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Development and validation of a neurotoxicological test battery for neurotoxicity risk assessment

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Summary

The present study was conducted as part of the research project "Tox-Box: securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany", funded by the Federal Ministry of Education and Research (02WRS1282G). The main fields of research within this project were endocrine disruption, genotoxicity, germ cell toxicity and neurotoxicity. The central task was to provide a compilation of methods to gain information about emerging pollutants to rank these in the health-related indicator value (HRIV) supported by the Federal Environmental Agency. The thesis, embedded in the field of neurotoxicity, aims at developing and validating a test battery for neurotoxicity, based on zebrafish embryo within scope of the 3R principle. The test systems comprised: (1) an extended embryo toxicity test (FET) to assess the general toxicity of substances and sediment extracts as well as to discriminate between neurotoxic and general physiological effects, (2) the acetylcholinesterase activity, (3) the lateral line system, (4) the olfactory epithelium and (5) the retina of zebrafish embryos. The methods were evaluated by screening test with various substances, namely amidotrizoic acid, caffeine, cypermethrin, 2,4-dichlorophenol, 2,4-dinitrotoluene, dichlorvos, 4-nonylphenol, paraoxon-methyl, perfluorooctanoic acid and perfluorooctane sulfonic acid. The extended embryo toxicity test proved useful to detect physiopathological effects and led to the determination of the EC_{10} value, which was used in subsequent neurotoxicity test to exclude side effects by physiological influences. The acetylcholinesterase test detects both, inhibition (dichlorvos / paraoxon) and activation (cypermethrin) and can be used as an *in vivo* and *in vitro* assay, as well as for pure substances and sediment extracts. A few substances were positive in one method exclusively, either *in vivo* or *in vitro*. Three out of nine substances triggered the test system at all, but, far below the EC_{10}, constituting a high sensitivity and high specificity. The neuromast assay proved to be more sensitive than most comparable studies. Observing anterior and posterior neuromasts separately, led to a reduction of false-negative results. Additionally, 80 % of the substances showed effects at the EC_{10}, which documents a high sensitivity, but limited specificity. Furthermore, being affected by endocrine disruptors (nonylphenol, perfluorooctansulfonic acid) as well, this methods also covers effects outside neurotoxicity. Specific olfactory antibodies (Golf-, ANO2-, GAP43-Antibody) have been detected in the olfactory system. Anti-Golf was detected in the cilia of ciliary receptor neurons, leading to the use of Golf as a marker in the olfactory epithelium. The combination of wholemount staining and cryosectioning led to a sufficient structure resolution. Since only the positive control zinc sulfate and none of the standard substances had effects in the olfactory epithelium, the use in a test battery is not yet given. For the presentation of histopathological effects in the retina, a classic hematoxylin / eosin staining of paraffin sections has proved useful. However, effects in layer arrangement could be detected for any of the substances tested at the EC_{10}. Therefore, the retina is quite insensitive for neurotoxicity testing in the range of EC_{10}.

In summary, the combination of embryo toxicity testing, the acetylcholinesterase assay (*in vivo* and *in vitro*) and the neuromast assay, seems suitable for a neurotoxicity test battery.

Zusammenfassung

Die vorliegende Arbeit entstand im Rahmen des durch das Bundesministerium für Bildung und Forschung geförderten Projekts „Gefährdungsbasiertes Risikomanagement für anthropogene Spurenstoffe zur Sicherung der Trinkwasserversorgung (Akronym: Tox-Box)“. Hauptziel dieses Projekts war die Formulierung einer Testbatterie, niedergeschrieben in einem Leitfaden für den Umgang mit anthropogenen Spurenstoffen im Trinkwasser, deren Datenlage keine Risikobewertung erlaubt. Im Verlauf des Projekts sollte durch Etablierung und Evaluierung von Testmethoden aus den Bereichen endokrine Disruptoren, Gentoxizität, Reproduktionstoxizität und Neurotoxizität eine Testbatterie entstehen. Diese soll dazu dienen Grenzwerte für diese Substanzen im Rahmen des Gesundheitlichen Orientierungswert (GOW) festzulegen. Die vorliegende Dissertation hatte im Rahmen des Teilbereichs Neurotoxizität das Ziel, neurotoxische Methoden im Zebrabärblingsmodell zu etablieren oder bereits etablierte Methoden anzupassen, um sie auf Ihre Verwendbarkeit an ausgewählten Monosubstanzen zu prüfen. Bei der Etablierung wurde speziell auf eine Reduzierung der benötigten Individuenzahl und die Verwendung von Alternativmethoden im Rahmen des 3R Prinzips Wert gelegt.

Als Testsysteme wurden zunächst ein (1) erweiterter Embyrotoxizitätstest verwendet. Dieser diente zur Bestimmung der allgemeinen Toxizität der verwendeten Monosubstanzen. Die verwendeten neuronalen Systeme im Zebrabärblingsembryo wurden aus verschiedenen Organisationsstufen ausgewählt: (2) Acetylcholinesteraseaktivität (molekularer Mechanismus, ausgewertet mittels Enzymkinetik), (3) das Seitenliniensystem (Zellviabilität, ausgewertet mittels Fluoreszensintensität), (4) das olfaktorische Epithel (molekularer Marker, Anti-GSolf und Histopathologie, ausgewertet mittels immunhistochemischer Methoden) sowie (5) die Retina des Zebrabärblingsembryo (Histopathologie, ausgewertet mittels verschiedener unspezifischer Färbemethoden) verwendet. Nach Etablierung der Methoden wurden diese in Screeningversuchen mit den Modellsubstanzen Paraoxon-methyl, 2,4-Dichlorophenol, 2,4-Dinitrotoluene, 4-Nonylphenol, Koffein, Cypermethrin, Dichlorvos, Amidotrizoesäure, Perfluoroctansäure, und Perfluoroctansulfonsäure validiert.

Der durch subletale Parameter erweiterte Embryotoxiziätstest zeigte sich für die Darstellung von physiologischen Effekten im Embryo als sehr hilfreich. Bei der Ermittlung der Embryotoxizitätsdaten wurde der EC_{10} Wert ermittelt, welcher in nachfolgenden Neurotoxizitätstest als Höchstkonzentration verwendet wurde. Der EC_{10}-Wert diente hierbei dazu, die Expositionskonzentration in einem möglichst niedrigen Bereich anzulegen um eine Unterscheidung zwischen physiologischen und spezifisch neurotoxischen Effekten zu erleichtern.

