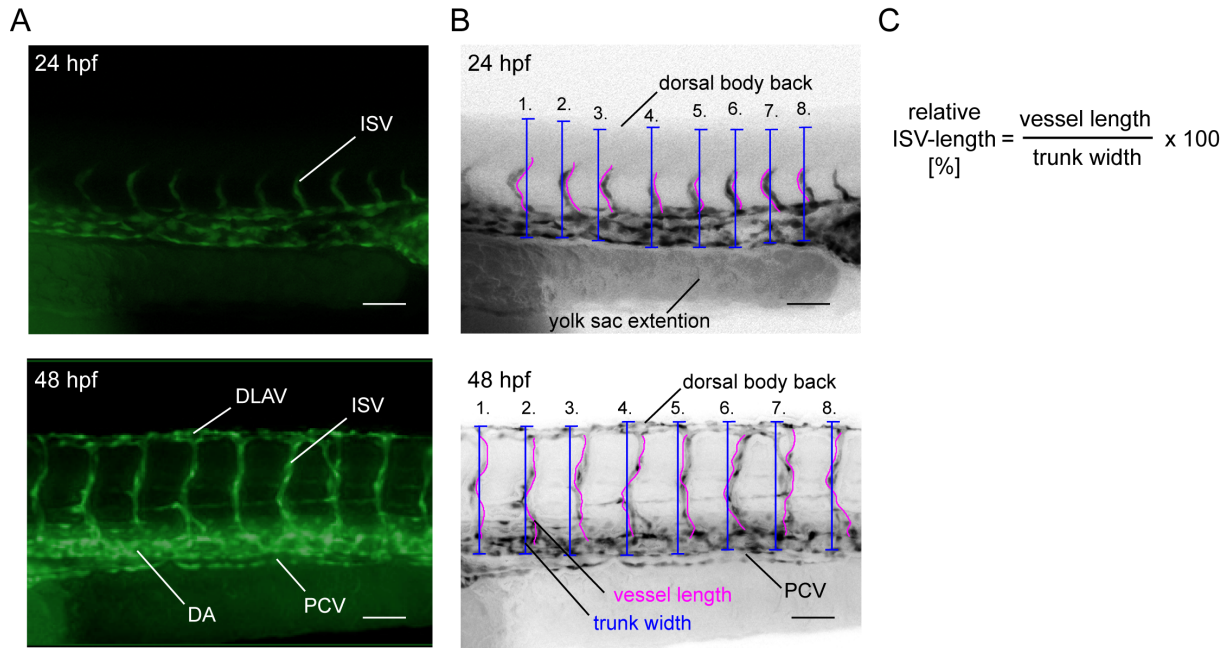


Fig. 7: Blood vessels of control *Tg(fli1:EGFP)^{y1}* embryos (A) and image analysis of the relative ISV-length by comparison of the vessel length and trunk width (B + C) (methods 2. 4 and 2. 7).

(A) Fluorescence full-focused images of the EGFP signal in the trunk of *Tg(fli1:EGFP)^{y1}* embryos at 24 hpf (upper panel) and 48 hpf (lower panel). (B) Images were processed by ImageJ into inverted black and white images, which were used for measuring the relative ISV-length of 24 and 48 hpf embryos. Therefore, individual vessel lengths (violet lines) were measured with a tracing tool of NeuronJ (plugin of ImageJ) and the trunk width (blue line), which was determined between the dorsal margin of the yolk sac extension (24 hpf) respectively the PCV (48 hpf) and the dorsal body back in the area of every ISV with the segmented line tool of ImageJ. The ISV-length and trunk width were measured for 8 ISVs per embryo. (C) For analysis of the normalized vessel development, the relative ISV-length was calculated by the ratio of vessel length and trunk width, which is shown by the formula. Images were obtained by standard fluorescence microscopy. All panels are oriented with anterior towards the left. DA, dorsal aorta; DLA, dorsal longitudinal anastomic vessel; PCV, posterior cardinal vein; ISV, intersegmental vessel. Scale bar: 50 μ m.

3.1.2 Neurotoxicity endpoint

The GFP signal of *Tg(gfap:GFP)* embryos is confined to the CNS, where the neural tube shows consistent GFP intensity, which can decrease with chemical exposure. The neural tube has a clearly defined, unstructured shape compared to the ISVs. Hence, I selected no dimensional measurement but the overall fluorescence intensity of the neural tube as an endpoint for detecting chemical-induced defects.

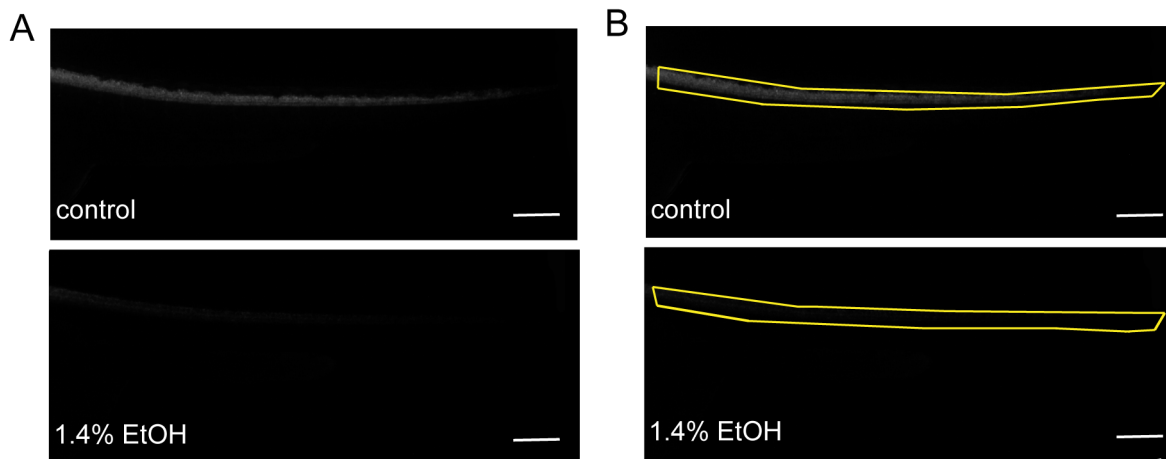


Fig. 8: Image analysis of the fluorescence intensity of the neural tube in *Tg(gfap:GFP)* embryos 48 hpf with ImageJ (methods: 2. 4 and 2. 7).

(A) Fluorescence full-focused image of the trunk of *Tg(gfap:GFP)* control embryo and embryo exposed to 1.4% (v/v) ethanol after 48 h (method: 2.3). Images were obtained by standard fluorescence microscopy always under the same settings: exposure 1.3 sec., gain 1.6 and intensity 5. (B) For analysis of the fluorescence intensity the obtained images were not further processed. With the ImageJ polygon-working tool (yellow line) the region of interest (ROI) of the neural tube was selected and the integrated density (IntDen = fluorescence intensity) was measured. Anterior is towards the left in all panels. Scale bar: 100 μ m.

For the assessment of chemical effects on RGCs, the *Tg(gfap:GFP)* embryos were photographed always using the same microscopic settings (exposure: 1.3 sec; gain: 1.6; intensity: 5), and the obtained full-focused images were not further processed. This was to ensure direct comparability of fluorescence intensity values between different fish. For measurement, the ROI was selected with the polygon selection tool (Fig. 8 B). The fluorescence intensity was then determined as integrated density/area (IntDen/area) in ImageJ. The integrated density determines the ratio of the area and the mean gray value, i.e., the sum of the gray-values of all pixels within the ROI. Readings were provided as percentages of control density. Therefore, the mean integrated density/area of all untreated embryos at 48 hpf was defined as 100%, and all the other integrated density/area values were set in relation to the control.

3.1.3 Myotoxicity endpoint

For the analysis of the muscle morphology of zebrafish embryos, whole-mount immunostainings with monoclonal antibody F59 were performed as described in chapter 2. 5. Zebrafish embryos have slow, tonic skeletal muscle fibers (red), which are active during slow swimming and fast, twitch muscle fibers (white), which are active during fast swimming (Buss and Drapeau, 2002). The secondary fluorescent antibody binds the antibody F59, which is specific to myosin and adaxial muscle cells and stains the red muscle fibers in zebrafish (see methods 2. 5). This enabled the detection of muscle abnormalities such as truncated fibers, disorganized fibers and altered angles between dorsal and ventral hemi-segments of myotomes caused by chemicals (Sylvain et al., 2010). In normally developed embryos both hemi-segments stand in a defined angle to each other and an aberration indicates disruption of embryogenesis thus, a teratogenic effect (Sylvain et al., 2010). Hence, I selected the angle between the two hemi-segments of somites to be measured to identify chemicals impacting on muscle morphology.

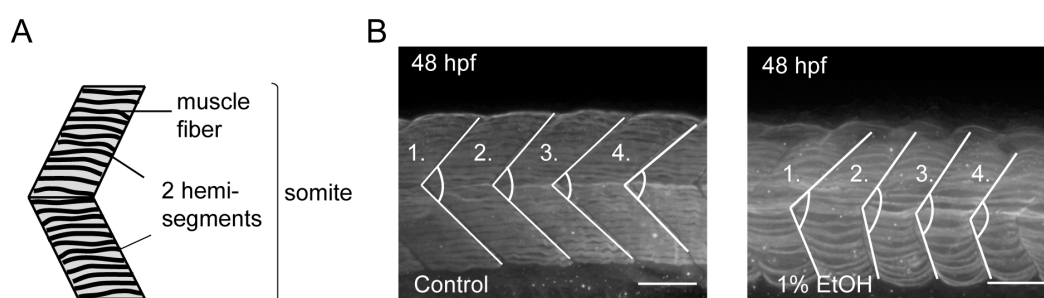


Fig. 9: Image analysis of whole-mount immunostaining with antibody F59 of the muscle morphology in zebrafish embryos 48 hpf with ImageJ (methods: 2. 5 and 2. 7).

(A) Detailed sketch of the zebrafish embryo somite structure. The myotomal segment is composed of two hemi-segments, the dorsal and ventral myoseptum, which are innervated by organized muscle fibers. (B) Full-focused images of zebrafish embryos 48 hpf stained with specific monoclonal antibody against myosin (F59) after no exposure (control) and 1% (v/v) ethanol exposure (method: 2. 3), obtained by fluorescence microscopy. With the help of the angle tool of ImageJ the angle between the two hemi-segments of 4 segments was measured. Anterior is towards the left in all panels. Scale bar: 50 μ m.

To assess defects of the myotome, 3 – 5 embryos per treatment were laterally photographed (z-stack images). The generated full-focused images were not further processed and directly used to measure the angle between the dorsal and ventral hemi-segment. It has been proven that in control embryos the values of the angles between the hemi-segments were only constant in the region above the yolk sack extension. Hence, the angles of 3 – 5 randomly chosen segments above the yolk sac extension were measured as it is demonstrated in figure 9

B. The angle between the two hemi-segments of normally developed 48 hpf zebrafish embryos was around $98^{\circ} - 100^{\circ}$ (Fig. 9 B).

3.1.4 Apoptosis endpoint

Apoptotic cells were visualized by acridine orange staining and analyzed in full-focused images of 3 – 5 embryos per treatment. To discriminate the fluorescent cells from background fluorescence, a threshold was manually set for each picture (Fig. 10 B). The threshold is used to classify image points as objects and to distinguish between focused pixels or background (noise signals) according to their intensity values (Gerlich et al., 2003), resulting in binarized images. After conversion into binary images, the region of the trunk and tail without the yolk sac extension was selected as ROI using the polygon tool of ImageJ (Fig. 10 C). The fluorescent (apoptotic) cells were counted with the tool '*Analyze Particles*' after defining '*Outlines*' additionally to the default settings. This approach enables counting of small single fluorescent cells (Fig. 10 D). In control embryos, the amount of apoptotic cells was $27 (\pm 2)$ cells per region of interest (ROI).

In case of a high density of substance-induced apoptotic cells, I measured the integrated density of the converted binary images. Using the polygon tool of ImageJ, the same region of the trunk and tail without the yolk sac extension, like in the cell counting, was selected as ROI. The integrated density/area (IntDen/area) of 3 – 5 embryo per exposure were measured (see 3.1.2: Neurotoxicity endpoint). Measurements are presented as percentages compared to control. Whereas the mean integrated density/area of all untreated embryos at 48 hpf was defined as 100% and all the other integrated density/area values set in relation to the control.

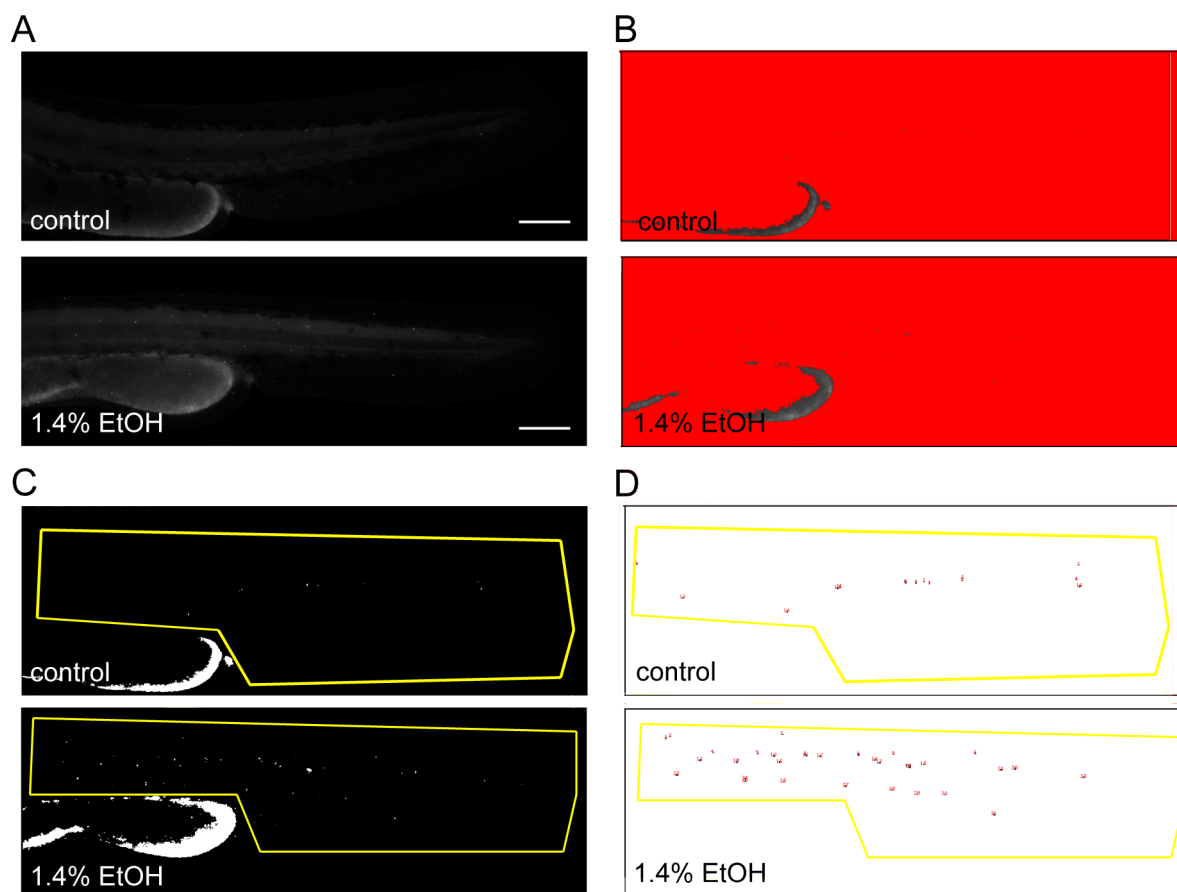


Fig. 10: Image analysis of vital staining of apoptosis with acridine orange in zebrafish embryos 48 hpf with ImageJ (methods: 2. 6 and 2. 7).

(A) Fluorescence full-focused image of wild type zebrafish embryos 48 hpf after no exposure (control) and 1.4% (v/v) ethanol exposure (method: 2.3), which were additionally stained with 2 $\mu\text{g/ml}$ (w/v) acridine orange, respectively. (B) For analysis of the stained apoptotic cells, an individual threshold (red color) was manually set in ImageJ. (C) The images were converted into binary images, where the region of interest (ROI) was selected with the polygon tool (yellow line). After executing 'analyze particles' in ImageJ, the output of the number of particles (= fluorescent cells) was given, displayed as an image (D) and data table (not shown). The number of particles equals the number of apoptotic cells. Images were obtained by standard fluorescence microscopy. Anterior is towards the left in all panels. Scale bar: 100 μm .

3. 2 Proof-of-principle study

In a proof-of-principle study, exposure of triclosan, genistein and cartap in comparison to reference (control) chemicals (ethanol, SU4312 and pentoxifylline) were used to demonstrate the validity of the newly developed fluorescence endpoints. The toxicity of these chemicals was initially assessed with bright field microscopy (3.2.1) and subsequently analyzed applying the new fluorescence endpoints (3.2.2 - 3.2.5). In terms of ethical and economic reasons, I conducted the morphological bright field assessment (zFET) and the immunostainings, as well as the vasotoxicity assessment on *Tg(fli1:EGFP)^{y1}* embryos. The neurotoxicity assessment was done with *Tg(gfap:GFP)* and the apoptosis assessment with

wild type embryos. The vital staining of the apoptotic cells had to be performed on wild type embryos due to the overlapping fluorescence signals of the AO staining and the EGFP/GFP of the transgenic lines. Thus, I was able to gain a maximum amount of data with minimal number of animals. Additionally, it had contributed to minimize the time and effort of the assessment.

3.2.1 Morphological phenotype determined by bright field microscopy

For general toxicological characterization of the effects of ethanol, SU4312, triclosan, genistein, and cartap on zebrafish embryos, standard 48 h zFETs were conducted and assessed by bright field microscopy. For all substances, a concentration-dependent increase in the effects was established (Fig. 12 and Fig. 13). Zebrafish embryos exposed to ethanol, SU4312, triclosan, genistein and cartap displayed a broad range of morphological abnormalities compared to the normal phenotype (Fig. 12). In figure 12 are next to the mortality, sublethal effects decoded, which can be linked to substances' vasotoxic potential (reduced blood circulation, edema), neurotoxic potential (malformation of notochord) and myotoxic potential (malformation of tail) after 48 h of substance exposure. The decoding of all sublethal effects after 24 h of exposure is not shown since they can not be directly correlated to the tissue-specific toxicity classes, except of the endpoint no spontaneous movement. This effect is only recorded after 24 hpf and indicates a neurotoxic disruption (Irons et al., 2010), which is why it is additionally shown in figure 12. The summarized effects (lethal and sublethal) recorded after 24 h and 48 h of exposure can be found in figure 13. Typical effect phenotypes are depicted in figure 11. The results of the negative control of blood vessel development, pentoxifylline, are described separately in sub-chapter 3.2.2.

The bright field analysis showed that ethanol mainly reduced blood circulation and caused pericardial edema at concentrations $\geq 0.71\%$ (Fig. 12 A). I observed malformations of tail at the highest test concentration of 2% (v/v) ethanol only. The sum of all morphological effects determined by bright field microscopy is displayed in figure 13. Ethanol concentrations $\geq 1.41\%$ (v/v) caused significant effects compared to the control ($p = 0.01$) after 48 h of exposure (Fig. 13 A). Assessment of 24 h ethanol treatment was not recorded.

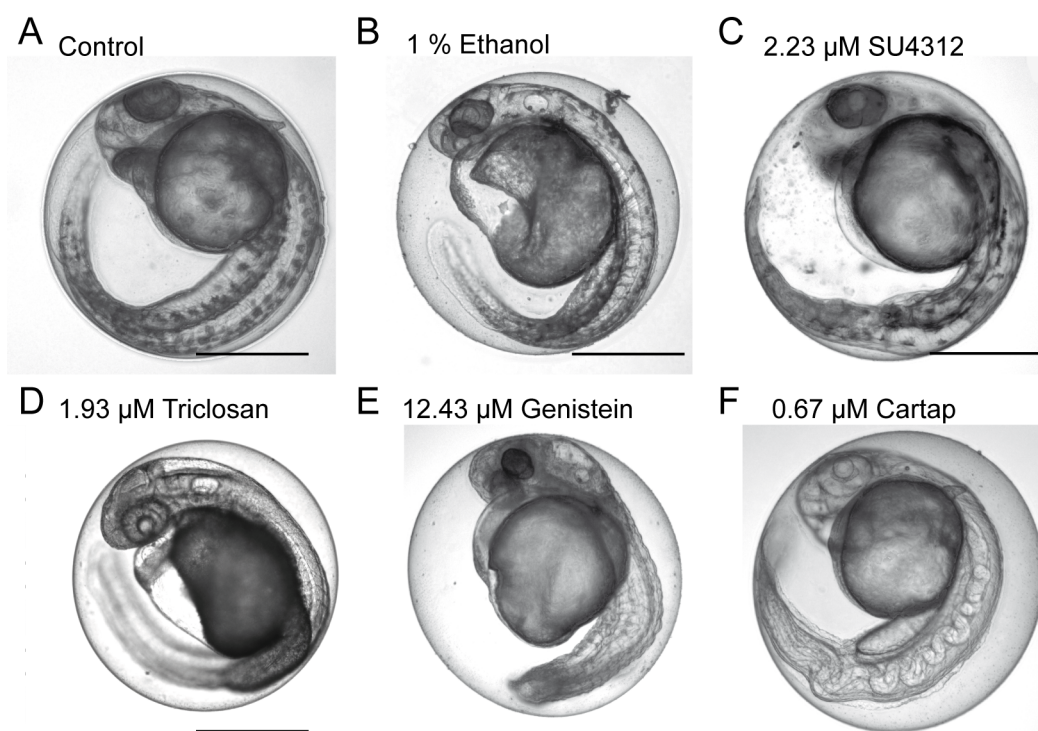


Fig. 11: Morphological phenotype of control and exposed 48 hpf-zebrafish embryos (method: 2. 3).

Exemplary bright field micrographs of a normally developed control zebrafish embryo (A) and embryos displaying different effects due to exposure to nominal 1% (v/v) ethanol (B), 2.23 μM SU4312 (C), 1.93 μM triclosan (D), 12.43 μM genistein (E) and 0.67 μM cartap (F). Images were obtained by standard bright field microscopy. Live embryos were photographed in 96 well plates along their z-axis using the LAS-AF software of the Leica microscope. The obtained z-stacks were processed into full-focused images using ImageJ (methods analog to 2.7). The exposed embryos showed typically malformations of the heart and/or notochord and pericardial edema. Scale bar: 500 μm .

The antiangiogenic control substance SU4312 caused a significant increase in morphologically impaired embryos from 1.44 μM after 24 h ($p = 0.05$) and 2.23 μM after 48 h ($p = 0.01$) of exposure (Fig. 13 B). SU4312 exposure primarily caused coagulation of the eggs (mortality). The mortality increased with increasing concentration up to 39% at a concentration of 3.48 μM of SU4312 (Fig. 12 B). After 24 h, a significant 30% of embryos of the 1.44 μM treatment were coagulated and after 48 h the significance threshold was at 2.23 μM corresponding to 52% of the exposed embryos. The most apparent effects associated with vasculo- and angiogenesis, i.e. reduced blood circulation and heart malformation, only occurred at higher test concentrations and did not exceed 20% of the embryos (Fig. 12 B). The highest SU4312 concentration of 3.48 μM affected 64% of the embryos.

Triclosan and genistein caused a wide range of morphological effects. In triclosan-exposed embryos, I observed a reduced blood circulation, often in combination with blood accumulations in the tail, and malformations of the tail, as well as pericardial and yolk sac

edema at 48 hpf and a lack of spontaneous movement at 24 hpf (Fig. 12 C). Additionally, general growth retardation was recorded (Appendix 8. 1, Table 8). Lethal effects occurred after 48 h of exposure at a concentration of $\geq 2.45 \mu\text{M}$, and at $2.76 \mu\text{M}$ 35% of the embryos were found dead. Cumulative effects showed significant differences to the control at $2.45 \mu\text{M}$ after 24 h of exposure ($p = 0.05$), and after 48 h of exposure at the highest test concentration of $2.76 \mu\text{M}$ ($p = 0.01$; Fig. 13 C).

Genistein caused a strong induction of sublethal effect, whereas lethality was only observed in 28% of the embryos at the highest test concentration of $14.80 \mu\text{M}$ after 48 h of exposure (Fig. 12 D). A prominent sublethal effect was the malformation of tail, which occurred in 88% of the embryos treated with $14.80 \mu\text{M}$ of genistein. Further, I detected reduced blood circulation and edema (Fig. 11 E). 48 h genistein treatment resulted in a significant impairment of zebrafish embryo development from a concentration of $\geq 10.47 \mu\text{M}$ ($p = 0.01$; Fig. 13 D). After 24 h of genistein exposure, only the highest test concentration of $14.80 \mu\text{M}$ caused a significant increase in embryos affected ($p = 0.01$), with 92% of embryos showing morphological abnormalities (Fig. 13 D).

Cartap treatment mainly affected the notochord morphology (Fig. 11 F). At a concentration of $0.55 \mu\text{M}$, 8% of the embryos displayed a slightly undulating notochord. At cartap concentrations of $0.84 \mu\text{M}$, 88% of embryos displayed severe notochord deformations (Fig. 12 E). Additionally, loss of pigmentation was observed after 48 h (Annex 8. 1, Table 10). No effects on the blood circulation or heart morphology could be detected. After cartap exposure, fewer than 10% of the embryos died (Fig. 12 E). I determined significant morphological effects caused by cartap exposure (24 and 48 h) at concentrations of 0.67 and $0.84 \mu\text{M}$ ($p = 0.01$; Fig. 13 E).

The results demonstrated that all substances but cartap affected the blood circulatory system of the zebrafish embryos.

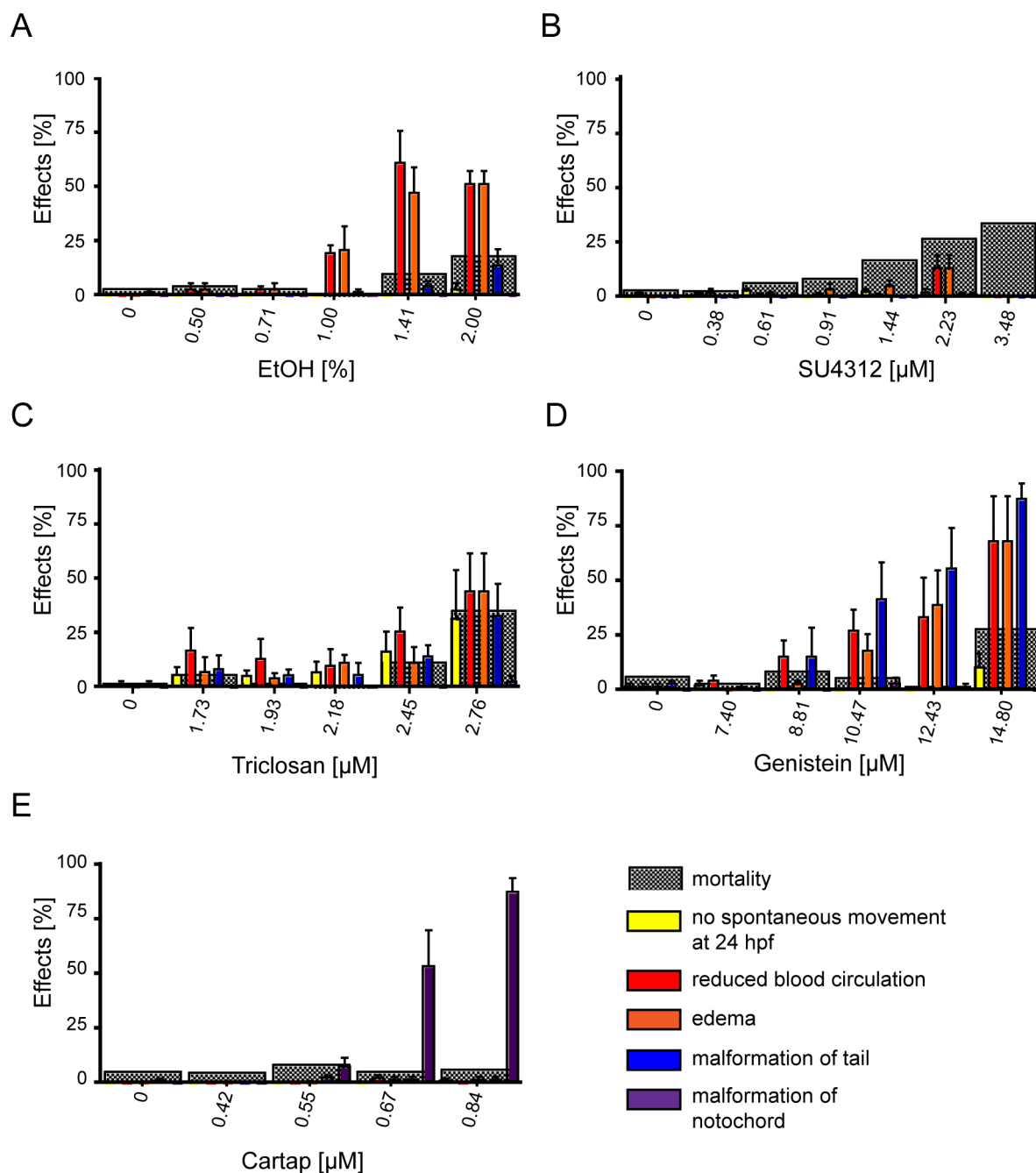


Fig. 12: Lethal and sublethal morphological effects occurring in zebrafish embryos exposed for 48 h to different concentrations of (A) ethanol, (B) SU4312, (C) triclosan, (D) genistein or (E) cartap (method: 2. 3).

Cumulative morphological effects according to OECD draft guideline (OECD TG 2006) and background paper of Braunbeck and Lammert (2006) were determined by microscopic bright field assessment (zFET; mean + SEM of 3 independent experiments with $n = 21 - 50$ embryos per concentration and experiment). Lethal effects (coagulation, no detachment of tail, no somites, no heartbeat and no differentiation) were summarized as mortality. Sublethal effects, which can be linked to substances' vasotoxic potential: reduced blood circulation, edema; neurotoxic potential: no spontaneous movement at 24 hpf, malformation of notochord and myotoxic potential: malformation of tail, are displayed. All effects displayed were recorded after 48 h of exposure, except the observation no spontaneous movement at 24 hpf. This additional endpoint was included as it represents an apical effect, which typically indicates a neurotoxic potential.

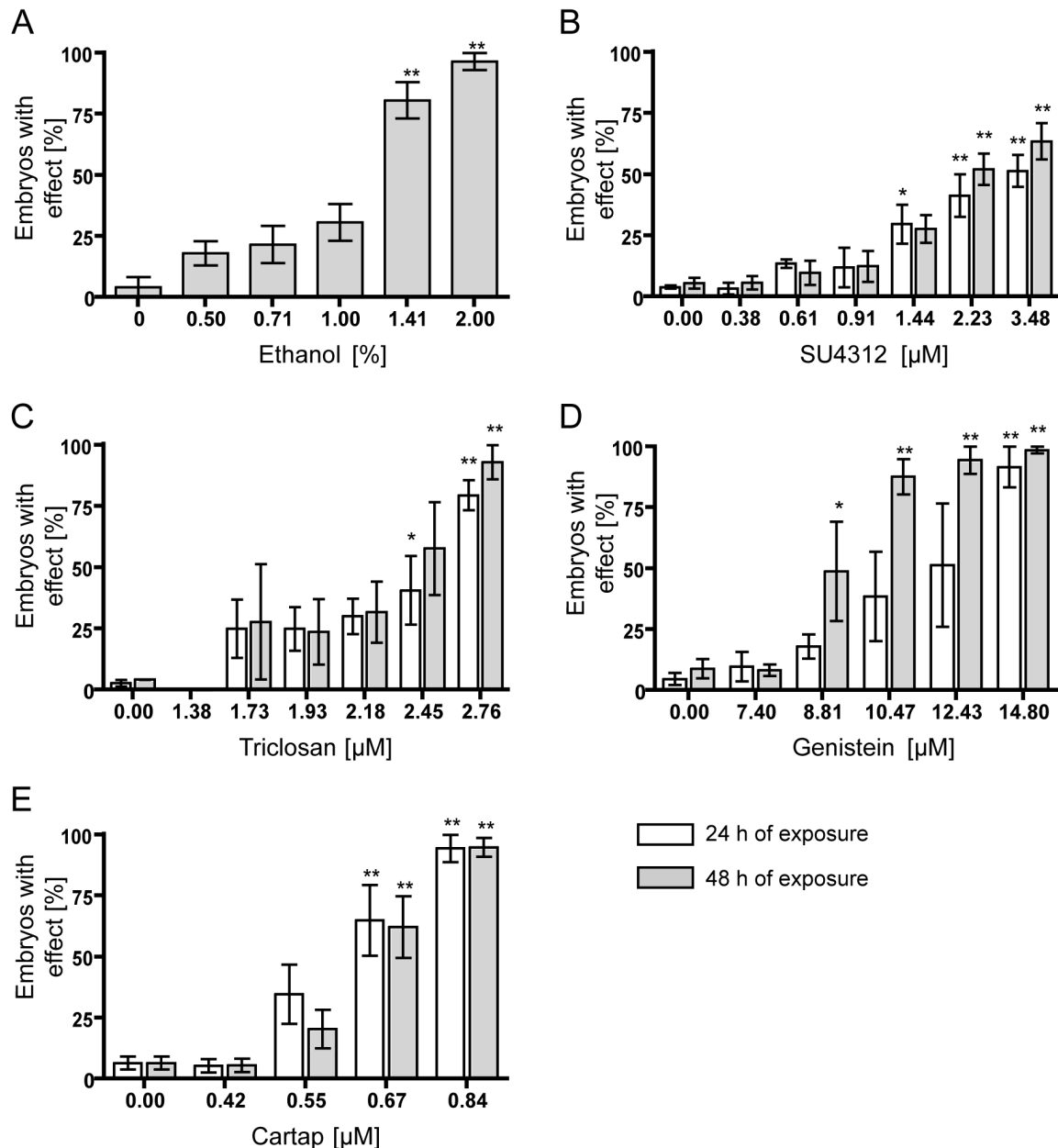


Fig. 13: Summarized morphological effects in zebrafish embryos treated with (A) ethanol, (B) SU4312, (C) triclosan, (D) genistein and (E) cartap analysed by bright field microscopy (method: 2.3).

Concentration-dependent increase in the proportion of embryos showing any (cumulative of lethal and sublethal) morphological effects (%) after exposure to different substances for 24 and 48 h. Effects were determined according to OECD draft guideline (OECD TG 2006) and background paper of Braunbeck and Lammert (2006). Significant differences to the control are indicated with asterisks (mean $\pm$ SEM of 3 independent experiments with $n = 21 - 50$ embryos per concentration and experiment; * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with post-hoc Dunnet's multiple comparison test).

3.2.2 ISV assessment of *Tg(fli1:EGFP)^{y1}* after triclosan, genistein and cartap exposure

The utility of fluorescence endpoints for the microscopical evaluation of effects in the zFET was scrutinized in comparison to standard bright field microscopy. The vasotoxic potential of

triclosan, genistein and cartap was assessed qualitatively (ISV defects) and quantitatively (ISV-length). SU4312 and pentoxifylline were used as control compounds. The results of the negative control pentoxifylline are described separately.

Comparison of the morphological bright field (MBFA) and the ISV fluorescence effect assessment (ISVFA)

To evaluate the application of *Tg(fli1:EGFP)^{y1}* embryos for toxicity assessment, the MBFA was compared with the ISVFA in terms of sensitivity. As a benchmark to directly compare the two qualitative effect assessment methods, the effect concentration (EC) values for the MBFA and ISVFA were used. The EC₅₀ values, obtained for each test substance, were used as estimates of the method's sensitivity to indicate developmental toxicity. The concentration-effect curves and the resulting EC₅₀ values are displayed in figure 14.

For the known vasotoxic control substance SU4312, a slight shift of the ISV concentration-effect curve to the left along the x-axis was observed, resulting in a lower EC₅₀ of 1.85 μ M compared to the EC₅₀ of 2.46 μ M for the MBFA. Thus, a higher sensitivity for vasotoxicity was indicated (Fig. 14 A). For triclosan, a similar trend was found (Fig. 14 B), and the EC₅₀ value for ISVFA of 1.64 μ M suggested a higher sensitivity of vascular than of general developmental toxicity effects (MBFA-EC₅₀ of 2.16 μ M). On the contrary, genistein displayed a slightly rightwards-shifted ISVFA concentration-effect curve, resulting in a minimally higher EC₅₀ of 9.38 μ M than for the MBFA (EC₅₀ of 9.03 μ M; Fig. 14 C). A tendency towards an equal or lower vasotoxic potential was therefore indicated. For cartap, the EC₅₀ values of both assessment methods were 0.15 μ M. Although, the concentration-effect curves of morphological malformations in the MBFA showed a steeper slope than those of the ISV defects (Fig. 14 D). As a consequence, minimal changes in the concentrations of genistein and cartap led to big differences in the bright field morphology effect levels. Even though the EC₅₀ values of genistein for both assessment methods were almost identical, the 100% effect level of the MBFA was reached at a lower concentration of 12.43 μ M than for the ISVFA, which reached 74% at the highest concentration of 14.80 μ M (Fig. 14 C). Overall, for genistein and cartap a higher sensitivity of the ISVFA was indicated at lower concentrations, while at higher test concentrations, the MBFA was the more sensitive method.

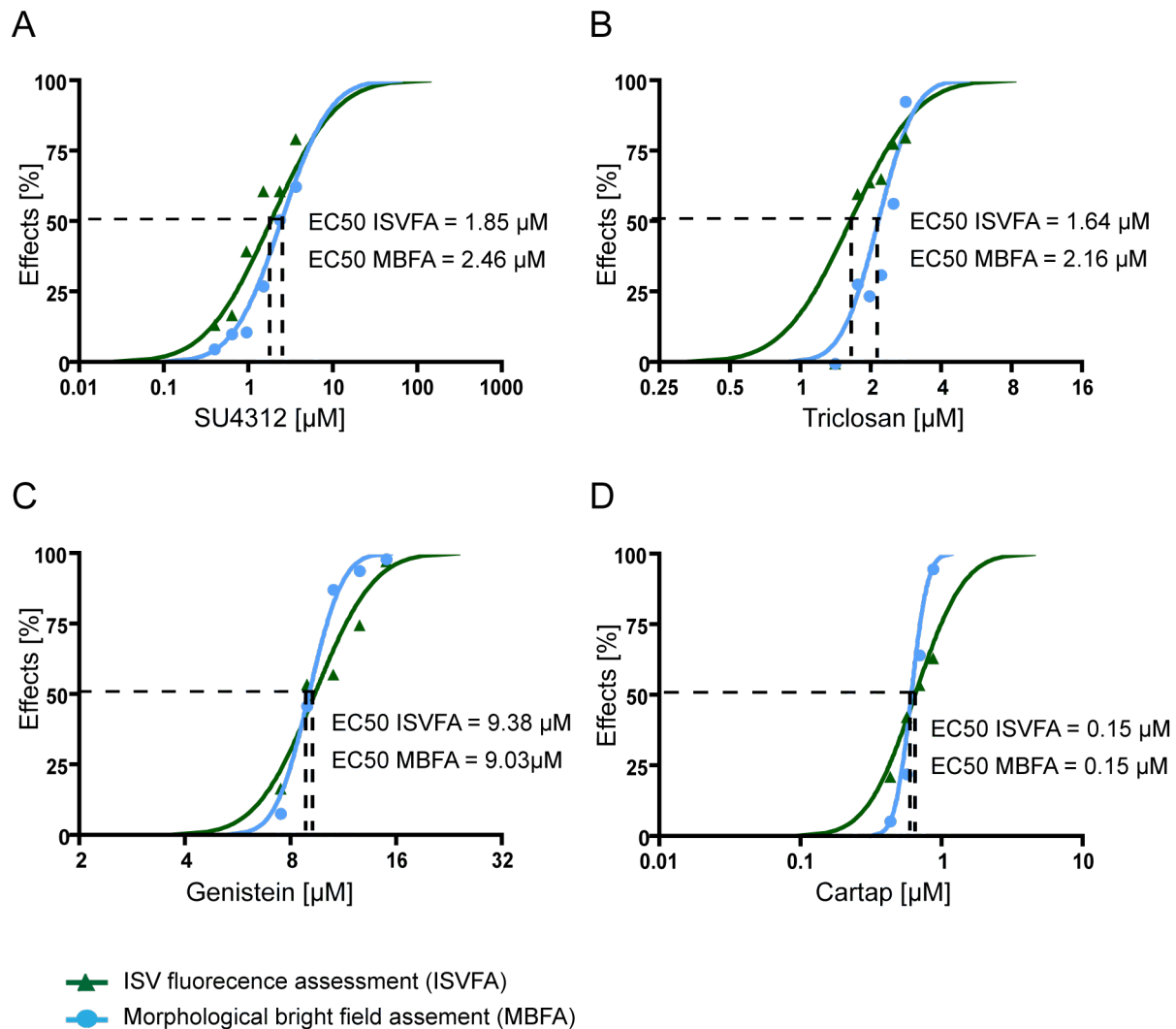


Fig. 14: Concentration-effect curves and EC50 values of (A) SU4312, (B) triclosan, (C) genistein and (D) cartap, derived from 48 hpf morphological bright field assessment (MBFA) (blue) and ISV fluorescence assessment (ISVFA) (green) (methods: 2.3 and 3.1.1).

Leftward shift of the EC-curves indicates a more sensitive effect detection. Curves and EC50 values were calculated by probit analysis using ToxRat® Professional 2.10. Three independent experiments with $n = 21 - 50$ embryos per concentration and experiment were merged for the calculation of the concentration-response curves based on the zFET (MBFA) and $n = 40 - 56$ ISVs per concentration and experiment for the calculation of the dose-response curves based on the ISV defects assessment (ISVFA).

Intersegmental vessel (ISV) length enables effect quantification

The relative length of the ISVs in dependence to substance concentration was determined to evaluate the vasotoxic potential of the substances and to obtain a quantitative test endpoint additionally to the ISVFA (defect assessment and corresponding effect threshold determination).

In accordance with the literature, SU4312 exposure inhibited the angiogenic vessel growth and specifically blocked the ISV outgrowth in the zebrafish embryos. I observed truncation of the ISVs with increasing concentration until a complete lack of ISVs occurred as a final stage.