Der Endpunkt Acetylcholinesteraseaktivität zeigte sich nützlich im Nachweis von Inhibierung (Dichlorvos/Paraoxon) und Aktivierung (Cypermethrin), als einsetzbar für *in vivo* und *in vitro* Testverfahren, sowie für Monosubstanzen und Sedimentextrakten. Da Effekte aufgrund verschiedener Mechanismen nur entweder *in vivo* oder *in vitro* auftraten, muss eine kombinierte Verwendung beider vorgeschlagen werden. Da, sowohl *in vivo* als auch *in vitro*, der Test bei 3 von 9 Substanzen anschlug, muss zusätzlich eine hohe Spezifität festgestellt werden. Da für Cypermethrin Effekte im ng/L Bereich und für Dichlorvos und Paraoxon signifikante Effekte weit unterhalb des EC_{10} gezeigt werden konnten, stellt sich dieses System jedoch zusätzlich als hochsensitiv dar.

Die Verwendung von 4 Embryonen mit jeweils 9 Neuromasten und einem 4-schrittigen Bewertungssystem von Effekten zeigte sich als sensitiver als die meisten vergleichbaren Studien. Da Neuromasten aus dem anterioren (ALL) und posterioren (PLL Teil des Seitenliniensystems unterschiedliche Reaktionen auf verschiedene Substanzen und Expositionszeiten zeigten, wird mit der vorgestellten Methode die Möglichkeit falsch-negativer Befunde minimiert. 80 % der Substanzen zeigten Effekte im Bereich des EC_{10}, dies belegt eine hohe Sensitivität und, da die

getesteten Substanzen aus verschiedenen Stoffklassen stammen, einer weniger stark ausgeprägte Spezifität. Weiterhin entstanden, durch Effekte von endokrinen Disruptoren (Nonylphenol, Perfluoroktansulfonsäure) Möglichkeiten einer Effektdarstellung außerhalb des Neurotoxizitätsspektrums.

Im olfaktorischen System ließen sich die Erkennungssequenzen für die molekularen Marker GSolf, ANO2 und GAP43 nachweisen. GSolf wurde dabei in den Zilien ziliarer Rezeptorneurone detektiert und diente dadurch als Marker für Schädigungen in diesem Riechzelltyp. Da bei einer Wholemountfärbung das basale Epithel und bei der Färbung von Kryoschnitten die Zilien nicht darstellbar waren, stellte sich eine Kombination beider als notwendig heraus. Da zwar für die Positivkontrolle Zinksulfat, aber nicht für die getesteten Monosubstanzen Effekte im Bereich des EC_{10} dargestellt werden konnten ist ein Einsatz in einer Testbatterie noch nicht gegeben. Um eine mögliche Insensitivität gegen eine hohe Spezifität abzugrenzen, sind weitere Monosubstanztests nötig.

Für die Darstellung histopathologischer Effekte in der Retina erwies sich eine klassische Hämatoxilin / Eosin Färbung von Paraffinschnitten als geeignet. Jedoch konnte für keine der verwendeten Substanzen im Bereich des EC_{10} ein Einfluss auf die Retina nachgewiesen werden. Demnach ist die Retina für Testungen mit den hier ausgewählten Monosubstanzen im Bereich des EC_{10} als insensitiv einzuschätzen.

Zusammenfassend zeigte sich die Zusammenstellung aus Embryotoxizitätstest und der Aktivität der Acetylcholinesterase (*in vivo* und *in vitro*) sowie der Zellvitalität von Neuromasten (PLL und ALL) als vielversprechende Kombination in der Verwendung als Neurotoxizitätstestbatterie. Da Substanzen teilweise nur in einem der Testsysteme Effekte auslösten, ist die kombinierte Verwendung verschiedener unabhängiger Systeme notwendig.

Im Vergleich der Effektkonzentrationen und Spezifitäten der verschiedenen Testsysteme zeigt sich mit steigendem Organisationsniveau des Testsystems, von molekular zu organismisch, eine Verringerung von Sensitivität und Spezifität. Daher sind für Aussagen über die Relevanz von Effekten, Tests aus unterschiedlichen Organisationsniveaus zu kombinieren. Da Substanzen auch in mehreren Testsystemen Effekte zeigten, liefert die Kombination zusätzlich mögliche Aussagen über das „wahre“ Wirkungsspektrum von neurotoxischen Stoffen.

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Introduction

Neurotoxicity

Adequate neuronal function is essential for animals of every systematic and organizational level. Main functions of neuronal systems are perception, transmission and processing of information. These characteristics ensure senses to interact with the surrounding environment and to receive information which leads to specific behavior. However, the neuronal system is not only of concern for signal transduction within one organism, but also leads to specific and interactive social behavior patterns, which are essential for survival, such as feeding, mating, migrating and predator-prey-interaction. Such behavioral patterns are thus based on intra- and inter-species communication and involve functional senses. Perception of environmental information generally takes place on the body-surface of organisms, which directly exposes sensory neurons to the surrounding environment. These sensory neurons are strongly susceptible to neurotoxic influences. Neuronal systems can be affected at multiple organizational levels, for example neurochemically (e.g. transmitters), neurophysiologically (e.g. cellular function), neuroanatomically (e.g. organ structure) or neurobehaviorally (e.g. predator-prey-interaction). These alterations may appear on a small scale in mechanisms of molecular gene regulation or affect whole population dynamics by altering individual behavior. However, besides being directly affected, the function of neuronal systems may be altered by non-neuronal organs. Since regulatory neuronal systems are closely linked to non-neuronal organs, non-neurotoxic effects might lead to structural and functional abnormalities in neuronal tissues or functions and *vice versa*. Thus, the detection of neurotoxicological effects is complex and needs a precise definition of neurotoxic endpoints to exclude effects of indirect (secondary) nature.