Embryos exposed to SU4312 showed a decrease in the ISV-length compared to untreated embryos already at 24 hpf (Fig. 15 A and Fig. 16 A). There, I observed a significant difference at a SU4312 concentration of 0.61 μM ($p = 0.05$). Fig. 15 A shows images of truncated ISVs in 48 hpf embryos exposed to low (0.91 μM) and high (3.48 μM) concentrations of SU4312. At this time point, a significant increase in ISV developmental defects was observed also at a SU4312 concentration of 0.61 μM , which resulted in a 10% reduced ISV-length (Fig. 16 A). At 3.48 μM of SU4312, the mean relative ISV-length was 58% of control.

The patterns of the ISV effects in embryos exposed to triclosan were inconsistent and variable between individuals of the same treatment. In some embryos, triclosan exposure caused truncated and missing ISVs while in others it resulted in diffuse EGFP-signals, independently of the applied concentration. Fig. 15 B shows an embryo treated with 2.76 μM of triclosan, where no clear ISV-structure was detected. The ISVs appeared disrupted, as did the EGFP signal. However, I determined significant differences in the relative ISV-length (Fig. 16 B). After 24 h of triclosan exposure, a significant decrease in the ISV-length compared to the controls was observed from $\geq 1.73 \mu\text{M}$ ($p = 0.01$), but further concentration increase did not result in further decrease in the relative ISV-length. Relative ISV-lengths of between 46 – 50% were measured for these triclosan concentrations. After 48 h of triclosan exposure, only the two highest concentrations of 2.45 μM and 2.76 μM had significant effects on the ISV development ($p = 0.001$), reducing the relative ISV-lengths to 62 and 64%, respectively (Fig. 16 D). In genistein-exposed embryos, I observed smaller vessels and irregularities in the spacing between the ISVs (Fig. 15 C). All ISVs appeared thinner and with increasing concentration, also the degree of truncations and malformations increased. Consequently, the relative ISV-length decreased with increasing genistein concentration and exposure time (Fig. 16 C). The 24 hpf assessment showed no effects on ISV development for the first three concentrations. At a concentration of 12.43 μM , the differences in ISV-length became significant ($p = 0.01$), with the relative ISV-length reaching 28%. After 48 h of genistein exposure, significant ISV-length differences ($p = 0.001$) were caused by all concentrations, starting from 7.4 μM with a relative ISV-length of 81% (Fig. 16 D).

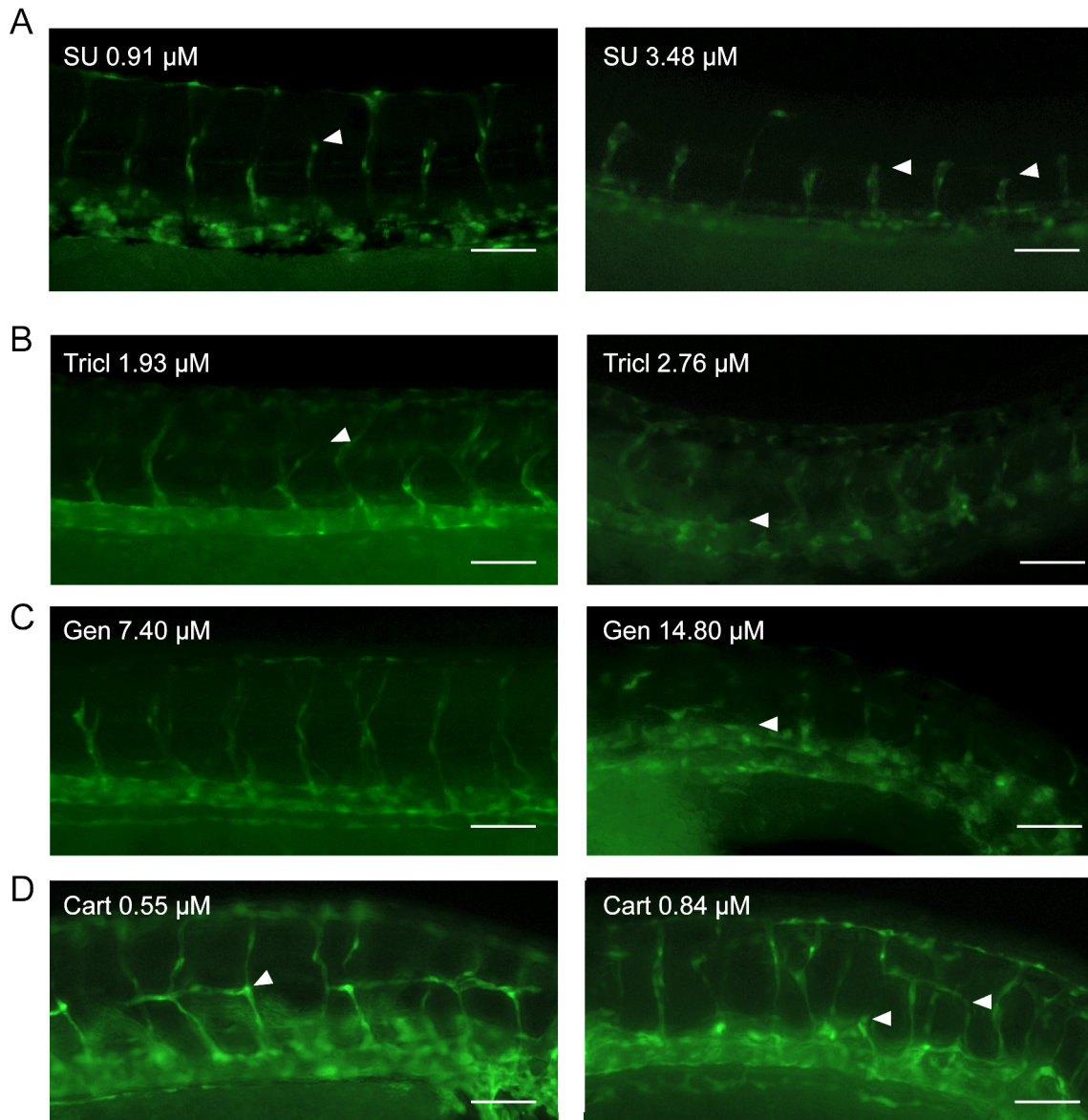


Fig. 15: Phenotype of the intersegmental blood vessels (ISVs) of *Tg(fli1:EGFP)^{y1}* embryos at 48 hpf after exposure to different substances (methods: 2. 7 and 3.1.1) .

Images showing EGFP expression in the ISV of *Tg(fli1:EGFP)^{y1}* embryos exposed to increasing concentrations of (A) SU4312 (0.91 and 3.48 μM), (B) triclosan (1.93 and 2.76 μM), (C) genistein (7.40 and 14.80 μM) and (D) cartap (0.55 and 0.84 μM) (method: 2. 3). ISVs show irregularities and an aberrant morphology (indicated by arrowheads). Images were obtained by standard fluorescence microscopy. Anterior is towards the left in all panels. Scale bar: 50 μm .

Embryos exposed to cartap displayed a characteristic ISV-phenotype, which was in accordance with published results (Zhou et al., 2009). ISVs were often looped and irregularly branched (Fig. 15 D), and a straight trajectory of the ISVs from the ventral to dorsal axis instead of the characteristic S-pattern was typically observed. The straightness of the ISVs caused a decrease in the relative ISV-length down to 72%, but did not decrease further with increasing cartap concentrations (Fig. 16 D). For the 24 hpf assessment point, I observed only

concentration-independent changes in the relative ISV-length, mainly displayed by ISV-spacing irregularities. At 48 hpf significant abnormalities in ISV development occurred from a concentration of 0.55 μM ($p = 0.001$).

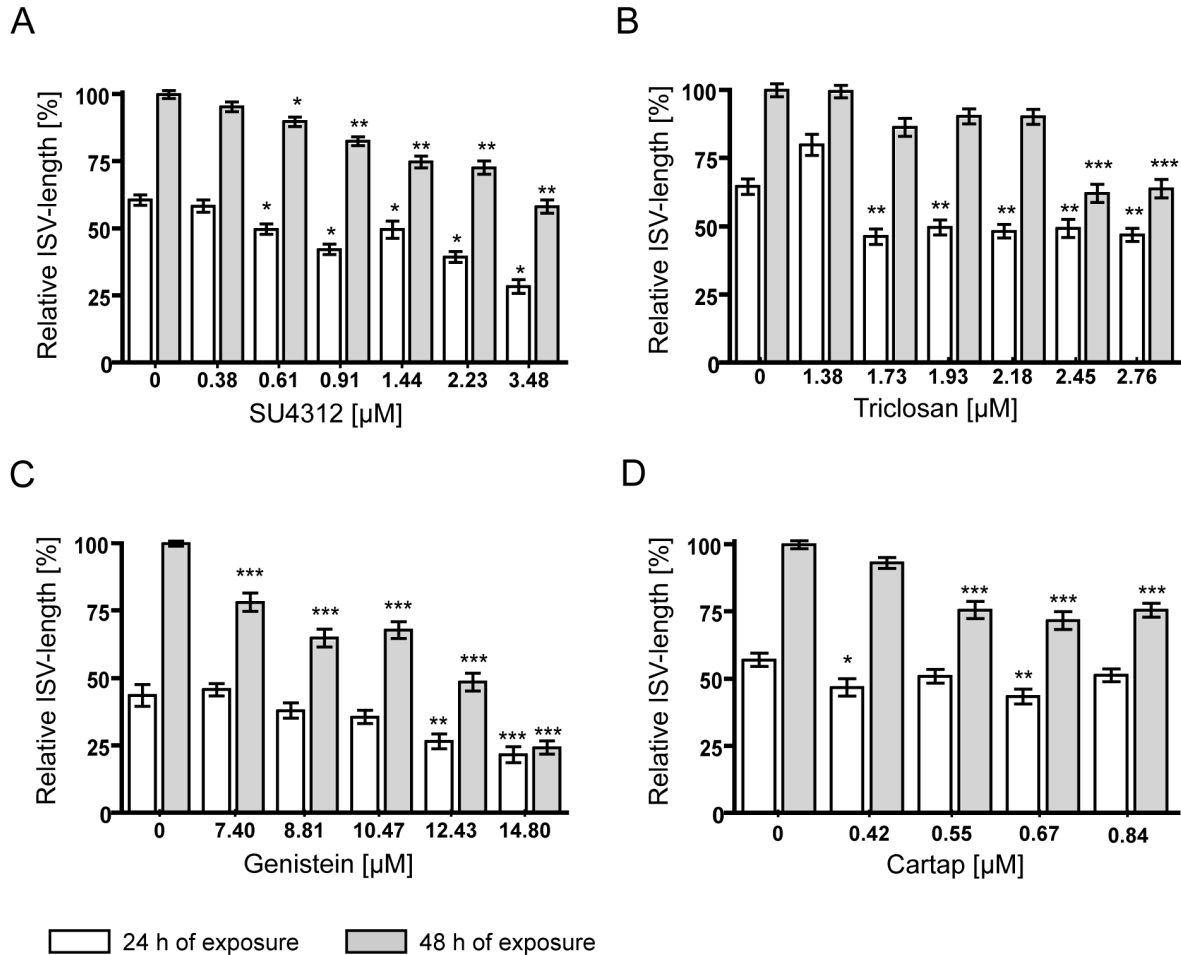


Fig. 16: Concentration-dependent effects on intersegmental blood vessel (ISV) development and growth of *Tg(fli1:EGFP)^{y1}* embryos after treatment with different substances (method: 3.1.1).

Measurement of the relative ISV-length (%) after 24 and 48 h of exposure to (A) SU4312, (B) triclosan, (C) genistein and (D) cartap (method: 2. 3). Significant differences of the ISV-length to the control group are indicated (mean $\pm$ SEM of 3 independent experiments; 8 ISVs of 3 – 6 embryos per concentration and experiment were measured with ImageJ and NeuronJ; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with post-hoc Dunnet's multiple comparison test).

Negative control substance pentoxifylline induced systemic embryo toxicity

Pentoxifylline was tested as a negative control substance for vascular defects, as it is known to improve blood flow by increasing the flexibility of the erythrocytes and leukocytes (Bessler et al., 1986; Semmler et al., 1993). However, 48 h exposed zebrafish embryos showed slow blood circulation at concentrations $\geq 593.22 \mu\text{M}$. Besides, malformations of the heart and the notochord occurred in the two highest exposure concentrations (Fig. 17 B). Pericardial and/or yolk sac edema were also recorded in pentoxifylline treated embryos (Appendix 8. 1, Table 7). The cumulative EC50 for all morphological effects (MBFA) at 48 hpf was $395.66 \mu\text{M}$ (Fig. 17 C). A concentration-response curve for ISVFA could not be established since the cumulative effect level did not exceed 25% within the range of concentrations tested. No significant impairment of the ISV development was observed in all but the highest test concentration. At nominal $1019.44 \mu\text{M}$ few embryos showed some effects on the ISV development, reducing the relative ISV-length to 90.1% (Fig. 17 D). Thus, a very weak response of the ISV development was detected, most likely as a secondary effect of the general toxicity caused by the highest pentoxifylline concentration.

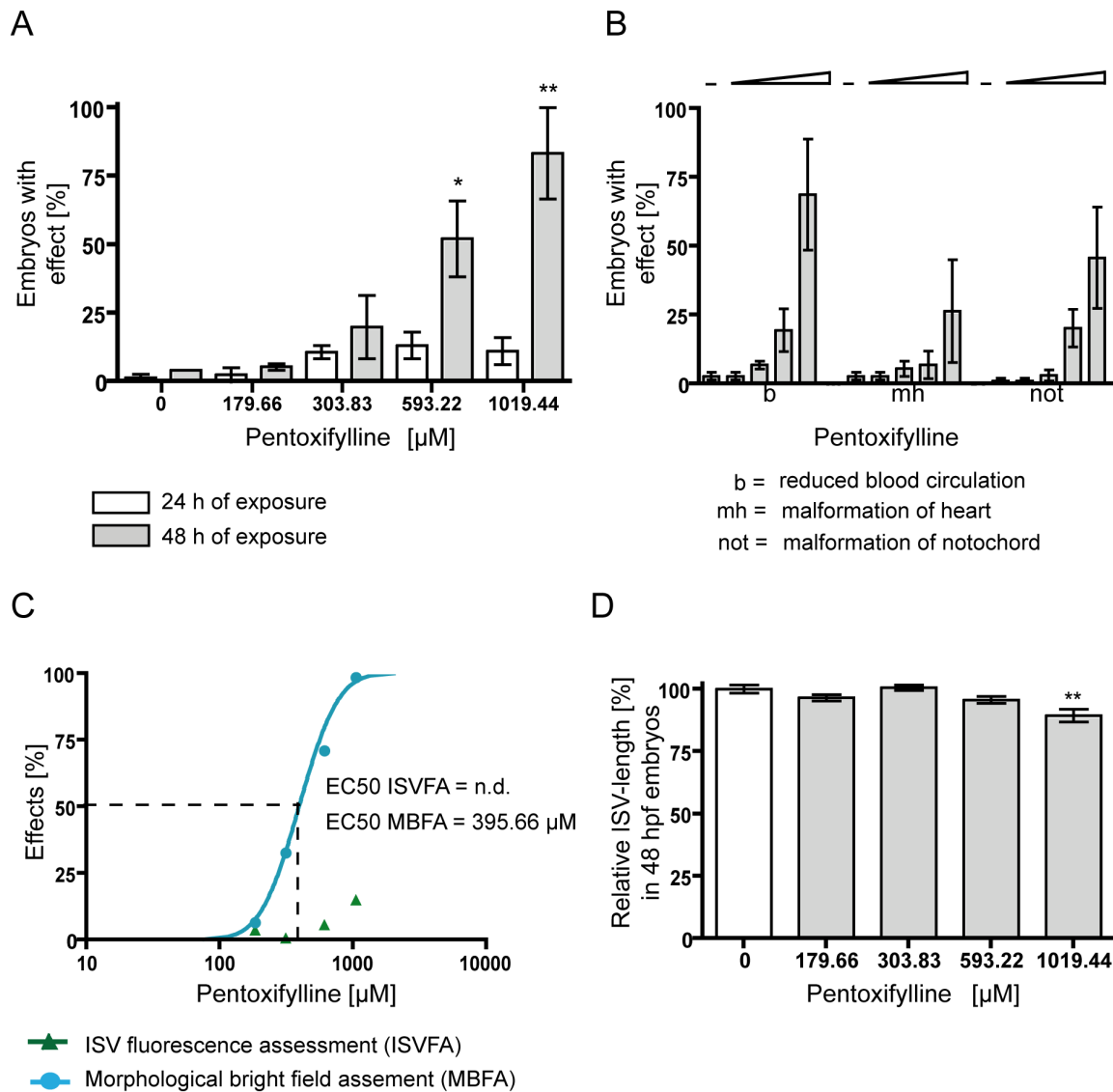


Fig. 17: Effects of exposure of zebrafish embryos to negative control pentoxifylline assessed by bright field (A+B) and fluorescence microscopy (D) (methods: 2. 3 and 3.1.1).

(A) Proportion of embryos (%) with morphological effects in total per treatment after 24 h and 48 h exposure of pentoxifylline (mean values $\pm$ SEM of 3 independent experiments with 24 – 51 embryos). (B) Percentage of embryos exhibiting specific effects (reduced blood circulation (b), malformation of heart (mh) or malformation of notochord (not)) after 48 h of increasing pentoxifylline concentrations. (C) Comparison of concentration-effect curves and EC50 values (probit analysis using ToxRat® Professional 2.10), based on morphological bright field assessment (MBFA) (blue) and ISV fluorescence assessment (ISVFA) (green) after 48 h. Data of 3 independent experiments with $n = 24 - 51$ embryos each were pooled for the calculation of the MBFA- and $n = 40 - 56$ ISVs each for the calculation of the ISVFA-concentration-response curves. (D) Relative ISV-lengths (%) compared to the controls measured in *Tg(fli1:EGFP)^{y1}* embryos after 48 h of exposure (mean values $\pm$ SEM of 3 independent experiments; 8 ISVs of 3 – 6 embryos per concentration). Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with post-hoc Dunnett's multiple comparison test).

3.2.3 Fluorescence intensity of *Tg(gfap:GFP)* embryos as a measure of neural development effects

The GFP expression of *Tg(gfap:GFP)* embryos 48 hpf enabled the visualization of the neural tube, as shown in Fig. 18. The fluorescence intensity in the neural tube was dependent of the chemical treatment, which can also be seen in Fig. 19, depicting the measurement values against the treatment concentrations.

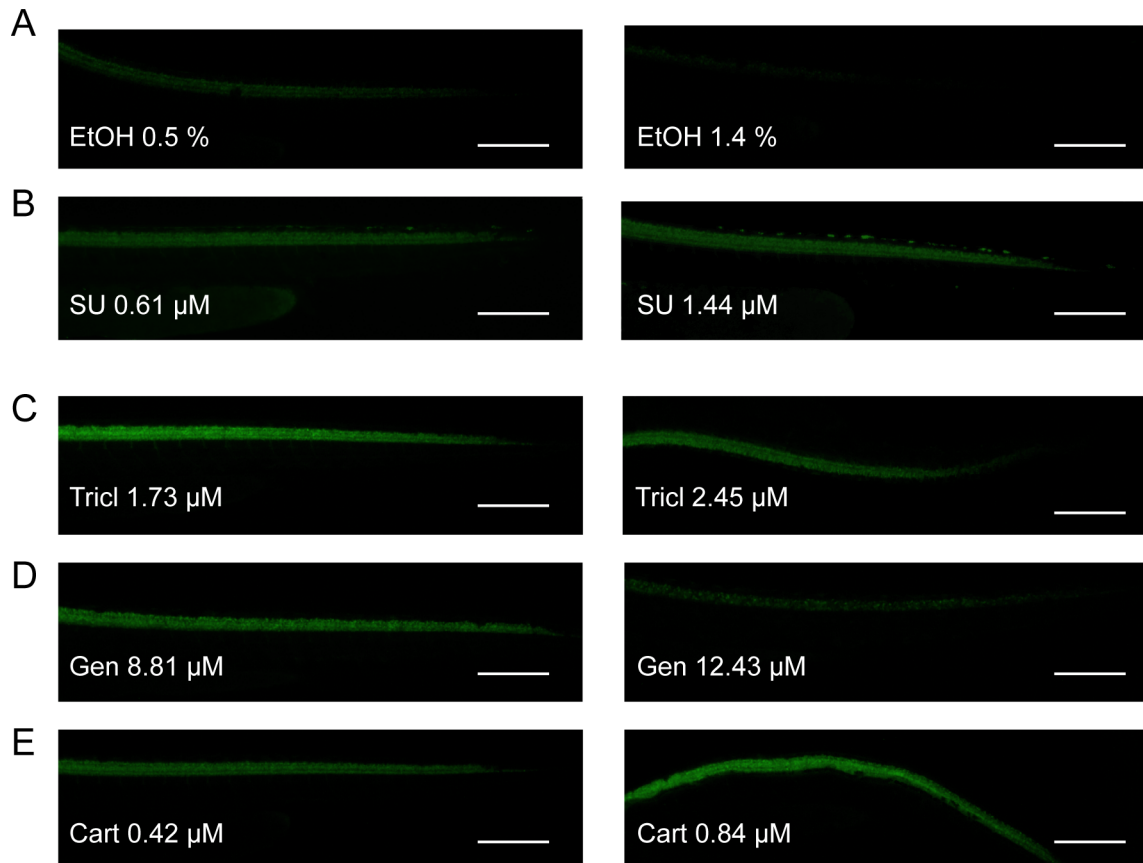


Fig. 18: Fluorescence signal in the trunk of *Tg(gfap:GFP)* embryos 48 hpf after exposure to different substances (methods: 2. 7 and 3.1.2).

Images showing GFP expression in radial glial cells and astrocytes present in the neural tube of *Tg(gfap:GFP)* embryos treated with (A) positive (0.5 and 1.4% (v/v) ethanol) and (B) negative control (0.61 and 1.44 μ M SU4312). (C) GFP expression in the neural tube of *Tg(gfap:GFP)* embryos exposed to increasing concentrations of triclosan (1.73 and 2.45 μ M), (D) genistein (8.81 and 12.43 μ M) and (E) cartap (0.42 and 0.84 μ M) (method: 2. 3). Reduced GFP expression, resulting in decreased fluorescence intensity, indicates a neurotoxic potential of the substance. Images were obtained by standard fluorescence microscopy. Anterior is towards the left in all panels. Scale bar: 250 μ m.

Ethanol is known to act as a neurotoxin (Muth-Köhne et al., 2012; Zhang and Gong, 2013) and was thus used as positive control for the assessment of GFAP expression alterations. As expected, ethanol impaired the development of the RGCs in the zebrafish embryo, resulting in

a concentration-dependent decrease of the fluorescence intensity (Fig. 18 A and Fig. 19 A). Already 0.5% (v/v) of ethanol caused a significantly decreased fluorescence intensity of 28% ($p = 0.01$) compared to control embryos. For the highest tested ethanol concentration of 2% (v/v), only 6.6% (v/v) of the maximum fluorescence signal remained.

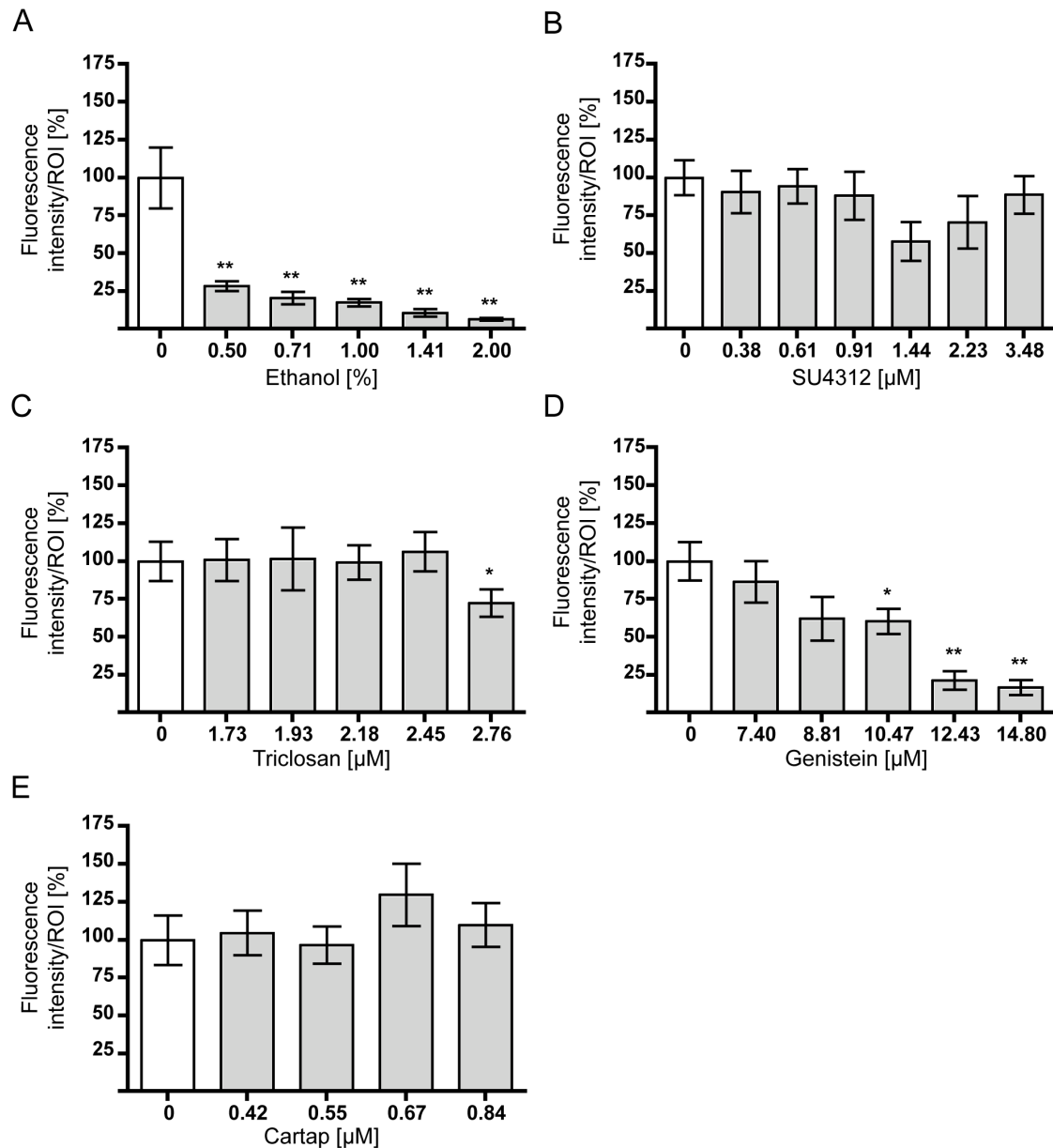


Fig. 19: Fluorescence intensity [%] of GFP in the neural tube of *Tg(gfap:GFP)* zebrafish embryos 48 hpf exposed to different chemicals (method: 3.1.2).

Fluorescence intensity after exposure to (A) positive control ethanol, (B) negative control SU4312, (C) triclosan, (D) genistein and (E) cartap (method: 2. 3). Significant differences in the fluorescence intensity [%] to the control group are indicated (mean $\pm$ SEM of 3 independent experiments; Fluorescence intensity/ROI of 5 – 6 embryos per concentration and experiment was measured with ImageJ; * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with post-hoc Dunnett's multiple comparison test).

In addition to ethanol, genistein showed a concentration-dependent impact on the fluorescence intensity in the neural tube (Fig. 18 D and Fig. 19 D). Significant effects

compared to the control were assessed from a genistein concentration of 10.47 μM ($p = 0.05$), which resulted in a remaining fluorescence intensity of 20.5% at 14.80 μM ($p = 0.01$). This corresponded to a loss of the fluorescence signal of 79.5% (Fig. 19 D). With the exception of the highest triclosan concentration, triclosan and cartap had no effect on the fluorescence intensity of the neural tube of zebrafish embryos (Fig. 19 B, C, E), although an aberrant morphology of the neural tube was observed (Fig. 18 C+E).

3.2.4 Angle aberrations between myotome hemi-segments indicate effects on muscle morphology

The impact on the muscle fiber morphology was analyzed by whole-mount immunostainings and measurements of the angle between the hemi-segments as shown in Fig. 21. The image analysis showed disorganized muscle fibers in embryos exposed to high concentrations of ethanol, triclosan and genistein (Fig. 20 A, C, D). Severe aberrant muscle fibers were observed in particular in the embryos exposed to genistein (Fig. 20 D).

Ethanol was used as positive control, as it was described by Sylvian et al. (2010) to cause severe effects on myotome development. In the present study, an increase in the mean angle between the hemi-segments with increasing concentrations of ethanol, genistein and cartap was determined (Fig. 20 A, D, E and Fig. 21 A, D, E). SU4312 and triclosan had some but no concentration-dependent impact on the muscle morphology (Fig. 21 B+C). Ethanol caused significant effects on the muscle morphology from concentrations $\geq 0.71\%$ (v/v) ($p = 0.01 - 0.001$). A mean angle of $106^\circ (\pm 1.6^\circ)$ between the hemi-segments in embryos treated with 2% (v/v) of ethanol was measured. Genistein-exposed embryos showed an increase in the angle between the hemi-segments up to $123^\circ (\pm 1.8^\circ)$, with significant differences compared to the control from 10.47 μM ($p = 0.01$; Fig. 21 D). Zebrafish embryos exposed to cartap showed significant myotomal effects at the two highest test concentrations of 0.67 μM ($110^\circ \pm 1.9^\circ$; $p = 0.01$) and 0.84 μM ($116^\circ \pm 2.4^\circ$; $p = 0.001$) (Fig. 21 E).

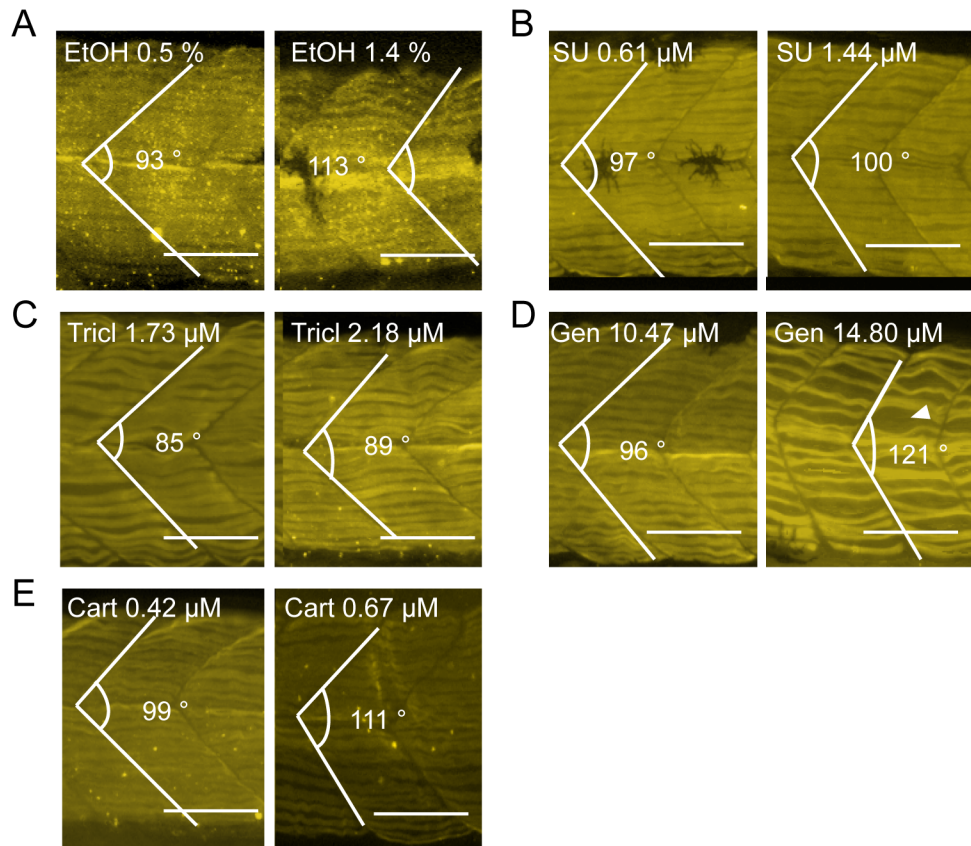


Fig. 20: Muscle morphology of zebrafish embryos 48 hpf, whole-mount immunostained with monoclonal antibody F59 after exposure to different substances (methods: 2. 5, 2. 7 and 3.1.3).

Images showing representative angles between hemi-segments of exposed embryos to (A) positive (0.5 and 1.4% (v/v) ethanol) and (B) negative control (0.61 and 1.44 μM SU4312). (C) Muscle morphology of embryos exposed to increasing concentrations of triclosan (1.73 and 2.18 μM), (D) genistein (10.47 and 14.80 μM) and (E) cartap (0.42 and 0.67 μM) (method: 2. 3). The fluorescence signal of the secondary antibody DyLightTM (549-conjugated) and the binding specificity of the primary antibody F59 against myosin and axial muscle cells visualized the muscle fibers in the fixed embryos. The angle between the two hemi-segments was measured with the angle tool of ImageJ. If the angle between two hemi-segments increases to $\geq 100^\circ$, a chemically induced myotoxic effect was indicated. Disorganization of fiber morphology is marked by arrowhead. Images were obtained by standard fluorescence microscopy. Anterior is towards the left in all panels. Scale bar: 50 μm .

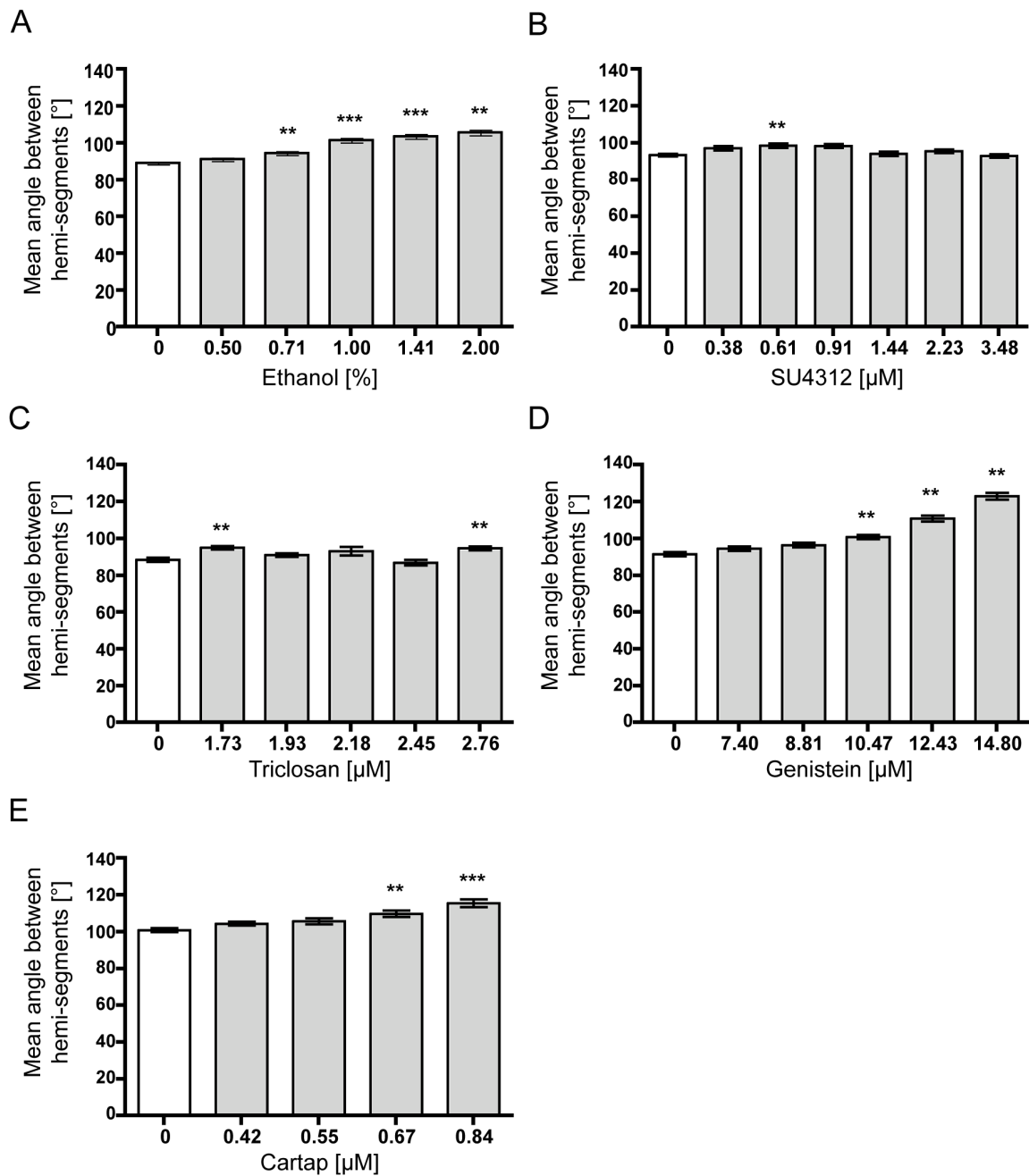


Fig. 21: Mean angles between hemi-segments [°] of myotome segments of zebrafish embryos 48 hpf after exposure to different chemicals (methods: 2. 5 and 3.1.3).

Effects on muscle morphology after exposure to (A) positive control ethanol, (B) negative control SU4312, (C) triclosan, (D) genistein and (E) cartap (method: 2. 3). Measurements of the mean angle between hemi-segments were provided by the image analysis of the whole-mount immunostainings with F59. Significant differences of the mean angle between hemisegments [°] to the control group are indicated (mean $\pm$ SEM of 3 independent experiments; 3 – 5 angles of hemi-segments of 3 – 6 embryos each per concentration and experiment were measured with ImageJ; * p <0.05, ** p <0.01, *** p <0.001; one-way ANOVA with post-hoc Dunnett's multiple comparison test).

3.2.5 Impact on apoptosis

The vital staining with AO provided information on the chemical-induced impact on the apoptosis rate in zebrafish embryos. Ethanol and genistein increased apoptosis in 48 hpf zebrafish embryos, as shown in figure 22 (A+D) and Fig. 23 (A+D). The apoptotic cells in embryos exposed to genistein occurred abundantly in the region of the neural tube (Fig. 22 D). Ethanol-induced apoptotic cells were less frequent and more scattered across the trunk region (Fig. 22 A).

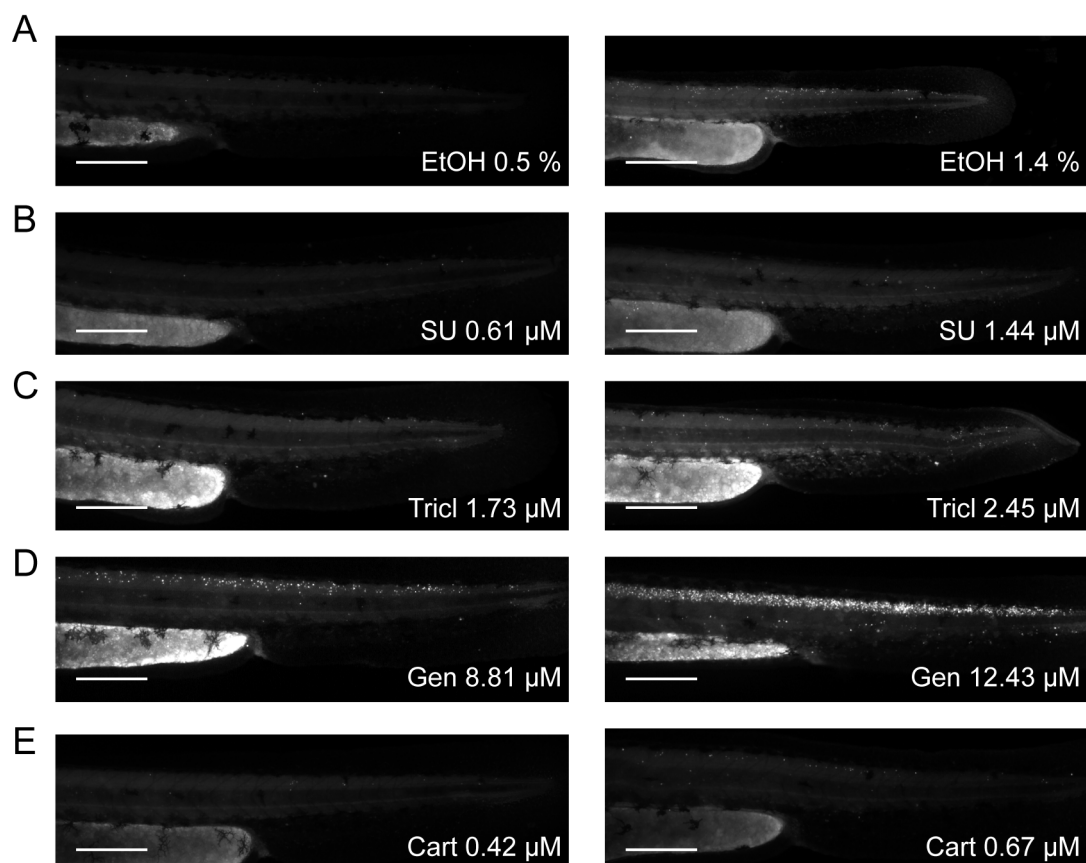


Fig. 22: Trunk region of zebrafish wild type embryos 48 hpf, vital stained with acridine orange after exposure to different substances (methods: 2. 6, 2. 7 and 3.1.4).

(A) Images showing the fluorescent acridine orange (AO) staining of apoptotic cells in the trunk region of zebrafish embryos exposed to positive (0.5 and 1.4% (v/v) ethanol) and (B) negative control (0.61 and 1.44 μ M SU4312). (C) Fluorescent, i.e. apoptotic cells in the trunk region of embryos treated with 1.73 and 2.45 μ M triclosan, (D) 8.81 and 12.43 μ M genistein and (E) 0.42 and 0.67 μ M cartap (method: 2. 3). AO staining enabled the detection of chemically induced apoptosis: number of apoptotic cells increased with increasing exposure concentrations of ethanol and genistein. Images were obtained by standard fluorescence microscopy. Anterior is towards the left in all panels. Scale bar: 250 μ m.

I determined an increase in the number of apoptotic cells in the ROI of zebrafish embryos exposed to ethanol, triclosan and genistein (Fig. 23 A, C, D). Triclosan caused a slight concentration-dependent increase in apoptotic cells at concentrations ≥ 2.45 μ M with up to 95

(± 21) apoptotic cells/ROI ($p = 0.01$, Fig. 23 C). Ethanol and genistein showed a stronger influence on apoptosis, as 176 (± 32) apoptotic cells/ROI were counted in embryos exposed to 2% (v/v) ethanol and 151 (± 31) apoptotic cells/ROI in embryos exposed to 8.81 μM genistein (Fig. 23 A+D).