Regulatory institutions, for instance the Organization for Economic Co-operation and Development (OECD), have defined neurotoxicity in neuronal systems of single individuals e.g. test guideline TG 424:

> *"Neurotoxicity is defined as an adverse change in the structure or function of the nervous system that results from exposure to a chemical, biological or physical agent" (OECD TG 424, OECD (1997))*

This definition, however, is assigned to the use in toxicity testing limiting effects to a specific region of interest and neglecting the population relevance and developmental influences of neuronal systems. Harry et al. (1998), therefore, added developmental and environmental aspects to this definition:

> *"Neurotoxicology is the study of the adverse effects of chemical, biological, and certain physical agents on the nervous system and/or behavior during development and in maturity" (Harry et al. 1998).*

A controversial supplement to recent definitions hardly feasible of being integrated into standardized methods within the scope of neurotoxicity risk assessment was suggested by Chepelev et al. (2015), who include indirect (secondary) effects:

"*It is important to note that "neurotoxicity" is a broad term describing a multitude of effects triggered by chemicals acting*

(i) *directly on the central nervous system (brain, spinal cord, optic nerves),*

(ii) *and/or directly on the peripheral nervous system (motor, sensory and/or autonomic components and end organs),*

(iii) *and/or indirectly via a peripheral organ such as the liver, where abnormal function can trigger abnormal brain activity (e.g., hepatic encephalopathy)*" (Chepelev et al. 2015).

By defining organismal targets and following endpoints in neurotoxicity, substances affecting these endpoints are therefore called neurotoxicants:

> *"Neurotoxicant is any chemical, biological or physical agent having the potential to cause neurotoxicity" (OECD TG 424, OECD (1997))*

Neurotoxicants can affect neuronal systems in various ways and originate from multiple sources. Commonly known neurotoxicants such as heavy metals mainly act on a molecular level, for example through the induction of oxidative toxicity and the binding to proteins of metal transport (Tomás 2012, Wright and Baccarelli 2007). Besides molecular effects on a single cell level, these effects may lead to consequences at the brain function level. Lead, for example, has been assumed to have neurotoxic effects in environmentally relevant concentrations leading to neurodegenerative diseases and reduced IQ values in human children (Lee and Freeman 2014). Copper, manganese and iron are supposed to have an influence in initiating Parkinsons`s disease (Dusek et al. 2015). By effecting molecular mechanisms, neurotoxicants are, therefore, also able to change cognitive functions. However, besides heavy metals, long-established pesticides, mostly organophosphates and pyrethroid insecticides, have also been shown to act neurotoxic. Organophosphates, for instance, have shown to induce loss of hearing in humans (Gatto et al. 2014) which has formerly been described for pharmacy such as neomycin and cisplatin in the lateral line of fish (Forge and Schacht 2000, Ton and Parng 2005).

Pharmaceutical drugs, if excreted, appear in the water cycle and become environmental pollutants (Pruvot et al. 2012); in fact some have been detected in increasing concentrations (Bergmann et al. 2011). These pollutants may affect animals as well as humans at the specific neuronal mechanisms which they were designed for. For example, the psychoactive drug fluoxetine (antidepressant) has been shown to affect specific behaviors involved in reproduction, feeding and predator avoidance in the fathead minnow (*Pimephales promelas*) at environmentally relevant concentrations (Weinberger and Klaper 2014). The antidepressant venlafaxine affected fish brain serotonin levels leading to altered predation behavior (Bisesi et al. 2014). These emerging anthropogenic pollutants with, among others, have received increasing public concern.

In a cross-linked, urbanized world, there is a growing pressure on natural resources, forcing governments to change regulations. Water is the essential resource and the preservation and

allocation of high-quality drinking water is a major challenge for economy and science. A wide range of substances which are capable of entering groundwater systems are thought to have adverse neurodevelopmental effects (Grandjean and Landrigan 2006). Additionally, evidence is rising that chronic exposure to neurotoxicants (i.e. by diet) may cause neurodevelopmental disorders (i.e. autism, attention-deficit hyperactivity disorder, dyslexia or others; Fox et al. (2012), Grandjean and Landrigan (2014)). Considering an increasing number of emerging compounds in drinking water, which evidently affect human health, research programs have to directly aim at a hazard-based risk assessment. As a consequence, the development of acute and mechanism-specific bioassays to identify the putative damage potential has become a major subject of current research in neurotoxicity.

European regulations

This thesis is embedded in a joint research program, "Risk management of emerging compounds and pathogens in the water cycle (RiSKWa)"which has been established by the Federal Ministry of Education and Research (BMBF; Huckele and Track (2013b)). One branch of this program deals with the development of a harmonized testing strategy applying an exposure- and hazard-based risk management of anthropogenic trace substances. This branch (ToxBox: securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany; Grummt et al. (2013)) deals with the establishment of standardized methods, combined in a test battery concerning endocrine effects, neurotoxicity, genotoxicity and germ cell damage. As a result, the supported test battery is supposed to gather information about substances with scarce toxicological data. This information is supposed to be used for the ranking of these substances in the Health-Related Indicator Value (HRIV) approach suggested by the German Federal Environment Agency (UBA 2003) ensuring water quality.

As detailed above, neurotoxicity has an adequate scientific background, has become a topic in research programs and has been suggested to be a legal supplement in the regulation of chemical risk assessment (Pugh and Tarazona 1998) by the current REACH Directive (EU 2006a). Apart from this, neurotoxicity is not a part of the legal framework in the EU.

The REACH regulation (EU 2006a), in fact supports optional tests for delayed neurotoxicity of organophosphorus substances, as evaluated by the following OECD Guidelines:

- TG 426-Developmental neurotoxicity test,
- TG 424-Neurotoxicity in rodents,
- TG 418-Delayed neurotoxicity of organophosphates,
- TG 419-Delayed neurotoxicity of organophosphates, 28 days repeated dose.

Thus, REACH does not demand information about neurotoxic effects induced by chemicals which are produced at more than 10 tons per year (EU 2006a). Since developmental neurotoxic effects of industrial chemicals indicate adverse effects on human health (Grandjean and Landrigan 2014, Schapira 2010), gaining information on neurotoxicants should be mandatory

in chemical regulation.

Given the mandatory character of neurotoxicity testing in chemical regulation, ethical issues in the establishment of appropriate methods have to be considered. In their presentation of the 3R principle (Reduce, Refine, Replace), Russell and Burch (1959) suggested considerations of reduction of individuals, refinement or replacement of existing methods to account for these ethical issues. Therefore, a reduction in the number of necessary individuals and the overall use of fish embryos was a central task in the present thesis. The use of zebrafish embryos, which are not protected according to current EU legislation, can be considered as an alternative method to replace standard adult models.

Model organism and neuronal systems used in the present thesis

Zebrafish and embryo toxicity test (FET)

The zebrafish (*Danio rerio*, Fig. 1) is a tropical freshwater fish which belongs to the family of Cyprinidae and lives in shallow water, e.g. rice fields or rivers in Southeast Asia, (e.g. Thailand, Burma, India, Pakistan and Bangladesh; Laale (1977)). The zebrafish is a frequently used model organism due to special characteristics: it is inexpensive, easy to maintain and reaches maturation in less than 4 months. Egg production is independent of the season under appropriate conditions (Lele and Krone 1996). Zebrafish lays fully transparent eggs, in which every developmental step and possible disruption can easily be observed by microscopic inspection. Among other reasons, the fish embryo toxicity test (FET) can, therefore, be suggested as a usable tool for the exposure to neurotoxic substances (Braunbeck et al. 2014).

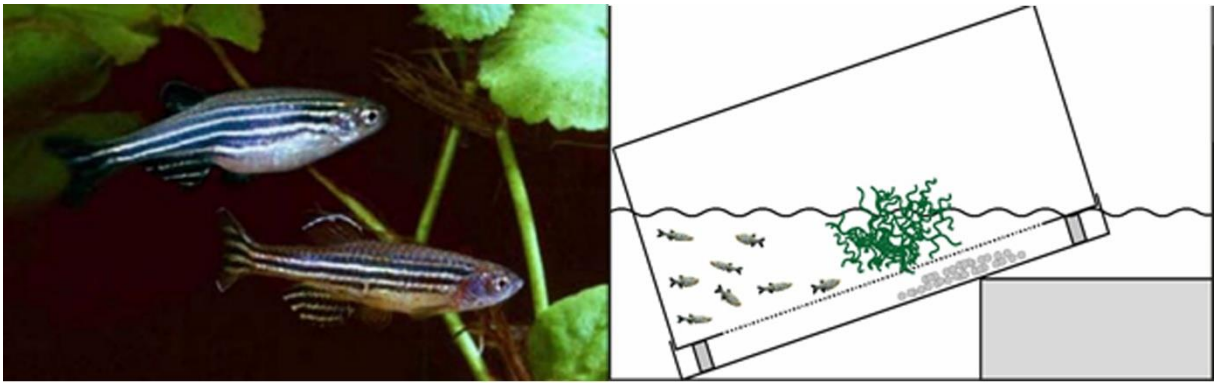

Figure 1: Left side, adult zebrafish (Danio rerio). Female: upper individual, Male: lower individual (left picture. Picture by Erik Leist); Right side, diagram of the used spawning method according to Sessa et al. (2008; right picture).

By acknowledging these benefits of zebrafish embryos, the fish embryo toxicity test (FET), as an alternative to acute toxicity testing with adult fish (Lammer et al. 2009b), has been adapted as test guideline no. 236 by the OECD (OECD 2013). Due to all these advantages, the zebrafish embryo has been suggested as a screening model for endocrine (Lohr and Hammerschmidt 2011, Segner 2009) and neurotoxicity research (de Esch et al. 2012, Froehlicher et al. 2009b, Küster 2005).

The FET also serves as a basic embryo lethality test, which can be supplemented by additional

sublethal effects (Lammer et al. 2009b). These effects, observed after exposure to sublethal concentrations, involve abnormalities such as malformations of the head, tail, notochord and yolk as well as retarded growth (Nagel 2002). In toxicological risk assessment and chemical regulation, LC_{50} values (OECD 2013) and, to a smaller extent, EC_{50} data have for long been used to compare the toxic potentials of chemicals. In addition, acute and sub-acute toxicity data have been used to select concentrations for subsequent more-in-depth testing and higher-tier testing. However, screening for specific modes of action at such high concentrations is debatable, since secondary (indirect) effects on the specific endpoints are likely to occur – independent of the specific mode of action. Endocrine systems, especially those related to general stress response systems, will almost inevitably respond, if only test concentrations are high enough (Tyler et al. 1998, US EPA 2007). Likewise, inappropriately high test concentrations have been shown to mask specific adaptive responses such as the induction of various forms of cytochrome P450. As a consequence, given that the search for responses of specific toxicological endpoints is driven by the attempt to reveal sublethal modes of action, the *highest* test concentration for such screening exercises should at best be within the range of lowest observed effect or EC_{10} concentrations. In the present thesis, EC_{10} concentrations were picked, since at this concentration only limited adverse impact on general physiology may be expected, but, based on experience, EC_{10} concentrations still represent concentrations high enough for both adaptive reactions and induction of biochemical responses (Braunbeck 1992, 1994, 1998).

The neurotransmitter acetylcholine

Neurons transmit signals from one cell to another. Since this process is necessarily linked to specific proteins and associated regulatory mechanisms, these proteins may serve as molecular biomarkers in ecotoxicology. Besides gamma-aminobutyric acid (GABA), dopamine, serotonin, adrenaline and others, the transmitter acetylcholine (ACh) plays a major role in the central and peripheral nervous system. Released by presynaptic cholinergic neurons *via* vesicular excretion, ACh causes a postsynaptic potential by binding to the acetylcholine receptor (AChR) at the postsynaptic membrane. After triggering the AChR, ACh is released from the receptor into the synaptic cleft and binds to additional receptors or to the acetylcholinesterase enzyme (EC 3.1.1.7; AChE). While ligated to the AChE at the postsynaptic membrane, ACh becomes inactivated by cleavage into choline and acetate, which stops the postsynaptic signal. Choline is transported in the presynapse *via* choline carriers and reactivated to acetylcholine with acetyl CoA by the choline acetyl transferase (Figure *2*, Massoulié et al. (1993), Silver (1974)).

Even if the underlying mechanisms are not fully understood, AChE also plays an important role in neuronal and muscular development (Behra et al. 2002, Hanneman 1992), as well as in axonal outgrowth (Hanneman and Westerfield 1989). Therefore, the activity of the AChE serves as a marker for alterations in neuronal development, as well as for mechanisms that are associated with locomotion. AChE transcripts can be observed in zebrafish embryos at 4 hpf. However, the activity of acetylcholinesterase remains low the following 4 hours (Allebrandt et al. 2005), but strongly rises from 24 hpf to 48 hpf in development. This rise in activity has been shown to be sufficient to detect AChE-inhibitors (Küster 2005, Küster and Altenburger 2006,

2007).