In case of a dense accumulation of apoptotic cells, another assessment method had to be applied since counting of individual cells was not possible anymore. This alternative assessment method was compared to the cell counting (see sub-chapter 3.1.4).

By measuring the area-wide fluorescence intensity in the ROI, effects were determined in embryos treated with ethanol and genistein (Fig. 24 A+D). A significantly increased fluorescence intensity was determined for genistein at concentrations $\geq 10.47 \mu\text{M}$ ($p = 0.05$), increasing with concentration (Fig. 24 D). In contrast to the fluorescent cell counting, the area-wide fluorescence intensity of ethanol-exposed embryos determined significant effects only in the highest test concentration of 2% (v/v) ethanol ($p = 0.05$, Fig. 24 A). The apoptotic impact of triclosan was too minimal to be detected by measuring the fluorescence intensity of the ROI (Fig. 24 C). SU4312 and cartap had no effects on apoptosis, independently of the assessment method (Fig. 23 B+E).

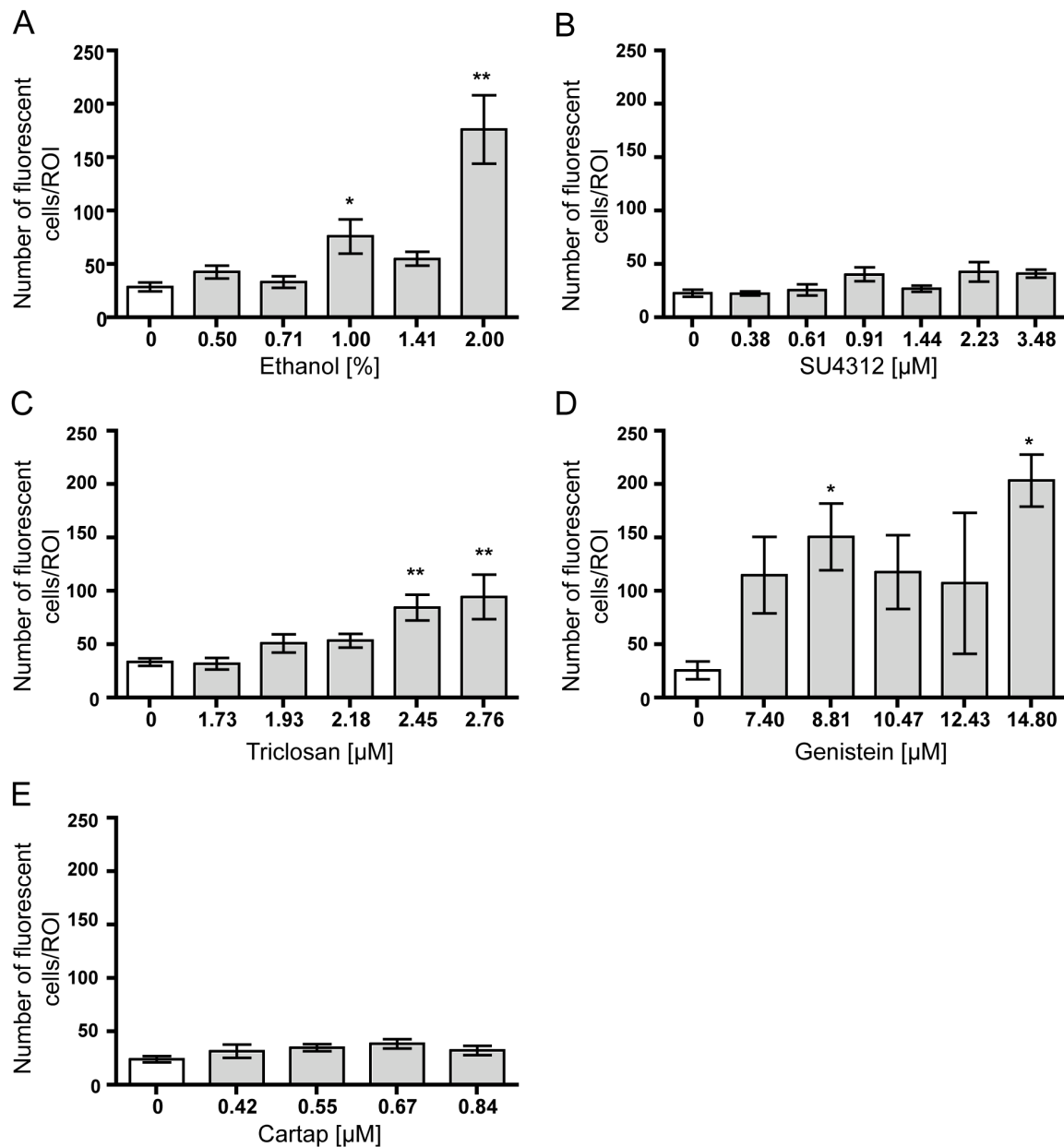


Fig. 23: Number of apoptotic cells in the trunk region of acridine orange (AO) stained zebrafish embryos 48 hpf exposed to different chemicals (methods: 2. 6 and 3.1.4).

The number of fluorescently labeled cells present in the trunk region (= ROI: region of interest) of exposed embryos was assessed by the ImageJ tool *Analyze particles*, counting individual single apoptotic cells. The embryos were exposed to different concentrations of (A) ethanol (positive control), (B) SU4312 (negative control), (C) triclosan, (D) genistein and (E) cartap (method: 2. 3). Significant differences in the number of fluorescent cells/ROI to the control group are indicated (mean $\pm$ SEM of 3 independent experiments; number of fluorescent cells/ROI of 3 – 4 embryos per concentration and experiment was measured. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with post-hoc Dunnett's multiple comparison test).

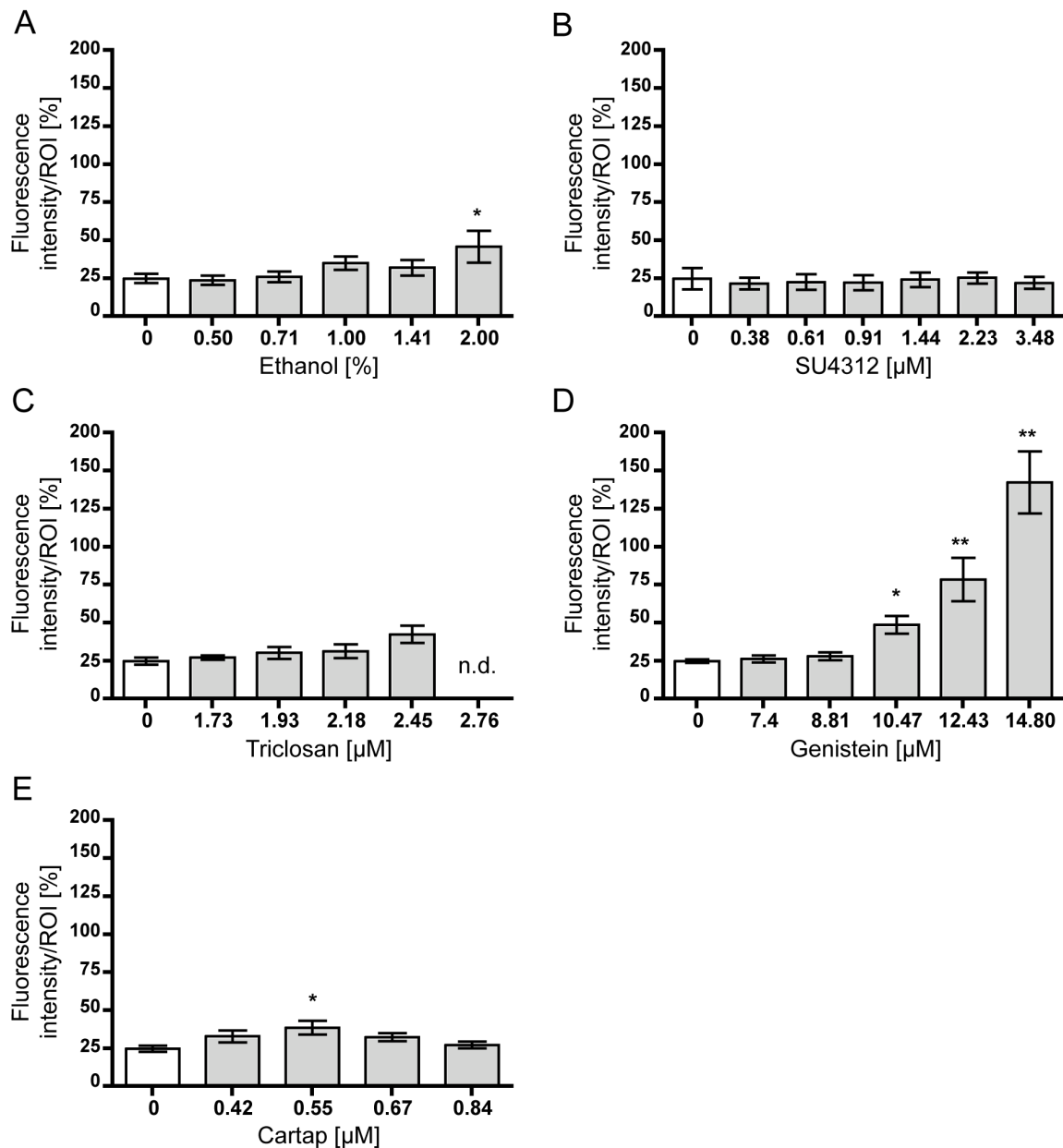


Fig. 24: Fluorescence intensity [%] in the trunk region of acridine orange (AO) stained zebrafish embryos 48 hpf exposed to different chemicals (methods: 2. 6 and 3.1.4).

The fluorescence intensity (IntDen/area) of the trunk region (= ROI: region of interest) of the same embryos, which were used for the counting of fluorescent cells (Fig. 23) was assessed. The embryos were exposed to different concentrations of (A) ethanol (positive control), (B) SU4312 (negative control), (C) triclosan, (D) genistein and (E) cartap (method: 2. 3). Significant differences of the fluorescence intensity [%] to the control group are indicated (mean $\pm$ SEM of 3 independent experiments; Fluorescence intensity/ROI of 3 – 4 embryos per concentration and experiment was measured. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with post-hoc Dunnett's multiple comparison test).

3.3 Comparative evaluation of the microscopic bright field and fluorescence endpoints

To evaluate the specificity and sensitivity of the toxicity analysis using the two different microscopic assessment methods, all 48 h non-fluorescence endpoint (A) and newly established fluorescence endpoint data (B – F) were contrasted in Fig. 25. For an easy comparison of the response and sensitivity of each method, frames (red and green) were included indicating the significant effect thresholds.

Ethanol exposure caused significant effects for all tested endpoints. It resulted in significant vaso- and neurotoxicity effects already at the lowest test concentration of 0.5% (v/v), as determined by the image analysis of the corresponding transgenic lines (Fig. 25 B+C). The results of the SU4312 exposure confirmed the specific inhibition of the VEGF pathway, as only the MBFA and the ISV-length assessment showed significant and concentration-dependent effects (Fig. 25 A+B). The ISV-length was more sensitive, indicating significant effects already at the second test concentration (0.61 μ M).

The results of the triclosan treatment revealed significant effects for the ISV-length assessment and for apoptosis at a concentration of 2.45 μ M (Fig. 25 B and Fig. 25 E+F). For the MBFA significant effects were detected only at 2.76 μ M (Fig. 25 A). The only substance, which showed concentration-dependent effects in all tested endpoints in this study, was genistein. However, the vasotoxicity endpoint responded most sensitive at a test concentration of 7.4 μ M (Fig. 25 B). In contrast, cartap caused an effect response only for MBFA and the vaso- and myotoxicity endpoints, with the ISV-length assessment being the most sensitive endpoint (significant from 0.55 μ M) (Fig. 25 A, B, D). Significant effects on the angle between the two hemi-segments occurred at the same concentration as the cumulative morphological effects (0.67 μ M).

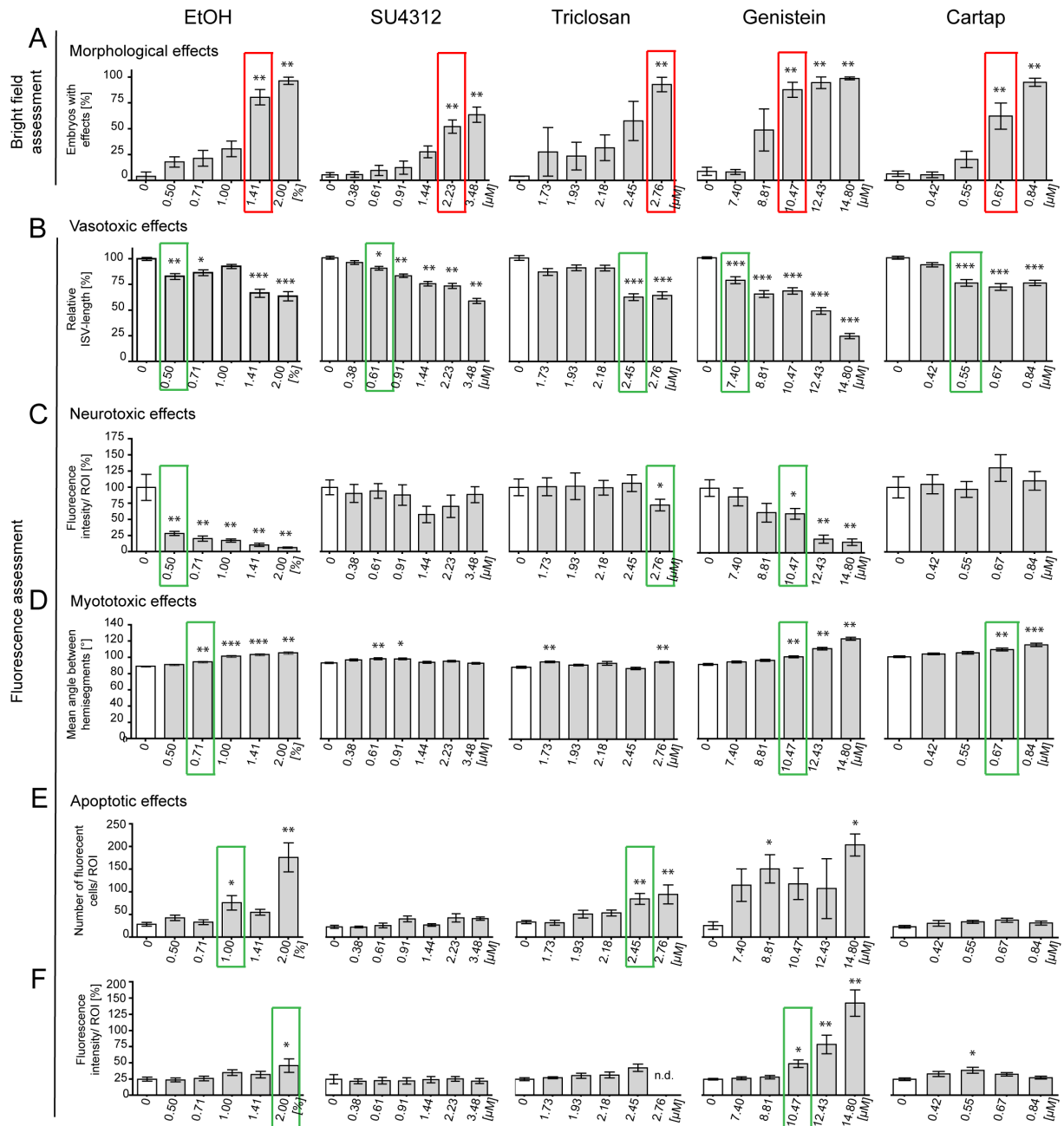


Fig. 25: Summary of bright field (A) and fluorescence endpoint data (B – F) for zebrafish embryos 48 hpf after exposure to different chemicals (methods: 2. 3, 3.1.1, 3.1.2, 3.1.3 and 3.1.4).

(A) Morphological, (B) vaso-, (C) neuro-, (D) mytotoxic effects and (E + F) apoptotic effects of ethanol, SU4312, triclosan, genistein and cartap ((A) mean $\pm$ SEM of 3 independent experiments with 21 – 50 embryos each, (B) 8 ISVs of 3 – 6 embryos each, (C) 5 – 6 embryos each, (D) 3 – 5 angles of hemi-segments of 3 – 6 embryos each and (E + F) 3 – 4 embryos per treatment; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; one-way ANOVA with post-hoc Dunnett's multiple comparison test). Frames indicate the first significant effect of the corresponding endpoint (red: MBFA, green: fluorescence assessment).

3. 4 Linkage between transgene phenotype and gene expression

To investigate the correlation between the endothelial marker gene expression and ISV developmental impairments, the expression of *flila* was analyzed by quantitative RT-PCR. Additionally, the endothelial marker genes *cdh5* and *flt4* were measured to investigate their value as early markers of vascular defects. To find a linkage between the effect phenotype of the *Tg(gfap:GFP)* embryos and the corresponding molecular initiating event, the expression rate of the neuromarker *gfap* was assessed as well.

For each substance, two concentrations were tested, one resulting in no morphological effects and a second causing significant morphological effects compared to the control. However, the results of the PCRs showed no statistically significant changes in mRNA-expression ratios of both, the endothelial and neuronal marker genes (Fig. 26). However, even though statistically non-significant, *flila* expression hinted towards a slight concentration dependency for e.g., in triclosan, genistein and cartap treatment (Fig. 26 A, C, E) and *gfap* expression for ethanol, SU4312 and cartap treatment (Fig. 26 A, B, E).

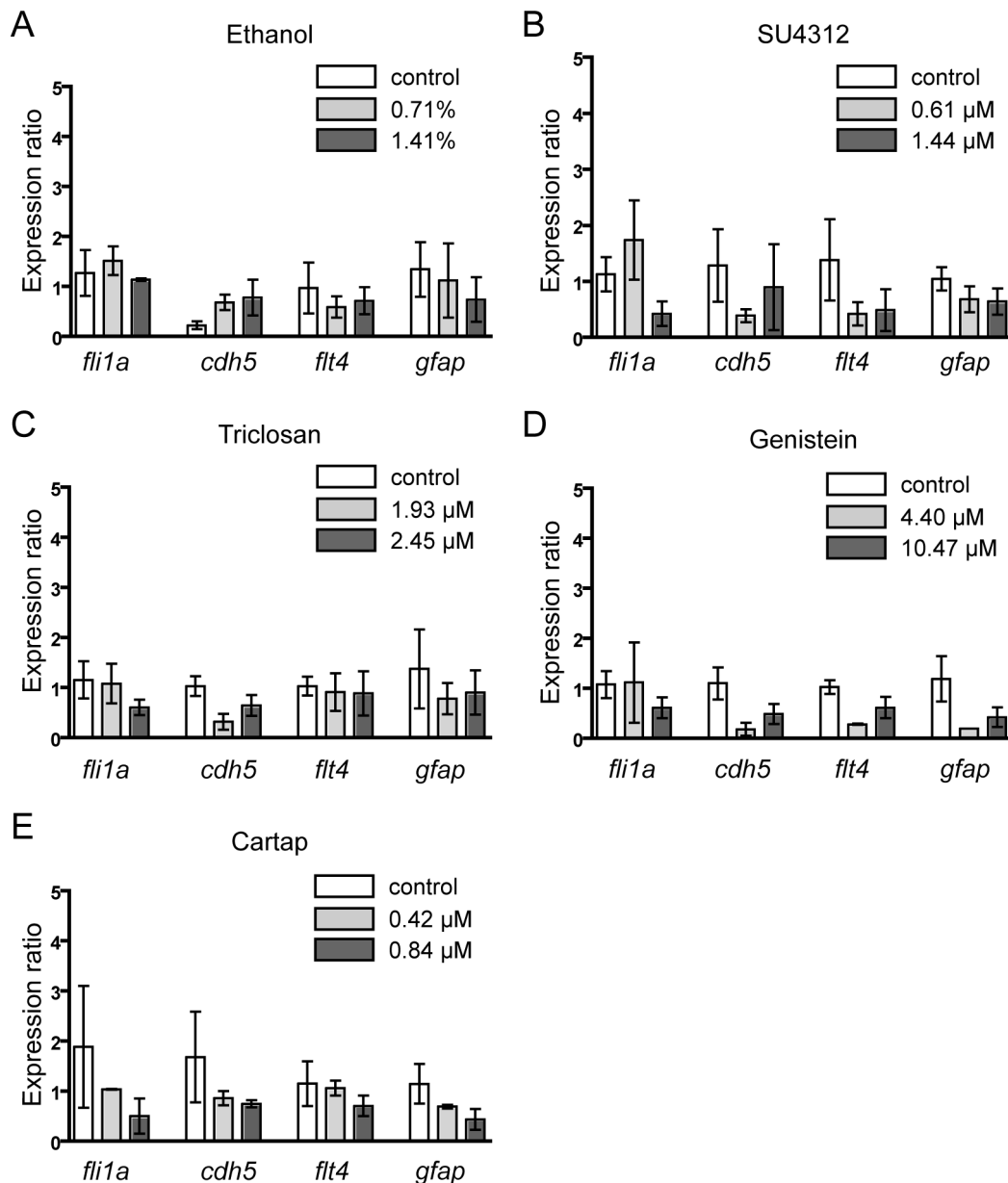


Fig. 26: mRNA-expression ratios of specific cell marker genes in substance-treated zebrafish embryos of 48 hpf, compared to the mRNA-expression ratio in control embryos (method: 2. 8).