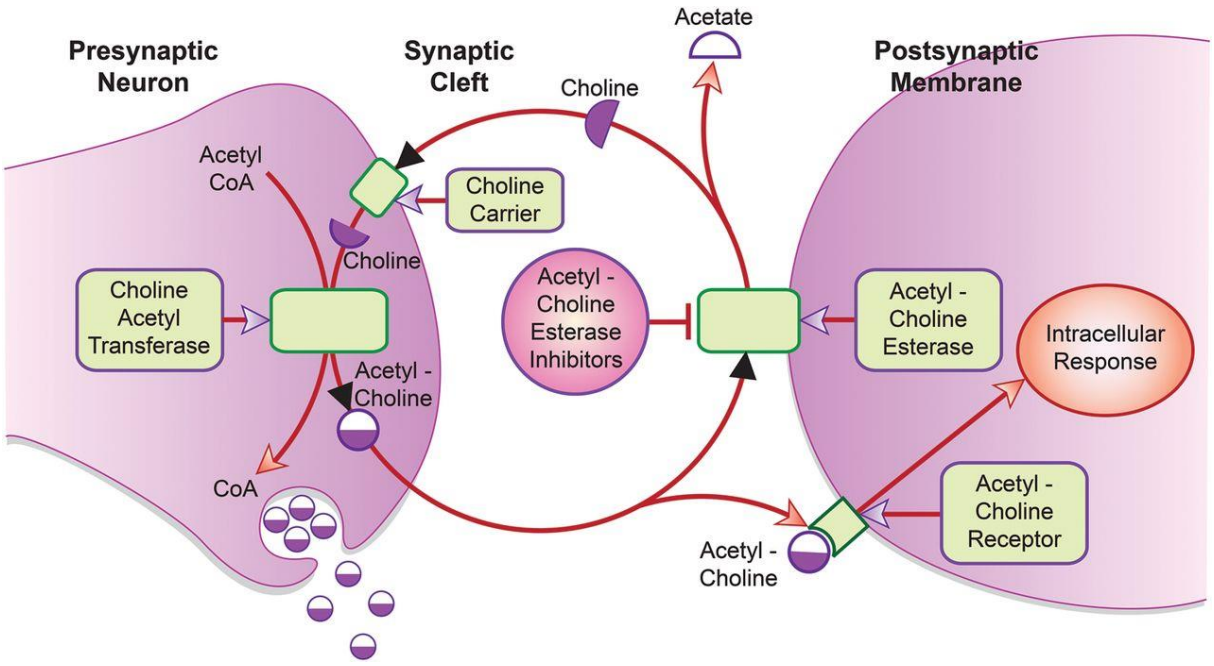

Figure 2: Figure of the Acetylcholine transmission cycle by Jeger (2013)

Within the scope of the detection of neurotoxic effects, molecular markers have to be based on specific neuronal mechanisms. Hence, focusing on these mechanisms, for instance, neuronal transmission and associated enzymes, receptors or transporters, exclude the detection of effect. A more detailed view on the strength and limitation of these neurochemical based biomarkers including cholinergic, dopaminergic, GABAergic, glutamatergic signal transmission and others, as well as a critical review on effect determination has been given for multiple model organisms by Basu (2015) and specifically for fish by Rico et al. (2011) and Scott and Sloman (2004).

In the present thesis, the inhibition of the acetylcholinesterase has been chosen on the basis of its wide scientific background to serve as an endpoint for neurotoxicity. The inhibition of AChE has been widely used as a biomarker for the neurotoxic potential of pure substances and environmental samples in different model organisms and tissues (De Domenico et al. 2013, Jung et al. 2012, Kais et al. 2015b, Kopecka-Pilarczyk and Correia 2011, Oropesa et al. 2007, Rodrigues et al. 2013, Xu et al. 2013). Specifically, AChE inhibition has been shown to be a reliable tool to detect organophosphates and carbamates *in vivo* (Küster 2005, Küster and Altenburger 2006, 2007). These classes of pesticides inhibit the AChE by binding to the catalytic center of the enzyme. As a result, ACh remains in the postsynaptic cleft and leads to overstimulation of the following neuron and subsequently to seizures and paralysis of the target organism. Besides organophosphates and carbamates, additional substance classes are able to affect AChE-activity by inhibition or activation of the enzyme. Polycyclic aromatic hydrocarbons (PAHs; Kang and Fang (1997)) or dioxins (Xu et al. 2013) have been shown to reduce

enzyme activity, whereas zinc, copper (Gioda et al. 2013) or glyphosate (Prevot-D'Alvise et al. 2013) have shown to increase enzyme activity. If exposed to substance mixtures, effects on AChE activity can be altered by synergistic (Chen et al. 2014a), additive (Mwila et al. 2013) or antagonistic mechanisms (Perez et al. 2013). The method to measure enzyme activity, as applied in this thesis, has been established by Ellman et al. (1961) and adopted to microplate use by Guilhermino et al. (1996). Following the 3R principle of Russell and Burch (1959) and given that zebrafish (*Danio rerio*) has become an important model in ecotoxicology, Küster (2005) adapted this approach for the alternative use of zebrafish embryos, and Perez et al. (2013) reduced the number of individuals required.

Sensory systems and their benefits

Sensory systems, for example vision, olfaction and tactile sense, interact with the surrounding environment to gather information necessary for survival. Given the near-surface localization of these senses this direct exposure to the surrounding environment leads to an immediate risk of harm. However, this direct exposure of sensory systems to the surrounding medium has a striking advantage or ecotoxicology. While neurons are directly and acutely exposed to chemicals, incidence of effects can be assumed to be earlier if compared to “in-body” neuronal organs. Therefore, for the present thesis, three different sensory systems, namely the lateral line system, vision and olfaction, were selected for the potential use in a test battery.

Lateral line system

The lateral line system is a mechanical sense in aquatic vertebrates, that detects weak water movements and pressure gradients in the surrounding medium (Bleckmann and Zelick 2009, Thomas et al. 2015). Its functional unit, the neuromast, comprises sensory hair cells which perceive water movements (Fig. 3). The neuromasts are interconnected to build up the lateral line system (Fig. 4), which is able to perceive hydrodynamic information that provides the basis for many behavioral decisions (Bleckmann and Zelick 2009), for instance, prey detection and predator avoidance, intraspecific communication, schooling, object discrimination, entrainment and rheotaxis (Bleckmann 1986, Montgomery et al. 1994).

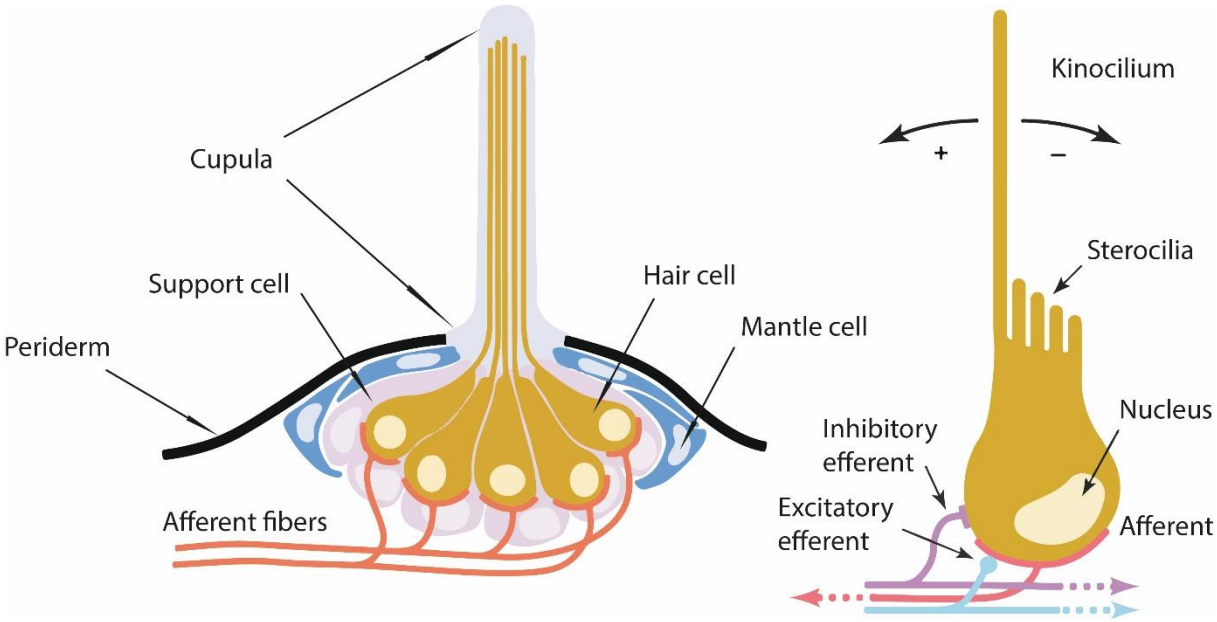

Figure 3: Lateral line neuromast (left) and a single hair cell (right) with according nervous innervation. From Ghysen and Dambly-Chaudiere (2007).

At 96 hpf, the lateral line system is divided into two major line systems, the anterior lateral line system (ALL), mostly located around the head, and the posterior lateral line system (PLL) mostly located at the trunk (Fig. 3, Bleckmann and Zelick (2009), Ghysen and Dambly-Chaudiere (2004), Metcalfe et al. (1985), Raible and Kruse (2000)).

Development of the lateral line has mostly been described for the PLL (Chitnis et al. 2012, Ghysen and Dambly-Chaudiere 2004, 2007, Gompel et al. 2001, Sarrazin et al. 2010). The PLL derives from the PLL placode, a thickening in the cranial epidermis, from which a bulk of 100 cells build a cluster, the primordium. At 20 hpf, the primordium migrates subdermally along the horizontal myoseptum and reaches the caudal fin at 40 hpf. While migrating, the primordium periodically deposits proneuromast at defined locations (Metcalfe et al. 1985). These proneuromasts differentiate into functional neuromast a few hours later (Ghysen and Dambly-Chaudiere 2007). About 20 cells stay back in the placode to build the PLL ganglion, which sends axons to follow the primordium and to innervate the neuromasts (Gilmour et al. 2002, Metcalfe et al. 1985). Functional and fully differentiated neuromasts consist of 15 – 20 hair cells, encircled by two layers of support and mantle cells (Fig. 3, Hernandez et al. (2007)).

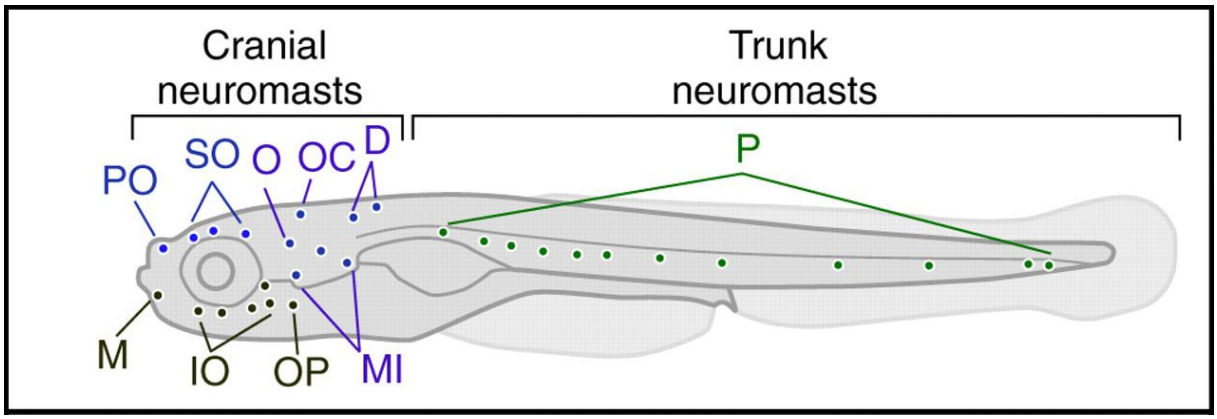

Figure 4: Localization of neuromasts in a 96 h zebrafish (*Danio rerio*) embryo according to the terminologies by Metcalfe et al. (1985), Raible and Kruse (2000) and Harris et al. (2003). Picture from Van Trump and McHenry (2008); supraorbital region (blue) including preoptic (PO) and supraorbital (SO) neuromasts; infraorbital region (black) including, mandibular (M), infraorbital (IO) and opercular (OP) neuromasts; caudal–cranial region (purple) including the otic (O), occipital (OC), dorsal (D) and middle (MI) neuromasts; posterior (P) neuromasts in the trunk region (green).