Bar chart depicting the mRNA-expression ratios of *fli1a*, *cdh*, *flt4* and *gfap* in embryos exposed to (A) 0.61 μ M and 1.44 μ M of SU4312, to (B) 1.93 μ M and 2.45 μ M of triclosan, to (C) 4.40 μ M and 10.47 μ M of genistein and to (D) 0.42 μ M and 0.84 μ M of cartap compared to untreated control embryos, respectively. No significant changes in expression ratios were found (the Students t-test was performed). All mRNAs were quantified by real time RT-QPCR and normalized to rpl8 and s18 rRNA expression according to the $2^{-\Delta\Delta Ct}$ method of Pfaffl, 2004 (mean $\pm$ SEM of 3 independent experiments).

4. Discussion

In the present thesis, I investigated the potentials of fluorescence microscopy to enhance the zFET. The zFET finds application in environmental and chemical hazard assessments as an alternative test system for the assessment of acute toxicity. The conventional test only accounts for lethal morphological endpoints while disregarding any sublethal effects, which may provide additional information on the toxicity. Consequently, limiting the zFET to acute toxicity diminishes the informative value as well as the specificity of the test. Hence, I developed an approach, which considers all sublethal morphological effects occurring in the embryos, and, in parallel, uses cell-specific fluorescent labels to gain supplementary information on the toxicity. The *Tg(fli1:EGFP)^{y1}* transgenic embryos can reveal defects of blood vessel development and the *Tg(gfap:GFP)* embryos detect effects on glial cell development, fluorescent stainings with an anti myosin antibody allow conclusions on the myotoxic potential of substances. Lastly, acridine orange *in vivo* staining shows substance-induced changes in the apoptosis rate. The vascular system, the neural tube and the myotom of the zebrafish embryo are well-defined organs/tissues, which enable the detection of chemical-induced changes in morphology by fluorescence microscopy. For a proof-of-principle, three chemicals with different mode of actions and relevant occurrence in the aquatic environment, namely triclosan (Singer et al., 2002; Ruedel et al., 2013), genistein (Rearick et al., 2013) and cartap (Berg, 2001), were assessed. By combining different the phenotypic effect assessments, I achieved an increase in test sensitivity, widened the applicability and enhanced the explanatory power of the zFET.

4.1 Shortcomings of the MBFA

The determination of sublethal effects in the zFET by eye is prone to errors and requires some experience. The MBFA of malformations during a conventional zFET is usually carried out by a single examiner who evaluates the presence or absence of defined lethal and sublethal morphological endpoints in every single embryo. The outcome of the evaluation varies with the experience of the examiner and thus, reflects on the concentration-effect curves generated from the data. For instance, *reduced blood circulation* is a common sublethal effect. It is a good example for an endpoint difficult to assess for an untrained examiner. This sublethal effect can only be evaluated after 48 hpf and is therefore not a very sensitive parameter of the zFET. However, vasotoxic effects can in general be detected earlier since vasculogenesis in

zebrafish embryos drives the formation of the two main axial vessels, the dorsal aorta (DA) and more ventrally, the posterior cardinal vein (PCV) already within the first 24 hpf (reviewed in Baldessari and Mione, 2008). Also the blood flow starts at this time point, but is unambiguously observable in the DA and PCV not before 48 hpf (Kimmel et al., 1995). Overall, the blood circulation in the small vessels between the somites, the ISVs, is difficult to assess by bright field microscopy. The advantage of using fluorescent fish is that it allows illumination of cells or organs thereby embryonic structures, which would otherwise not be visible in bright field microscopy. This is for instance reflected in the SU4312 treatment, where the effect *disturbed blood circulation* was not observable until a concentration of 2.23 μM . In contrast to the fluorescence assessment, where already 0.61 μM of SU4312 showed significant truncations of the ISVs (Fig. 16 B). In this thesis, the illumination and visualization of the ISVs in the trunk of fluorescent embryos were exploited in favor of an earlier and specific vascular effect detection and facilitation of effect interpretations. Another example of an earlier and sensitive detection of effects by fluorescence microscopy was provided by the cartap exposure. Here, the sublethal endpoint *malformation of notochord* was recorded significant in the MBFA only at higher concentration than for the fluorescent endpoint ISV-length (Fig. 25). Additionally, the visualization of the neural tube in the *Tg(gfap:GFP)* line allowed a detection of the cartap-induced aberrant morphology of the neural tube (Fig. 18 E), an effect which could not be detected by the MBFA at all.

Effects, which are easily and unmistakably detectable by MBFA are usually well distinct in the embryo. Thus, it is easily possible to over-look subtle effects on the morphological level. And besides the need of an experienced and trained examiner, the standard zFET MBFA suffers from low objectivity. This is reflected by high standard deviations and a low reproducibility. Hence, the main challenges when using the zFET are inter-laboratory reproducibility as well as regulatory constraints in terms of data acceptance. Furthermore, the standard MBFA allows no graded evaluation of the impact. Only presence or absence of the individual effects (quality assessment) are recorded and evaluated, resulting in a loss of information. Consequently, additional endpoints are needed which are less objective and which can be quantified.

4.2 Specific toxicity detection

The EGFP-based examination of the ISV development provided a qualitative as well as quantitative additional endpoint for the zFET, which increases the test sensitivity for substances affecting the vascular development. The qualitative ISV defects in the

Tg(fli1:EGFP)^{y1} embryos (ISVFA) indicated a vasotoxic potential of substances with higher resolution and at lower exposure concentrations than the assessment by bright field microscopy. The effect thresholds of the ISVFA of the vasotoxic compounds tested were all below the MBFA (Fig. 14). However, the concentration-effect curves based on general morphology (MBFA) showed steeper slopes than the ISVFA curves i.e., a minimal increase in substance concentration caused huge differences in the effect level. This generally suggests a high toxic potency of a chemical. Here, the steepness of the slopes was caused also by the multiparametric effect assessment where the effect level is based on the sum of many different (morphological) effects. Contrary, the ISV assessment is a monoparametric assessment, causing the gradually increasing blood vessel development curves of cartap, genistein and triclosan.

The EGFP signal in *Tg(fli1:EGFP)^{y1}* embryos is suitable for a precise and sensitive detection of abnormal vasculature or blood circulation. Nevertheless, despite a higher sensitivity of the fluorescence, the ISVFA endpoint still suffers from the shortcomings of other morphological endpoints in bright field microscopy, like low objectivity, high error rate and sophisticated assessment, when used as a qualitative measure. I was able to improve the assessment within the *Tg(fli1:EGFP)^{y1}* embryos by including the measurement of the ISV-length. This parameter can be assessed and quantified even by untrained test evaluators. The quantification of the ISV growth in *Tg(fli1:EGFP)^{y1}* provides a robust and quantifiable vascular development endpoint after 24 and 48 h of substance exposure.

Furthermore, I was able to show that the ISV-length can also be used as a quantifiable measure of the developmental stage of the embryo and may therefore serve as an objective measure of pollutant-induced developmental delay. The identification of developmental stages in zebrafish embryos is otherwise estimated based on published staging series (Kimmel et al., 1995).

Besides the vasotoxicity assessment, I evaluated neurotoxic effects on the neural tube fluorescence of *Tg(gfap:GFP)* embryos. The *gfap*-expressing cells are mainly located in RGCs and astrocytes of the central nervous system (Bernardos and Raymond, 2006). The region of the neural tube was selected for measuring the fluorescence intensity as the *gfap*-coupled GFP-expression is strongest along the spinal chord of 24 and 48 hpf embryos (Bernardos and Raymond, 2006). The fluorescence intensity of the neural tube of 24 hpf embryos was deemed indeterminable due to the spontaneous movements at this time point. The GFP-signal of this transgenic line is not as strong as the EGFP-signal of *Tg(fli1:EGFP)^{y1}*

embryos and required longer exposure times (~ 1.3 sec) during z-stack image acquisition. Thus, the time required was too long to suppress the spontaneous movement of living 24 hpf embryos without risking the overdose of anesthesia. Therefore, a 24 h assessment endpoint proved not suitable for live embryos and an alternative method using fixed embryos was considered. However, the measurement of the fluorescence intensity of live 48 hpf *Tg(gfap:GFP)* embryos was suitable to measure a negative impact (weaker fluorescent signal) on the neural development by ethanol, triclosan and genistein exposure (Fig. 18 A+D and Fig. 19 A+D).

However, I was not able to confirm the fluorescence intensity of the *Tg(gfap:GFP)* embryos as a direct indicator of neurotoxicity, as no correlation of the intensity decrease with functional effects (e.g., no spontaneous movement at 24 hpf) could be found. Maybe with the help of behavioral assays a neurotoxicity correlation could be determined for *Tg(gfap:GFP)*. Although further research is still required, my data suggest that the fluorescence intensity of gfap-expressing cells is not an indicator of general neurotoxicity, but rather of a specific impact on neural development and thus, of neural development toxicity.

The whole-mount antibody staining method was an easy and fast method to visualize and locate specific tissues or cells in fixed organisms. In 24 hpf embryos the muscle morphology was not yet fully formed to give sharp contrasts of the single muscle fibers. This obscured the outline and thus, the visualization of the angle between the hemi-segments. Therefore, the myotoxic potential of the chemicals tested could be determined only after 48 h of exposure. With this fluorescence endpoint, I confirmed concentration-dependent aberrations in muscle fiber morphology of ethanol, genistein and cartap treated embryos (Fig. 20 A, D, E and Fig. 21 A, D, E). Aberrant muscle morphology affects the locomotion and impacts on survival of the fish due to hatching, foraging or escape difficulties. Moreover, the quantification of the angle between the two hemi-segments can specify the degree of teratogenicity, as aberrant muscle morphology displays embryo malformation (Sylvain et al., 2010). Effects, which indicated altered muscle morphology in the MBFA were *malformations of the tail* and an *undulating notochord* (Fig. 12 A, D, E), but these endpoints are not measurable. Hence, the immunostaining method provided a quantifiable endpoint what enabled the evaluation of the degree of teratogenicity.

Apoptosis is a highly conserved driving force of embryogenesis (Abrams et al., 1993). Developing tissues or organs produce excess cells, which are removed by apoptosis as an

important step in normal development. Environmental or substance-induced influences can impact apoptosis (Parg et al., 2002), what makes apoptosis an interesting endpoint in ecotoxicology. AO stains the nuclei of cells by interaction with the chromatin (Abrams et al., 1993). It has the advantage of a rapid analysis of apoptosis in live embryos, but it does not allow discrimination between apoptotic and necrotic cells. A combination of AO with ethidium bromide would have facilitated this differentiation, but was not conducted, as the assessment was aimed to be performed on living embryos. Hence, the results of the non-invasive AO staining method showed dead cells independently of induced necrosis or apoptosis. Since it was not possible to achieve this specific conclusion, the fluorescent cells were summarized as apoptotic cells.

Cole (2001) has described the normal patterns of apoptosis in various structures of the developing zebrafish embryos. The dynamics of apoptosis in zebrafish embryos follow a time course and mainly occur during neurulation and somitogenesis (Cole, 2001). By 20 hpf, apoptotic events are mostly completed and reduced to a basic level in the embryo (Cole, 2001). This is why I only determined the apoptosis rate of 48 hpf embryos. Cole (2001) discovered a small amount of apoptotic cells in the Rohon-Beard neurons, musculature, urogenital system and median fin fold in the trunk region of normally developed embryos 48 hpf. Based on the image analysis, I observed additional apoptotic cells, which were unspecifically scattered across the trunk, but also an increase in apoptotic cells mainly in the region of the neural tube, depending on the chemical tested (Fig. 22). Genistein exhibited the strongest impact, showing a dense area of apoptotic cells in the ROI (Fig. 22 A, C, D and Fig. 23 A). Consequently, the fluorescent cell counting of this apoptotic phenotype showed the need of a different sensitive assessment method since e.g., overlapping single cells were miscounted as one big cell, which caused an underestimation of the number of apoptotic cells. The application of an alternative quantification method became necessary and therefore the overall fluorescence was measured across the ROI instead (Fig. 23 A+B). For unspecifically induced apoptosis with apoptotic cells spread over a certain region, as in the case of ethanol and triclosan, counting of single fluorescent cells was more suitable and thus more sensitive, whereas area dense apoptosis was assessed with higher sensitivity by measuring the total fluorescence intensity (Fig. 23 and Fig. 24). Maybe a combination of both assessments would be the best method for the most significant and realistic analysis of chemically induced apoptosis. For realizing such a combined method, more data of more substances with a high and area specific increase of apoptotic cells, like genistein, should to be tested.

It should be noted that an automation of the assessment of apoptosis either based on cell counting or measuring total fluorescence intensity, would not provide information on apoptosis tissue specificity otherwise obtainable by the manual image-analysis. With regard to an automated image-analysis, it would be useful to integrate a tool for an automated localization of fluorescent cells.

4.3 Mechanism of toxicity

In hazard and risk assessments applied acute or chronic fish test data deliver little information about the mechanism of action of chemicals. However, such information is a prerequisite in order to reliably predict the potential toxicological impact on the environment as well as humans. In this study, anomalies of main embryonic structures were specifically detected and quantified with the help of fluorescence microscopy. To investigate if they enable a mechanistically specific toxicity assessment within the zFET, model compounds with different toxicity pathways had to be tested. Other studies already highlighted the multiplicity of possible actions of triclosan (Oliveira et al., 2009), genistein (Sassi-Messai et al., 2009) and cartap (Zhou et al., 2009), claiming they have more than one mode of toxic action. Hence, their effects were studied to investigate whether their differing modes of action impact differently on the newly established fluorescence endpoints and whether the results inform on the toxicity pathways.

For triclosan, effects on the ISV-length occurred before any other exposure effects. However, the lack of a concentration-dependency of the ISV-length together with the observed phenotype of a 10-somite staged embryo in the MBFA at 24 hpf (Appendix 8. 1, Table 8), led to the assumption that triclosan caused growth retardation rather than a specific vasculo- or angiogenic developmental delay. Even though the embryos appeared smaller than the control embryos, the mean angle between hemi-segements of the embryo was no indicator for growth retardation. In contrast, the vital AO staining showed triclosan-induced effects on the apoptosis rate in the 48 hpf embryos (Fig. 23 C). Triclosan caused an increase in apoptosis what could indicate growth retardation, as the amount of apoptotic cells should be reduced with progressed normal embryonic development (Cole, 2001). General growth retardation would also explain that the effect on ISV-length diminished at 48 hpf, where a significant reduction in ISV-length was only observed at triclosan concentrations of $\geq 2.45 \mu\text{M}$ (Fig. 16 B). A developmental delay in triclosan-treated embryos was described previously by a study

of Oliviera et al. (2009). I could confirm the typical effect phenotype of triclosan by the additional fluorescence endpoints.

Genistein induced impairment of the ISV outgrowth leading to thinner appearances and, with increasing concentrations, to stronger truncations and malformations (Fig. 15 C and Fig. 16 C). Kalin et al. (2009) identified a similar vessel phenotype in *Xenopus* larvae after genistein exposure. Genistein exhibits anti-angiogenic activity, mediated by a down regulation of VEGF gene expression due to inhibition of the activation of hypoxia-inducible factor-1 (HIF-1) (Büchler et al., 2004). HIF-1 mediates transcriptional activation of vessel dilation, angiogenesis and erythropoiesis via up-regulation of e-NOS, VEGF and erythropoietin (EPO) (Jensen, 2007). Genistein affected the ISV development differently to SU4312 since the embryos of both treatments displayed different ISV phenotypes. According to other described mechanism of action of genistein related to vasculo- and angiogenesis (Sassi-Messai et al., 2009; Schiller et al., 2013), inhibition of the VEGFR pathway is in fact only one of several possible mechanisms. SU4312 instead was specifically developed as a VEGFR antagonist for cancer treatment (Molina et al., 2007). I could further demonstrate that genistein induced apoptosis in a dose-dependent manner by vital AO staining (Fig. 24 D). Interestingly, no apoptotic cells were detected outside the CNS, an effect of genistein already described by Sassi-Messai et al. (2009). As suggested by Kanungo et al. (2008), genistein-induced apoptosis was likely not attributed to Rohon-Beard neurons as they are located in the dorsal part of the spinal cord (Kimmel et al., 1995) and normally occur only occasionally. Hence, the high number of apoptotic cells induced by genistein probably originated from cells abundantly present in the developing CNS, like the RGCs. Strengthening this assumption, genistein-treated *Tg(gfap:GFP)* embryos lost fluorescence intensity within the neural tube, which demonstrated the impact of genistein on *gfap*-expressing cells. The additionally observed increase of fluorescent cells in the neural tube by AO staining confirmed that genistein induced apoptosis of RGCs or/and astrocytes. Transcriptome analysis performed by Schiller et al. (2013), showed that genistein disrupts brain and nervous system development pathways of zebrafish embryos already at low concentration (EC20-concentration). Specific up-regulation of the gene *cyp19a1b* was found in genistein-treated embryos (Schiller et al., 2013). *Cyp19a1b* encodes a brain predominant isoform of aromatase (Fenske and Segner, 2004). It is expressed restrictively in RGCs and is strongly stimulated by estrogens (Le Page et al., 2005; Pelligrini et al., 2007; Xing et al., 2014). Kishida et al. (2001) suggested that *cyp19a1b* is necessary for CNS development and thus represents a reliable marker for testing neurotoxic effects of EDCs during embryogenesis. A linkage between the expression of

cyp19a1b and *gfap* seemed likely, but was not investigated. Brion et al. (2012) showed with the transgenic *Tg(cyp19a1b-GFP)* line that RGCs can be a direct target of many EDCs, thereby emphasizing the need for research on potential effects of EDCs on brain development. In this context, the embryos of the *Tg(gfap:GFP)* may be an additional tool for screening EDCs affecting neurogenesis.

In addition, Schiller et al. (2013) found a down regulation of genes responsible for the formation of the myelin proteolipid protein, which is the major component of myelin. This indicates that genistein not only affects GFAP-positive RGCs and astrocytes as shown in this study, but also other glial cells in the CNS like, e.g., the GFAP-negative oligodendrocytes, which are responsible for the myelination (Xing et al., 2014) (Fig. 5). Hence, the results of our lab on genistein provide some evidence that genistein impacts the development of the CNS. It would be interesting to see, if a later genistein exposure of *Tg(gfap:GFP)* embryos had no effects on the GFP-signal. This would indirectly confirm that the effect of genistein is most likely limited to the neurogenic phase of zebrafish embryos.

Cartap was the only substance without MBFA-detectable impairments of the blood circulatory system or malformations of the heart. Instead, concentration-dependent notochord deformations were observed (Fig. 12 E). Cartap is described as a neurotoxin, which can cause notochord deformation by inhibition of lysyl oxidase enzyme activity (Zhou et al., 2009). The inhibition of the lysyl oxidase destabilizes the cross-linkage of collagens and elastins of the notochord and results in a well-described undulating notochord phenotype, which cause malformations of the chevron-shaped somites (Anderson et al., 2007; Gansner et al., 2007; van Boxtel et al., 2010). I could confirm the disturbed chevron-shaped somites by the immunostaining of the muscle fibers (Fig. 21 E). These malformations were most likely the reason for the disorganized arrangement of the ISVs caused by cartap (Fig. 15 D). Both defects occurred concurrently with the ISV-length decrease and the increase in ISV spacing irregularities and in concordance with the progression of the notochord deformations. This suggested that the effects on ISV pattern formation were due to the irregular notochord development rather than a primary vasotoxic effect. The fluorescent endpoint ISV development therefore indirectly displayed the primary notochord effects of cartap at below EC50 concentrations. Interestingly, effects on the ISV development occurred at lower concentrations than effects on muscle morphology (Fig. 25 B+D). Thus, cartap was probably not primarily myotoxic as well. Furthermore, the impacted morphology of the neural tube was most likely a consequence of the wavy notochord, as it had quite similar deformations (Fig.

18 E). However, an effect of cartap on the fluorescence intensity in the neural tube of *Tg(gfap:GFP)* embryos was not detectable (Fig. 19 E). Hence, the GFAP-positive astrocytes or RGCs were unaffected by cartap treatment. The neurotoxic potential of cartap is presumably due to influences on the development of motoneurons (Muth-Köhne et al., 2012). Consequently, fluorescence intensity of *gfap*-expressing cells does not seem to be an indicator for general neurotoxicity, but rather for a specific impact on neural development, as already postulated.

4.4 Fluorescence endpoints influence test sensitivity

Fluorescence-based approaches have already demonstrated to be sensitive and reliable methods for the analysis of adverse effects in zebrafish embryos upon substance exposure (Dai et al., 2013). The studies of Zhang and Gong (2013) and Muth-Köhne et al. (2012), for instance, have shown that fluorescence-based methods proved to be more sensitive for the detection of embryo toxicity than the non-fluorescent morphological effect assessment. Zhang and Gong (2013) used a transgenic zebrafish line to measure the axon length in living embryos in order to identify neurotoxins. Their fluorescence endpoint '*axon length*' was about 10-times more sensitive than the most sensitive morphological bright field endpoint '*heartbeat*' (Zhang and Gong, 2013). Additionally, Muth-Köhne et al. (2012) demonstrated a sensitive detection of neurotoxins by fluorescence whole-mount immunostainings of motoneurons in zebrafish embryos.