The lateral line has striking advantages for ecotoxicological research; for example, direct contact of neurons to the surrounding medium leads to an early response to neurotoxic chemicals. The lateral line has also been shown to express estrogen receptors, which play an important role in lateral line development (Froehlicher et al. 2009a). This gives the possibility to also use the lateral line as a tool to determine neuroendocrine effects *in vivo* which has been suggested by Froehlicher et al. (2009b). Form and function of the lateral line hair cells have a close homology to hair cells in the inner ear of higher vertebrates including humans (Ton and Parng 2005). This homology of hair cells between the human inner ear and in the lateral line of fish implies the potential for a direct transfer of effects in fish to effects in humans. In contrast to human hair cells, lateral line hair cells are able to regenerate damage (Harris et al. 2003, Hernandez et al. 2007, Lush and Piotrowski 2014), making them a useful tool for biochemical research. Even if the lateral line systems has frequently been used in ecotoxicological studies (Hill et al. 2005, Scholz et al. 2008, Segner 2009) and neurotoxicology (Linney et al. 2004), comparable data for neurotoxicity are still scarce for aquatic ecosystems.

Methods to detect toxic effects in the lateral line vary in the number of neuromasts used, in staining methods, endpoints and exposure times. The number of screened neuromasts range from one single neuromast (Linbo et al. 2009) to all neuromasts present at a specific age (Harris et al. 2003). As well as from exclusively head neuromasts (Coffin et al. 2009) to exclusively trunk neuromasts (Montalbano et al. 2014). Most studies have been undertaken by specific staining of hair cells. DASPEI, a styryl dye, stains exclusively mitochondria (Bereiter-Hahn 1976, Bereiter-Hahn and Voth 1994) and is often used to visualize functional mitochondria in hair cells. The fluorescence of this staining is currently used to visualize damage of neuromasts indicated by reduced intensity (Buck et al. 2012, Murakami et al. 2003, Owens et al. 2009). A different approach uses fluorescence to count the number of stained hair cells, therefore assumed as viable (Linbo et al. 2009, Linbo et al. 2006, Owens et al. 2008). Additionally there

are approaches using both intensity and hair cell counts (Harris et al. 2003). Fluorescence intensity, which is the most frequently used method, has mostly been assessed by a 3-step scoring established by Harris et al. (2003) and was, therefore, also basis of the methodical approach used in this thesis. Since the lateral line system has been shown to regenerate after substance-induced hair cell loss (Harris et al. 2003, Hernandez et al. 2007, Lush and Piotrowski 2014), it is highly important to consider this characteristic while evaluating results. Since chemicals possibly affect regeneration, exposure to substances may transiently lead to altered effects, depending on exposure time or substance. However, since there is growing evidence that hair cells of specific neuromasts show substance-specific differences in their susceptibility to neurotoxic substances (Harris et al. 2003, Murakami et al. 2003), a standardization of the method becomes even more important.

Olfactory system

Olfaction plays a crucial role in the life of fish. Predator-prey relationships, feeding, mating, social behavior and migration require a functional olfactory system (Hara 1975, Spehr et al. 2006). Hence, changes in these essential traits are likely to alter population–based processes. The olfactory system of zebrafish consists of three functional units, the geminate olfactory epithelium, the olfactory nerve (*Nervus olfactorius*) and the olfactory bulb. The rosette-shaped olfactory epithelium lies in two unventilated pits, rostrally between the eyes and is covered by skin flaps directing water currents in the nostrils (Fig. 5, Hamdani and Døving (2007)).

In fish, the olfactory epithelium derives from the olfactory placode, which delaminates from the neural tube at 17 hpf (Whitlock and Westerfield 2000) and differentiates into basal cells, olfactory receptor neurons (ORNs) and supporting cells at 23 hpf (Lindsay and Vogt 2004, Whitlock and Westerfield 2000). ORNs project axons at 24 hpf into specific glomeruli of the olfactory bulb, which reach the bulb at 44 hpf. (Whitlock and Westerfield 2000). Since, first olfaction-dependent behaviors can be induced at 96 hpf (Lindsay and Vogt 2004), the presence of functionally matured neurons can be assumed at this age.

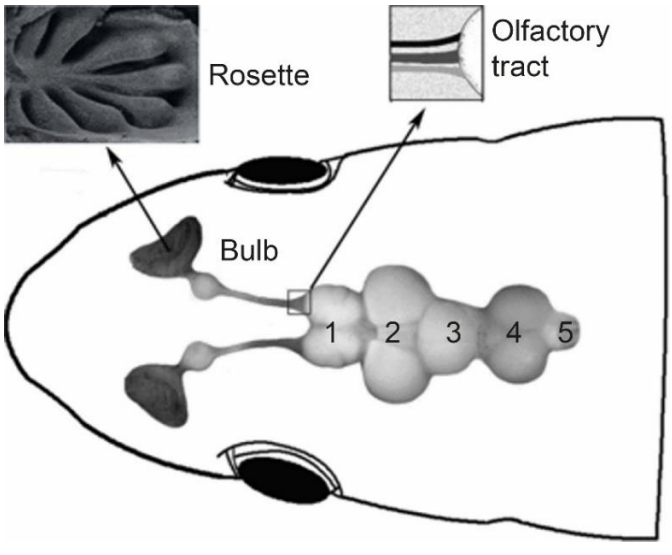

Figure 5: Olfactory system and brain structures of zebrafish from Dowing 2009 and Wullimann et al. 1996; 1 telencephalon, 2 tectum opticum, 3,4 cerebellum, 5 medulla spinalis

The main odorant-receiving units in the olfactory epithelium are the ORNs, which are divided in ciliary (cORN) and microvillus olfactory receptor neurons (mORN) as well as crypt cells (Byrd and Brunjes 1995, Doving and Lastein 2009) and derive from basal cells (Farbman 1994). These receptor neurons differ by either presenting microvilli (mORC), cilia (cORC) or both (crypt cells) and, additionally, by the location of their soma in the olfactory epithelium (Hamdani and Døving 2007). For use in ecotoxicology, there are a variety of possible molecular markers to detect specific structures or cell types in the olfactory epithelium. In the present study, a small selection (Gap 43, G*αS*/olf, G*αq,* ANO2) was chosen by scientific background.