Low substance concentrations may exert subtle and barely detectable effects on the morphological level, which could be missed in a test solely based on MBFA like in the case of the conventional zFET, as shown in Fig. 25. In this study, the fluorescence endpoints showed a higher sensitivity for a chemicals' primary toxic potential (vaso-, neuro-, myotoxic or apoptotic potential). Effects were determined earlier and/or at lower concentrations in the fluorescent test approach: I was able to show that embryos of *Tg(fli1:EGFP)^{y1}* allow an increase in test sensitivity for vasotoxic substances and the counting method of fluorescent cell/ROI in AO stained wild type zebrafish embryos showed also a sensitivity increase for detecting effects (Fig. 25 A, E, F). When significant effects occurred in the zebrafish embryos, then the fluorescence endpoints *fluorescence intensity of Tg(gfap:GFP)* and *the angle between hemi-segments* neither caused an increase nor a decrease in the test sensitivity for triclosan, genistein and cartap compared to the MBFA (Fig. 25 A, C, D). These two fluorescence endpoints did not enhance the identification of toxic actions of the standard

zFET for the tested model chemicals. I therefore concluded that none of the tested model chemicals were primarily myotoxic or neurotoxic.

In contrast to the ISV-length, which appeared to be a more sensitive indicator for vasotoxicity than examining morphological defects. This was demonstrated by decreased ISV-lengths occurring at lower exposure concentration than the morphological effects *reduced* or *missed blood circulation* or even the cumulative morphological effect threshold (Fig. 25 A+B). Also the direct comparison of the quantitative assessment methods, ISVA and MBFA underlined this assumption: For the positive control, the small molecule SU4312, the effect maximum at the highest test concentrations and also the steepness of the curves were similar for both ISVFA and MBFA (Fig. 14 A). At lower concentrations, the ISVFA was more sensitive confirming the primary vasotoxic potency of SU4312. Triclosan showed a higher sensitivity at the vasotoxic than the teratogenic level, as demonstrated by the lower EC50 value and effect thresholds (Fig. 14 B). In the case of genistein and cartap, it was difficult to decide on a more sensitive toxicity endpoint based on the effect curves. Due to the gradual effect increase in the ISVFA, very similar EC50s but higher EC80 values and lower EC10 values were derived than in the MBFA for genistein and cartap (Fig. 14 C+D). This means that the ISVFA allowed a more sensitive effect detection at lower concentrations than the MBFA and distinguished vasotoxic from non-vasotoxic substances below the 50% effect level. At low concentrations, the specific ISV effects were presumably not yet superimposed by systemic toxicity effects, which occurred increasingly upon treatment with increasing concentrations of genistein and cartap. Accordingly, the negative control pentoxifylline was classified as primarily non vasotoxic (Fig. 17 C) contrary to the positive control SU4312, which acts primarily vasotoxic (Molina et al., 2007; Tran et al., 2007). Due to the similarity of the ISVFA EC-curve to SU4312, triclosan was classified as a vasotoxicant, whereas genistein and cartap appeared primarily non vasotoxic (Fig. 14 C+D).

However, the most sensitive effect assessment for all tested chemicals was the ISV-length measurement (Fig. 25). Even for cartap the ISV-length was more sensitive than the MBFA of the standard zFET and the muscle morphology assessment (angle between hemi-segments). Surprisingly, no primary vasotoxic potential of cartap is described in literature (Sylvain et al., 2010). This implied that weak to severe ISV defects occur earlier or, like in the case of cartap, are more clearly to detect in embryos than weak notochord malformations (Fig. 16 and Fig. 13). The ISV-length may also be an early toxicity endpoint as their development starts very early in the embryogenesis and thus can be impacted by early chemical exposure.

Nevertheless, a prerequisite for endpoints assessing specific toxicity are appropriate control substances. The positive controls, ethanol and SU4312 were selected to achieve the most sensitive assessment based on at least one of the fluorescence endpoints. Upon the ethanol treatment all fluorescence endpoints responded earlier than the MBFA endpoints. Therefore, the neuro- and myotoxic potential of ethanol was confirmed, as well as the validity of the corresponding fluorescence endpoints. I could also show the impact of ethanol on the vascular development and apoptosis (Fig. 25). However, the apoptosis rate was not affected by ethanol in a clear concentration-dependent manner. Statistically, effects of ethanol were observed; hence, ethanol could be used as positive control for chemical-induced apoptosis, but as an unspecific one. For a more specific and thus sensitive control e.g., neomycin should have been selected. Neomycin is an antibiotic, which is known to induce apoptosis concentration dependently (Parnig et al., 2002).

In contrast to ethanol, SU4312 only induced concentration-dependent effects on the vascular system. This was determined with higher sensitivity by ISV-length measurements than by conventional zFET assessment methods. Due to its high specificity, as a VEGF antagonist (Molina et al., 2007), SU4312 was a suitable negative control for all other fluorescence endpoints. Pentoxifylline served as negative control for the assessment of a substance's impact on blood vessel development. As described before (chapter 3.1.1), pentoxifylline caused no significant effects on the ISV-length, except for the highest test concentration of 1019.44 μM (Fig. 17 D). As pentoxifylline exhibited moderate fish embryo toxicity in the MBFA, it was obviously not the best selection for a negative control in the fluorescence assessment. Besides, pentoxifylline-induced effects on the blood flow may have influenced the vessel outgrowth, as suggested by Yashiro et al. (2007). They showed that haemodynamics govern the VEGFR signaling and the aortic arch morphogenesis in mice (Yashiro et al., 2007). Disturbed blood flow was detected at low concentrations of genistein and triclosan (Fig. 12 D+C) along with effects on the relative ISV-length of the 48 hpf zebrafish embryos. It may therefore be speculated whether the positive hemorheological property of pentoxifylline would partially explain the absence of ISV defects in all but the highest exposure concentration (Fig. 17 D). My data could not unequivocally confirm that the ISV endpoint is not influenced by general toxicity. To substantiate this assumption further, other negative control substances have to be tested in the future. Nevertheless, the selected control substances proved useful in confirming general test validity of the fluorescence endpoints and the specificity of the particular parameters. A thorough validation, however,

will require more control (negative and positive) and test chemical exposures in more than one laboratory.

4.5 Phenotype versus gene expression

Molecular level parameters measured in ecotoxicological tests are characterised by a high sensitivity, specificity and speed but of low ecological relevance at the organism and population level. Gene expression analysis using PCR can measure the chemical-induced impact on a gene with high sensitivity in relatively little time. It was therefore hypothesised that measuring genes with PCR would reveal embryo toxicity earlier and quicker compared to the bright field and fluorescence endpoints.

In order to test this, early marker genes of angio- and vasculogenesis in zebrafish, *fli1a*, *cdh5* and *flt4* (Thompson et al., 1998; Covassin et al., 2006; Baldessari and Mione, 2008) and the neuromarker *gfap* were analyzed by quantitative RT-PCR. The aim was to compare the results of gene expression with the results of the ISV-length and *gfap*-transgenic assessment in terms of earlier detection and higher sensitivity. Yet, I could detect no substance-induced alterations in mRNA expression of the selected genes (Fig. 26). Since *fli1* and *cdh5* are not only expressed in the ISVs, but also in most other blood vessels (Thompson et al., 1998; Brown et al., 2000; Larson et al., 2004), it is possible that the downregulation related to the absence or truncation of ISVs in the trunk region was too minimal to be detected by RT-QPCR. The impact on blood vessels in the head region was not conspicuous and presumably neglectable and therefore not measurable in the RT-PCR despite amplification. Regarding the receptor tyrosine kinase *flt4*, presumably the wrong time point for the RT-PCR analysis was chosen since the expression evidently starts at 18 hpf and vanishes with further development and is no longer observable at 48 hpf (Thompson et al., 1998).

Evidently, the studied genes were deemed unsuitable as marker genes. To detect effects before they translate into a morphological phenotype more suitable genes will have to be chosen. Moreover, it should be considered that usually the course of molecular events is organized as cascades of aligned pathways defining the mechanism of action. Therefore, single genes are not always suited for early toxicity detection (Schiller et al., 2013). Nevertheless, these results highlight that quantitative RT-PCR approaches are not necessarily the most sensitive measure of exposure effects, in particular when they have already been translated into morphology and vice-versa. Instead, a fluorescence-based phenotypic assessment method using transgenic zebrafish lines like *Tg(fli1:EGFP)^{y1}* or *Tg(gfap:GFP)*, can directly link effects on gene expression with cellular and morphological impairments.

While techniques like quantitative RT-PCR or *in situ* hybridization are used for hypothesis-driven monitoring of expression changes of selected candidate genes, transcriptome analysis, like microarrays allow screening of large gene sets or entire transcriptomes for differentially expressed genes. However, neither RT-PCR nor transcriptome analysis provide information on effect localization. In addition, these methods are not able to detect effects in living organisms and deliver no information on time-related effects. Consequently, there is a need for methods providing mechanistic understanding and spatio-temporal resolution of effects.

In this study, fluorescent phenotypic endpoints have demonstrated to deliver a link between transcriptome effects and morphological impairments. For genistein for example, my results could show that apoptosis is in fact induced and that the nervous system development is impaired, while both effects were predicted by Schiller et al. (2012) based on transcriptome analysis data. Furthermore, the fluorescence endpoints enabled a localization of chemical-induced effects on the neural tube, more precisely on the RGCs and/or astrocytes. These fluorescence endpoints linked the disturbed neural development and increased cell death to the molecular initiating events. Only a robust correlation between molecular biomarkers and phenotypic endpoints can strengthen the acceptance and application of e.g., gene expression profile data in risk assessment (Calzolari et al., 2007; Ankley et al., 2010; Volz et al., 2011). And AOPs are intended to be used by regulatory agencies, which show their commitment by their active engagement in the AOP program activities of the OECD. Fluorescent phenotypic endpoint methods will play an important role in the recognition of key events and the development of key event assays within the new AOP (Adverse Outcome Pathway) driven paradigm in environmental risk assessment.

4.6 Application aspects and cost-benefit considerations

The combination of fluorescent and non-fluorescent, lethal and sublethal endpoints allowed an integrated effect assessment, which provided the ability to characterize chemicals by toxicity classes (e.g. neurotoxic, vasotoxic or teratogenic). The ultimate goal would be to apply fluorescence endpoint methods in screening applications. In a tiered toxicity testing approach, screening methods and endpoints are necessary to prioritize chemicals for further higher-tier testing. A resourceful screening battery with meaningful mechanistic and robust, reliable read-outs could contribute to the reduction of unnecessary follow-on tests on animals. An *in vivo* screening assay should fulfill the criteria of biological relevance, fast execution, standardization and acceptable personnel and financial expenditures. The zebrafish embryo provides a small transparent organism with a fast development and which can be obtained in

large quantities making it particularly well suited for a whole organism screening approach. A limitation of *in vivo* screening models with invertebrates (e.g., *Caenorhabditis elegans*), which are used in drug discovery (Segalat, 2007), is the fact that they lack many organs, which are important to vertebrate biology and physiology. Zebrafish instead possess most organs and functions also present in higher vertebrates, such as the cardiovascular system with a multi-chamber heart and circulation within arteries and veins, immune system and kidneys. Zebrafish embryos have most organs formed only within 48 hpf (Kimmel et al., 1995), which is hard to match by other vertebrates. Concurrently, they fit into microwell plates and can directly be exposed by immersion requiring low amounts of test compounds (Lieschke and Currie, 2007). These advantages have already been exploited by many investigators who have performed drug screening assays with the zebrafish embryo (Mattingly et al., 2001; Blechinger et al., 2002; Hill, 2003; Behra, 2004; Yu, 2005; Hernández and Allende, 2008; Chen et al., 2009). However, the majority of these studies had the shortcomings of a time-consuming assessment lacking automation of analysis. An automated and thus fast image analysis for the zFET is a fundamental requirement for potential HTS applications (the automation of the front-end workflow is another), which should be attractive for commercial use.

In this project, sublethal endpoints of specific toxicity were defined on the basis of fluorescence-labeled cells, tissues and anatomical structures, which were quantifiable and responsive to exposure. Besides, the application of fluorescence microscopy opened up many possibilities of computer-based image analysis to simplify and accelerate the test assessment and increase objectivity. The advantage of using fluorescence image analysis is the lower data volume of binary images compared to bright field images (Liu et al., 2012). The smaller amount of information stored for each pixel simplifies data processing and also the development of algorithms for automated computer-based image analysis.

The analysis of the fluorescence intensity of the *Tg(gfap:GFP)* embryos and of the AO staining were the most promising endpoints for an automated quantification. Parng et al. (2002) introduced a simple method to analyze fluorescence intensity of zebrafish embryos by using a fluorescence plate reader. However, I tried to analyze the fluorescence intensity of the *Tg(gfap:GFP)* and of the AO-stained embryos according to Parng et al. (2001), but neither a concentration dependency nor a statistically relevant difference could be determined. Interestingly, Parng et al. (2002) only showed for one substance (neomycin) the concentration-dependent results of the automated quantification. Furthermore, they could not determine any statistical differences to the control group (Parng et al., 2002). This led to the

assumption that the fluorescence plate reader might be a rather imprecise method and was therefore considered not appropriate for my purposes. Parng et al. (2002) provided only a basic screening method of AO-stained zebrafish embryos in a 96-well microplate fluorescence reader.

Besides automated image analysis, orientation and dechorination of the embryos is another challenging parameter in HTS applications (Liu et al., 2012). If the position of the embryo is not right, defects or malformations are not detectable. In this project, one initial idea to deal with the problem of an unfavorable position of the embryo was to process z-stack images into 3 D-projections, which would then enable a virtual rotation. A confocal microscopic system like the HTS OPERA System of Perkin-Elmer can provide high-resolution images for 3D-projection. However, previous application attempts with the in-house OPERA system had revealed problems concerning the available microscopic objectives and the illumination of the test vessels. Additionally, U-bottom well plates did not fit in the OPERA system, what would have complicated the right positioning of the embryo and hence, the assessment. For this project, it was decided that the resolution of these issues was too cost-intensive and the idea of using the OPERA system dismissed. Moreover, the assessment of the newly defined and developed fluorescence endpoints revealed an easily handled image acquisition, as the selected endpoints were all within the trunk/tail of the embryo, which is the most lateral region of the zebrafish embryo. Taking z-stacks of these regions ensured precise and good-definition images of the morphological details without the need of 3D-projection.

Nonetheless, the preparation of the embryos for image analysis was very time-intensive, not least due to the manual dechorination of embryos as well as due to the mounting procedure. The manual positioning makes the test design unfeasible for HTS. Letamendia et al. (2012) were able to develop a HTS platform, where manual manipulation of zebrafish embryos is not necessary, but only for a rough assessment. In their approach, the embryo dispensation, compound delivery, incubation, imaging and analysis were fully automated. However, neither the positioning of the embryos nor the suppression of embryo movement was described. And for a more detailed effect analysis they had to use a manual assessment as well (Letamendia et al., 2012). They could show that automated computer-based phenotypic analysis of zebrafish embryos is feasible and beneficial, but currently, there are still bottlenecks. Nevertheless, the application of an automated assessment method for the here defined fluorescence endpoints, opens up new possibilities for the application of the zFET also for HTS chemical or drug screens.

To get an idea about the personnel requirements and costs and an insight into optimization strategies of the zFET, a cost benefit analysis of the different toxicity assessment methods was performed. Exemplary costs of a fish embryo toxicity test comprising of ten dilution series of one sample from the environment (e.g., effluent from sewage) or of one unknown chemical, respectively, were listed to compare the different assessment methods: the standard zFET according to OECD 236 (test method I), fluorescence assessment using transgenic zebrafish embryos (test method II) or whole mount antibody staining (test method III) or vital staining with acridine orange (AO) (test method IV). The price of a fish embryo test according to the Fraunhofer IME price list (2012) was used as basis for the calculations. The principle test design was assumed to be identical for all methods. Thus, the amount of the consumables (basic material, like multi well plates, pipettes, etc.) as well as of the fixed overheads (e.g., energy costs: water, light; etc.) was the same for all calculations. Additionally, the cost calculation was performed for a manually performed method (A) and a computer/robotic automated method (B).

The manual standard test strategy according to the OECD 236, as considered for calculation A, was with 3750 € the most cost-efficient method compared to the fluorescence methods (3810 – 3950 €). For the manual fluorescence methods, the costs increased by ~ 1.6% performing vital staining with AO, by ~ 2% using transgenic zebrafish embryos and by ~ 5% performing whole mount antibody staining. When comparing the costs of each method with the manually (A) respectively automated (B) performance, the costs of the standard zFET method could only be reduced by 2%. The other methods would achieve a cost reduction of 4 – 6% by automating the steps of handling and assessment. Additional costs of the required tools (e.g., software for the automation) were not included in the calculation since they were difficult to estimate. However, due to the automation of the steps in the fluorescence assessment, the test method II and IV could become more cost-efficient as the conventional zFET.

To conclude, on the basis of this cost calculation, the fluorescence assessments within the zFET become only attractive with computer-based automation. Consequently, manually performed assessment of the fluorescence methods is unprofitable for regular use. The implementation of the fluorescence endpoints alone would not lead to any reduction in the cost of the zFET. However, if these endpoints were included in an automated screening approach, it would be a different matter, which suggested a significant reduction in costs. Thus, an automated image analysis would significantly improve the applicability of the zFET in screening assays.

This project developed robust fluorescence endpoints, which can be used as basis to fulfill the requirements of automation and enhancement of the microscopic assessment of the zFET. And progressing the zFET towards higher-throughput and higher tier content screening is of interest for both, regulatory agencies and industry.

Table 1: Costs of consumables, personnel and material for toxicity testing with zebrafish embryos according to the FET (DIN 15088-T6).

Costs of the FET according to DIN 15088-T6 (test method I) were calculated on the assumption of a simplified test strategy: one sample with ten dilution series, 10 eggs per concentration and two controls (positive and negative). The cost calculation was done for a manually (A) and for a computer automated (B) performed assessment method. BF: Bright field, n.a.: not available.

Test method I	Resource	Specification	Required units per test		Unit cost [€]	A) Cost per test [€] (manual)	B) Cost per test [€] (auto-mated)
			Manual	Auto-mated			
FET (DIN 38415-T5) (BF)	Fish husbandry	Eggs (piece)	120	120	1.58	190	190
	Personnel	Handling (sorting etc.) (h)	3	0,5	30	90	15
		Assessment (h)	4	n.p.	30	120	120
	Material	Basics (amount)	n.a.	n.a.	n.a.	1400	1400
Benefit	HTS-possible	No					
	Info	Mortality					
Fixed overheads (€)						1950	1950
Overall cost (€)						3750	3675

Table 2: Costs of consumables, personnel and material for a toxicity testing with transgenic zebrafish embryos.

Costs of using transgenic zebrafish (test method II) were calculated on the assumption of a simplified test strategy: one sample with ten dilution series, 10 eggs per concentration and two controls (positive and negative). The cost calculation was done for a manually (A) and for a computer automated (B) performed assessment method. FL: Fluorescence, n.a.: not available.

Test method II	Resource	Specification	Required units per test		Unit cost [€]	A) Cost per test [€] (manual)	B) Cost per test [€] (auto-mated)
			Manual	Auto-mated			
Transgenic fish line (FL)	Fish husbandry	Eggs (piece)	120	120	1.58	190	190
	Personnel	Handling (sorting, dechoriation, mounting etc.) (h)	4	1,5	30	120	45
		Assessment (h)	6	1,5	30	120	45
	Material	Basics (amount)	n.a.	n.a.	n.a.	1400	1400
Benefit	HTS-possible	Yes					
	Info	Target toxicity (Vaso-/ neurotoxic potential)					
Fixed overheads (€)						1950	1950
Overall cost (€)						3839	3629

Table 3: Costs of consumables, personnel and material for a toxicity testing with immunostained zebrafish embryos.

Costs of using whole mount antibody staining (method III) were calculated on the assumption of a simplified test strategy: one sample with ten dilution series, 10 eggs per concentration and two controls (positive and negative). The cost calculation was done for a manually (A) and for a computer automated (B) performed assessment method. FL: Fluorescence, n.a.: not available.

Test method III	Resource	Specification	Required units per test		Unit cost [€]	A) Cost per test [€] (manual)	B) Cost per test [€] (auto-mated)
			Manual	Auto-mated			
Whole-mount immuno-staining (FL)	Fish husbandry	Eggs (piece)	120	120	1.58	190	190
	Personnel	Handling (sorting, dechoriation, staining, mounting, etc.) (h)	6	1.5	30	240	45
		Assessment (h)	6	1.5	30	180	45
	Material	Basics (amount)	n.a.	n.a.	n.a.	1400	1400
		Staining material	16	16	0.01	0.16	0.16
		Antibody (primary)	1.92	1.92	26.04	50	50
		Antibody (secondary)	0.19	0.19	7.89	1.5	1.5
Benefit	HTS-possible	No					
	Info	Target toxicity (myotoxic potential)					
Fixed overheads (€)						1950	1950
Overall cost (€)						3951	3681

Table 4: Costs of consumables, personnel and material for a toxicity testing with acridine orange stained zebrafish embryos.

Costs of using vital staining with acridine orange (test method IV) were calculated on the assumption of a simplified test strategy: one sample with ten dilution series, 10 eggs per concentration and two controls (positive and negative). The cost calculation was done for a manually (A) and for a computer automated (B) performed assessment method. FL: Fluorescence, AO: Acridine orange, n.a.: not available.

Test method IV	Resource	Specification	Required units per test		Unit cost [€]	A) Cost per test [€] (manual)	B) Cost per test [€] (auto-mated)
			Manual	Auto-mated			
Vital staining with AO (FL)	Fish husbandry	Eggs (piece)	120	120	1.58	190	190
	Personnel	Handling (sorting, dechoriation, staining, mounting, etc.) (h)	4	1.5	30	120	45
		Assessment (h)	5	1.5	30	150	45
	Material	Basics (amount)	n.a.	n.a.	n.a.	1400	1400
		Staining material	16	16	0.01	0.16	0.16
		Acridine Orange	10	10	0.1	1	1
Benefit	HTS-possible Info	No Target toxicity (apoptosis rate)					
Fixed overheads (€)						1950	1950
Overall cost (€)						3810	3630

4.7 Conclusions and outlook

The results of the proof-of-principle study demonstrated that the combination of different fluorescence endpoints enables a multi-factorial-effect analysis and contributes to a better understanding of the toxic mechanism of substances. The possibility of double stainings with fluorescent dyes of different excitation and emission wavelength would further promote the applications of fluorescence microscopy within the zFET. However, more chemicals with known and unknown toxicity mechanism have to be assessed to strengthen this hypothesis. Of particular interest would also be to evaluate the practicality and validity of the new endpoint methods for toxicity assessments of samples from the environment and also of mixtures.

All in all, I could demonstrate that quantifiable fluorescence endpoints diminish the deficiencies of the traditional zFET endpoints, which are prone to low objectivity and high error rate and require sophisticated assessment. Additionally, a fluorescence-based analysis is more amenable to computer-based automation approaches, which could be far less time-consuming and promote HTS applications.

Long-term, it could be envisaged to apply the zFET also in screening approaches for environmental monitoring, pharmaceutical product validation and chemical hazard assessment.

5. Summary

The fish embryo toxicity test (FET) is currently one of the most advocated animal alternative tests in ecotoxicology. To date, the application of the FET with zebrafish (zFET) has focused on acute toxicity assessment, where only lethal morphological effects are accounted for. An application of the zFET beyond acute toxicity, however, necessitates the establishment of more refined and quantifiable toxicological endpoints. A valuable tool in this context is the use of gene expression-dependent fluorescent markers and staining methods that can even be measured *in vivo*.

In this thesis all sublethal morphological effects occurring in the embryos were determined by bright field microscopy, and, in parallel, fluorescent transgenic zebrafish embryos of *Tg(fli1:EGFP)^{y1}* were used to gain supplementary information on i) defects of blood vessel development, ii) *Tg(gfap:GFP)* embryos for detecting effects on radial glial cell development, iii) fluorescent staining with antibody against myosin to allow for conclusions on the myotoxic potential of substances and lastly, iv) an *in vivo* staining method in order to notice substance-induced changes in the apoptosis rate of the embryo. For a proof-of-concept study, the chemicals triclosan, genistein and cartap were selected as test compounds. On the basis of these quantifiable fluorescence endpoints, underlying toxicological mechanisms were elucidated what, in turn, proved to increase the sensitivity and specificity of the conventional zFET. In this study, the newly invented fluorescence endpoints showed a higher sensitivity of tested chemicals in accordance to their toxic potential (vaso-, neuro-, myotoxic or apoptotic potential), since effects were determined at lower concentrations in the test approach. The analysis of the ISV (intersegmental vessel)-length in *Tg(fli1:EGFP)^{y1}* enabled an *in vivo* detection of (vaso-) toxicity already in 24 hpf embryos. Moreover, two of the fluorescence endpoints methods (ii and iv) were able to link cellular effects of disturbed neural development and increased cell death to the molecular initiating events, what could contribute to the acceptance and application of gene expression profile data in risk assessment.

Furthermore, the results demonstrate that quantifying fluorescence endpoints prevents deficiencies of the traditional zFET, which are low objectivity, high error rate and sophisticated assessment. Additionally, the fluorescence analysis depicted the potential for computer-based automated image analysis, what would significantly improve the applicability of the zFET in industrial used high-throughput screenings.

6. Zusammenfassung

Der Fischembryotoxizitätstest (FET) wird in der Ökotoxikologie seit Jahren als vielversprechende Alternativmethode zum Tierversuch diskutiert. Bislang wurde der Einsatzschwerpunkt des FETs mit dem Zebrafisch (zFET) ausschließlich bei der Ermittlung akuter Toxizitätsdaten von Chemikalien angesehen, bei der nur letale morphologische Effekte zum Tragen kommen. Für die Anwendung des FETs bei der Bewertung toxikologischer Wirkungen von Chemikalien, die weit über die akute Toxizität hinausgeht, werden weiterreichende, spezifische und vor allem quantifizierbare toxikologische Testendpunkte benötigt. Genexpressionsabhängige Fluoreszenzmarker und Färbemethoden die u.a. eine *in vivo* Detektion ermöglichen, bieten solch eine Möglichkeit.

Innerhalb dieser Arbeit wurden alle in den Embryos auftretenden morphologischen Effekte mittels Hellfeld-Mikroskopie bestimmt. Gleichzeitig wurden anhand von fluoreszierenden, transgenen Zebrafischembryos der Linie Tg(*fli1*:EGFP)^{y1} zusätzliche Informationen zu i) Defekten auf die Blutgefäßentwicklung, ii) Tg(*gfap*:GFP) Embryos für die Detektion von Effekten auf die Radialglialzellen-Entwicklung, iii) Fluoreszenzfärbungen mittels spezifischer Antikörper gegen Myosin um Rückschlüsse auf das myotoxische Potential von Substanzen zu ermöglichen und zu letzt, iv) eine *in vivo* Färbemethode um Substanzinduzierte Veränderungen innerhalb der Apoptoserate der Embryos festzustellen, eingesetzt.

Für eine *Proof-of-concept*-Analyse wurden anschließend Triclosan, Genistein und Cartap als Modellsubstanzen gewählt. Anhand der Fluoreszenzendpunkte konnten zugrundeliegende toxikologische Wirkmechanismen aufgezeigt werden, was zu einer Steigerung der Sensitivität und Spezifität des konventionellen zFETs führte. Es konnten Effekte bereits in geringeren Konzentrationen festgestellt werden, was eine erhöhte Sensitivität gegenüber den getesteten Substanzen in Anbetracht ihres primär toxischen Potentials (vaso-, neuro-, myotoxisch oder apoptotisch) unter Beweis stellte. Anhand der ISV-Längenmessung der Tg(*fli1*:EGFP)^{y1} Linie konnte sogar eine *in vivo* Identifizierung von (Vaso-) Toxizität bereits in 24 hpf Embryos erfolgen. Ferner konnte mittels zweier Fluoreszenzendpunkt-Methoden (ii und iv) eine Verknüpfung von Effekten auf der zellulärer Ebene, wie die gestörte Neuronalentwicklung und der gesteigerte Zelltod, mit den dazugehörigen, sogenannten initiiierenden Ereignissen auf der molekularen Ebene, aufgezeigt werden. Dies untermauert die Anwendbarkeit von Genexpressionsprofilen in der Umweltrisikooanalyse.

Zudem zeigen die Ergebnisse, dass die Defizite des klassischen zFETs, wie die geringe Objektivität, die hohe Fehlerrate sowie die aufwändige Auswertung, durch die Quantifizierung der Fluoreszenzendpunkte vermindert werden können. Auch wird die

Möglichkeit für eine Computergestützte, automatisierte Bildanalyse aufgezeigt, was die Anwendbarkeit des zFET für industriell einsetzbare Hochdurchsatz-Screenings signifikant steigern würde.

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8. Appendix

8.1 Listing of all morphological effects recorded in the zFET

Table 5: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with ethanol [%].

Ethanol [%]		0.00	0.50	0.71	1.00	1.41	2.00
24 hpf	Normally developed eggs	0,0%	33,3%	30,6%	33,3%	19,4%	16,7%
	Eggs with any observed Effect	0,0%	0,0%	2,8%	0,0%	13,9%	16,7%
	Mortality*	0,0%	0,0%	0,0%	0,0%	2,8%	2,8%
	*Coagulated	0,0%	0,0%	0,0%	0,0%	2,8%	0,0%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%	2,8%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	2,8%	5,6%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%	2,8%
	Odema	0,0%	0,0%	2,8%	0,0%	8,3%	8,3%
	Malformation of Head	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	0,0%	0,0%	0,0%	2,8%
	Malformation of heart	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	2,8%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	62,5%	91,8%	90,3%	70,8%	22,2%	16,7%
	Eggs with any observed Effect [%]	4,2%	8,2%	9,7%	29,2%	77,8%	83,3%
	Mortality*	2,8%	4,1%	2,8%	0,0%	9,7%	18,1%
	*Coagulated	2,8%	4,1%	2,8%	0,0%	8,3%	1,4%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	0,0%	0,0%	0,0%	1,4%	11,1%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%	5,6%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	1,4%	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Odema	0,0%	2,8%	2,8%	20,8%	47,2%	59,7%
	Reduced Heart-beat/Blood circ.	0,0%	2,8%	2,8%	19,4%	61,1%	51,4%
	Pigmentation	0,0%	1,3%	0,0%	0,0%	0,0%	0,0%
	Malformation of Head	1,4%	0,0%	0,0%	0,0%	6,9%	13,9%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	1,4%	0,0%	0,0%	1,4%	4,2%	13,9%
	Malformation of heart	0,0%	0,0%	1,4%	5,6%	27,8%	44,4%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	2,8%	1,4%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 6: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with SU4312 [μM].

SU4312 [μM]		0.00	0.38	0.61	0.91	1.44	2.23	3.48
24 hpf	Normally developed eggs	96,1%	96,6%	86,4%	88,0%	70,3%	58,6%	48,6%
	Eggs with any observed Effect	3,9%	3,4%	13,6%	12,0%	29,7%	41,4%	51,4%
	Mortality*	3,0%	2,5%	5,6%	9,2%	18,9%	27,1%	37,3%
	*Coagulated	3,0%	2,5%	5,6%	9,2%	18,0%	25,0%	36,6%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	1,4%	0,7%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,7%	0,0%	0,9%	0,7%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,7%	0,0%	0,9%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,7%
	No spont. movement	0,0%	0,0%	3,0%	0,0%	2,3%	1,9%	0,0%
	Odema	1,9%	0,9%	5,2%	0,9%	5,3%	8,5%	9,8%
	Malformation of Head	0,0%	0,0%	0,0%	0,0%	0,9%	0,9%	0,0%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	0,0%	0,0%	0,9%	0,0%	0,0%
	Malformation of heart	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,9%	1,9%	0,0%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	0,9%	0,7%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	3,9%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,7%	0,0%	1,3%	0,0%	2,4%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	94,4%	94,2%	90,2%	87,6%	72,3%	47,8%	36,4%
	Eggs with any observed Effect [%]	5,6%	5,8%	9,8%	12,4%	27,7%	52,2%	63,6%
	Mortality*	3,0%	2,5%	6,3%	8,3%	16,8%	26,6%	33,9%
	*Coagulated	3,0%	2,5%	6,3%	8,3%	16,8%	25,9%	33,9%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,7%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,7%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Odema	0,0%	1,9%	1,4%	3,5%	4,9%	17,8%	17,8%
	Reduced Heart-beat/Blood circ.	1,7%	1,6%	0,7%	0,9%	0,7%	13,2%	10,9%
	Pigmentation	0,0%	0,0%	0,7%	0,0%	0,0%	8,1%	10,3%
	Malformation of Head	0,8%	0,0%	0,7%	2,8%	2,8%	0,0%	1,0%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,9%	1,0%
	Malformation of heart	0,0%	0,7%	1,4%	1,6%	2,1%	13,8%	6,3%
	Modified chorda structure	0,0%	0,0%	0,7%	0,9%	0,0%	0,9%	1,0%
	Yolk deformation	0,0%	0,0%	0,7%	0,9%	1,6%	8,3%	6,5%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	0,7%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,9%	2,9%	0,0%	1,6%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 7: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with pentoxifylline [μM].

Pentoxifylline [μM]		0.00	179.66	303.83	593.22	1019.44
24 hpf	Normally developed eggs	98,6%	97,4%	89,2%	86,8%	88,9%
	Eggs with any observed Effect	1,4%	2,6%	10,8%	13,2%	11,1%
	Mortality*	1,4%	2,6%	5,2%	9,7%	2,1%
	*Coagulated	1,4%	2,6%	5,2%	9,7%	2,1%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	1,4%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	8,3%	0,0%	4,2%
	Odema	0,0%	0,0%	5,6%	0,0%	0,0%
	Malformation of Head	0,0%	0,0%	0,0%	0,0%	0,0%
	Malform. of sacculi/otoliths	0,0%	0,0%	2,8%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	2,8%	0,7%	3,5%
	Malformation of heart	0,0%	0,0%	0,0%	0,0%	0,0%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,0%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	95,9%	94,7%	80,1%	47,9%	16,7%
	Eggs with any observed Effect [%]	4,1%	5,3%	19,9%	52,1%	83,3%
	Mortality*	1,4%	2,6%	5,2%	11,1%	3,5%
	*Coagulated	1,4%	2,6%	5,2%	9,7%	2,1%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	0,0%	0,0%	1,4%	1,4%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,6%	0,0%	0,0%
	Odema	0,0%	0,0%	1,3%	12,5%	45,8%
	Reduced Heart-beat/Blood circ.	2,8%	2,8%	6,9%	19,4%	68,8%
	Pigmentation	0,0%	1,3%	5,8%	8,3%	2,1%
	Malformation of Head	0,0%	0,0%	1,3%	0,0%	0,7%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	0,0%	4,2%	9,0%
	Malformation of heart	2,8%	2,8%	5,6%	6,9%	26,4%
	Modified chorda structure	0,0%	0,0%	1,3%	17,4%	44,4%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%

Table 8: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with triclosan [μM].

Triclosan [μM]		0.00	1.73	1.93	2.18	2.45	2.76
24 hpf	Normally developed eggs	97,2%	74,9%	75,0%	69,8%	59,6%	28,8%
	Eggs with any observed Effect	2,8%	25,1%	25,0%	30,2%	40,4%	62,8%
	Mortality*	1,4%	2,8%	1,4%	1,4%	1,4%	9,7%
	*Coagulated	2,8%	4,3%	2,8%	2,8%	2,8%	9,7%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	1,3%	1,4%	4,2%	4,2%
	No formation of Eyes	0,0%	2,8%	1,3%	1,4%	2,8%	1,3%
	No spont. movement	0,7%	5,6%	5,1%	6,8%	16,4%	31,5%
	Odema	0,0%	8,3%	5,4%	9,6%	8,4%	9,6%
	Malformation of Head	0,0%	5,6%	7,8%	6,9%	2,8%	20,4%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,7%	1,4%	5,4%	6,8%	4,3%	20,7%
	Malformation of heart	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Yolk deformation	0,7%	0,0%	0,0%	0,0%	1,5%	1,3%
	General growth retardation	0,0%	1,4%	2,6%	2,8%	2,9%	2,7%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	95,8%	72,2%	76,3%	68,1%	42,6%	6,9%
	Eggs with any observed Effect [%]	4,2%	27,8%	23,7%	31,9%	57,4%	93,1%
	Mortality*	1,4%	5,6%	1,4%	0,0%	11,2%	35,1%
	*Coagulated	2,8%	1,4%	2,8%	0,0%	2,8%	21,8%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	4,2%	0,0%	0,0%	7,1%	19,0%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	2,8%	2,7%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Odema	0,0%	6,9%	4,1%	11,1%	11,1%	19,3%
	Reduced Heart-beat/Blood circ.	1,4%	16,8%	13,0%	9,7%	25,7%	44,2%
	Pigmentation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of Head	0,0%	6,9%	5,3%	5,6%	15,7%	12,2%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	1,4%	8,3%	5,3%	5,6%	14,2%	32,9%
	Malformation of heart	0,0%	12,5%	14,5%	23,6%	39,1%	49,7%
	Modified chorda structure	0,0%	0,0%	0,0%	0,0%	0,0%	2,6%
	Yolk deformation	1,4%	8,3%	1,3%	2,8%	2,9%	11,1%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 9: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with genistein [μM].

Genistein [μM]		0.00	7.40	8.81	10.47	12.43	14.80
24 hpf	Normally developed eggs	97,1%	95,8%	83,3%	79,8%	70,8%	12,5%
	Eggs with any observed Effect	2,9%	4,2%	16,7%	20,2%	29,2%	87,5%
	Mortality*	2,9%	0,0%	8,3%	1,9%	0,0%	2,1%
	*Coagulated	2,9%	0,0%	8,3%	1,9%	0,0%	2,1%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	2,1%	0,0%	8,3%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	1,5%	2,1%	0,0%	0,0%	0,0%	10,4%
	Odema	0,0%	2,1%	6,3%	2,1%	14,6%	43,8%
	Malformation of Head	0,0%	2,1%	2,1%	18,3%	12,5%	64,6%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	0,0%	2,1%	0,0%	20,8%
	Malformation of heart	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Modified chorda structure	0,0%	0,0%	0,0%	4,2%	8,3%	31,3%
	Yolk deformation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	2,1%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	91,1%	91,7%	53,0%	12,4%	5,6%	1,4%
	Eggs with any observed Effect [%]	8,9%	8,3%	47,0%	87,6%	94,4%	98,6%
	Mortality*	6,1%	2,8%	8,5%	5,4%	1,4%	27,8%
	*Coagulated	4,7%	2,8%	8,5%	5,4%	1,4%	8,3%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	0,0%	0,0%	0,0%	0,0%	18,1%
	*No differentiation	0,0%	0,0%	0,0%	0,0%	0,0%	1,4%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	5,8%	13,9%	22,2%	48,6%
	No formation of Eyes	0,0%	0,0%	0,0%	19,4%	25,0%	33,3%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Odema	1,4%	0,0%	2,8%	17,9%	38,9%	72,2%
	Reduced Heart-beat/Blood circ.	1,4%	4,2%	15,3%	27,1%	33,3%	68,1%
	Pigmentation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of Head	1,4%	1,4%	22,2%	67,2%	86,1%	88,9%
	Malform. of sacculi/otoliths	0,0%	2,8%	0,0%	5,6%	4,2%	1,4%
	Malformation of tail	2,8%	1,4%	15,3%	41,6%	55,6%	87,5%
	Malformation of heart	0,0%	1,4%	5,6%	41,1%	56,9%	88,9%
	Modified chorda structure	0,0%	0,0%	0,0%	12,1%	25,0%	26,4%
	Yolk deformation	2,8%	0,0%	0,0%	2,8%	1,4%	1,4%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	1,4%	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%

Table 10: Mean percentage of morphological effects detected in the bright field assessment of the zFET according to the background paper of Braunbeck and Lammert (2006) for the treatment with cartap [μM].

Cartap [μM]		0.00	0.42	0.55	0.67	0.84
24 hpf	Normally developed eggs	94,2%	94,5%	65,2%	35,1%	5,6%
	Eggs with any observed Effect	5,8%	5,5%	34,8%	64,9%	94,4%
	Mortality*	4,7%	4,6%	8,5%	3,7%	7,4%
	*Coagulated	3,2%	4,6%	8,5%	3,7%	4,6%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	*No differentiation	0,5%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	No formation of Eyes	0,0%	0,0%	1,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,9%
	Odema	0,0%	0,0%	2,9%	0,0%	1,9%
	Malformation of Head	0,0%	0,0%	8,9%	18,8%	13,9%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,0%	0,0%	2,9%	0,9%	1,9%
	Malformation of heart	0,0%	0,0%	0,0%	5,6%	11,1%
	Modified chorda structure	0,0%	0,9%	19,2%	48,3%	73,6%
	Yolk deformation	0,0%	0,0%	1,0%	0,0%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%
48 hpf	Normally developed eggs [%]	93,5%	94,4%	79,6%	37,8%	5,1%
	Eggs with any observed Effect [%]	6,5%	5,6%	20,4%	62,2%	94,9%
	Mortality*	5,1%	4,6%	8,3%	5,1%	6,0%
	*Coagulated	3,2%	2,8%	8,3%	3,7%	5,1%
	*No detachment of tail	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Somites	0,0%	0,0%	0,0%	0,0%	0,0%
	*No Heartbeat	0,0%	0,0%	0,0%	1,4%	0,0%
	*No differentiation	0,5%	0,0%	0,0%	0,0%	0,0%
	*Exitus	0,0%	0,0%	0,0%	0,0%	0,0%
	Altered formation of Somites	0,0%	0,0%	0,0%	2,8%	15,3%
	No formation of Eyes	0,0%	0,0%	0,0%	0,0%	0,0%
	No spont. movement	0,0%	0,0%	0,0%	0,0%	0,0%
	Odema	0,0%	0,0%	0,0%	1,4%	1,4%
	Reduced Heart-beat/Blood circ.	0,0%	0,0%	0,0%	2,3%	0,0%
	Pigmentation	0,0%	0,0%	1,0%	52,0%	87,5%
	Malformation of Head	0,0%	0,0%	0,9%	0,9%	4,2%
	Malform. of sacculi/otoliths	0,0%	0,0%	0,0%	0,0%	0,0%
	Malformation of tail	0,9%	0,0%	2,3%	1,4%	1,4%
	Malformation of heart	0,0%	0,9%	2,3%	4,2%	2,8%
	Modified chorda structure	0,0%	0,0%	7,4%	53,5%	87,5%
	Yolk deformation	0,0%	0,0%	1,9%	0,9%	0,0%
	General growth retardation	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 1: ()	0,0%	0,0%	0,0%	0,0%	0,0%
	Other Effect 2: ()	0,0%	0,0%	0,0%	0,0%	0,0%

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