Gap 43 belongs to a family of growth-associated proteins closely related to axonal outgrowth in development and regeneration (Skene 1989). Increased Gap 43 expression, indicating an extensive fiber plasticity, could be observed in the olfactory bulb of coho salmon (*Oncorhynchus kisutch*) in the period of parr-smolt transformation. In this period, salmon pass the border from high to low salinity during the migration to their home rivers (Ebbesson and Braithwaite 2012, Ebbesson et al. 2003), which indicates changes in the olfactory epithelium to adapt to altered environmental conditions. In contrast, Udvadia et al. (2001) revealed Gap 43 expression in olfactory bulb, tract and epithelium of transgenic zebrafish larvae, expressing GFP under a rat Gap-43 promotor without changes of environmental parameters. However, such different result must not be conflictive, since the olfactory system of fish undergoes rapid turnover in degeneration and regeneration of neuronal cells (Altman 1969, Graziadei and Graziadei 1979, Lledo et al. 2006, Thornhill 1970, Zupanc 2001). Furthermore, this rapid turnover has the capability to restore function of the olfactory epithelium after lesions (Parisi et al. 2014, White et al. 2015). In this context, axons expressing Gap 43 reach out from the olfactory epithelium, targeting specific glomeruli of the olfactory bulb (Becker et al. 2005, Bettini et al. 2006) to regain olfactory function. Although Gap 43 has not been used in scope of detecting neurotoxic effects, the specific expression in olfactory regeneration makes the use in an ecotoxicological context promising.

A different biomarker, the G proteins, mediate olfaction signal transduction by initiating second messenger cascades in olfactory neurons. After binding to odorants, receptors couple to these G proteins to stimulate the adenylyl cyclase (Ronnett and Moon 2002), which releases the second messenger cAMP. Serveral G protein subunits which are specifically expressed in receptor neuron subtypes, have already been described for fish. According to Hansen and Zielinski (2005), ciliated ORCs express the G protein subunit G*α*olf/s, whereas microvillus or crypt ORCs express subunits G*α*0 and G*α*q/11. However, the expression profile may vary between different model species. Even if, and to the best of knowledge, G*α*olf/s and G*α*q/11 have not been used in an ecotoxicological context since G*α*olf/s and G*α*q/11 have been characterized in the olfactory epithelium of fish, these two markers were chosen to be tested in the present thesis.

Anoctamin 2 (ANO2, TMEM 16B) belongs to a family of transmembrane proteins building a calcium-activated chloride channel suggested to be involved in olfaction. In mouse, ANO2 is restricted to neurons, independent of neuron type (Billig et al. 2011, Hengl et al. 2010, Rasche et al. 2010), where it is localized in the cilia of ORNs and in the microvilli of the vomeronasal

organ (VNO, Dauner et al. (2012)). Although anoctamins have not been described in zebrafish so far, results from mouse indicate the presence of detectable antigens in fish.

The use of the olfactory system in ecotoxicology has a long tradition, and there are multiple methods still in use. An exhaustive review has been given by Tierney et al. (2010), which comprised multiple methods and test substance classes as well as environmentally relevant and laboratory studies. However, recent works focus on approaches combining different effect levels. Classical works on disrupted histology of the olfactory epithelium, for example, after exposure to methyl mercury (Ghosh and Mandal 2014) or uranium (Faucher et al. 2012) have been enhanced by mechanistical and behavioral studies. Even if effects derived from copper have been attributed to the olfactory system since 1973 by Gardner and LaRoche, in recent years, mechanism behind metal toxicity became more and more apparent. Azizishirazi et al. (2015) showed an interference of copper with Na/K-ATPase of rainbow trout (*Oncorhynchus mykiss*) and yellow perch (*Perca flavescens*) by a protection of olfactory function after dietary exposure to sodium, corroborated by extensive microarrays and electroolfactograms. Additionally, Wang et al. (2013) showed the involvement of supplemental biomarkers like OMP (olfactory marker protein) expression by rtPCR and loss of AC3 (Adenylate cyclase III) by immunohistochemistry. These insights show the complexity of possible mechanisms affecting the olfactory epithelium. However, besides various mechanisms, neuron subpopulations are able to react differently to chemical harm. Dew et al. (2014) showed a specificity in metal toxicity, In that copper and nickel affected different subpopulations of olfactory sensory neurons. Exposure to copper led to disruption of ciliary olfactory neurons and following predator-avoidance behavior. In contrast, exposure to copper nanoparticles eliminated predator avoidance without disrupting ciliary olfactory neurons, but significantly increased the oxidation state of brain glutathione. This suggests a change in behavior by a brain related mode of actions different from that of a metal ions (Sovova et al. 2014). Effects on a molecular level, determined under laboratory conditions, can provided a detailed insight into a single effect mechanism. However, effects in wildlife seem more complex by interaction of toxicants with toxic and non-toxic substances. Dissolved organic carbon (DOC) reduces effects by copper on olfactory behavior (Kennedy et al. 2012), whereas combined exposure of copper and nonylphenol reduced the effect of nonylphenol on olfactory behavior (Ward et al. 2013). Fish from metal-contaminated lakes show behavioral changes to skin odorants and effects as determined *via* microarray (Azizishirazi et al. 2014); nevertheless, they were able to recover (Azizishirazi et al. 2013). Even if olfactory toxicity has long been used, many questions still will have to be answered since mechanisms of olfactory toxicity were not fully understood.

Visual system

The sense of vision plays also a central role in the life of fish. The vertebrate retina is organized into defined layers, including three nuclear and two plexiform layers (Masland 2012), and is surrounded by a retinal pigmented epithelium (RPE). The nuclear layers are classified as the outer nuclear layer (ONL), containing the photoreceptor cells (cones and rods), the inner nuclear layer (INL) with horizontal, bipolar and amacrine neurons as well as Müller glia cells, and

the ganglion cell layer (GCL; Fig. 6). Multipotent progenitor cells give rise to all the neurons, photoreceptors and Müller glia cells during development (Sidman 1960). This allows to use the retina as a model for investigations of molecular mechanisms of neuronal cell fate (Livesey and Cepko 2001) as well as a system to study the interaction of chemicals and neurosensory development and function. Anatomical studies have shown that adult zebrafish possess three receptor cell types: short single (SSC), long single (LSC) and double (DC) cone types (Branchek and Bremiller 1984, Raymond et al. 1993), which allows to detect visible and ultraviolet light (Hughes et al., 1998). A special feature of fish and amphibians is that their retinas grow continuously throughout their lifespan. This occurs by addition of concentric rings of new retinal cells from the ciliary marginal zone (CMZ), which is the most peripheral region of the retina. It has been shown that the retinal marginal cells increase the production of new retinal cells after mechanical or neurochemical damage (Reh 1987, Reh and Tully 1986).

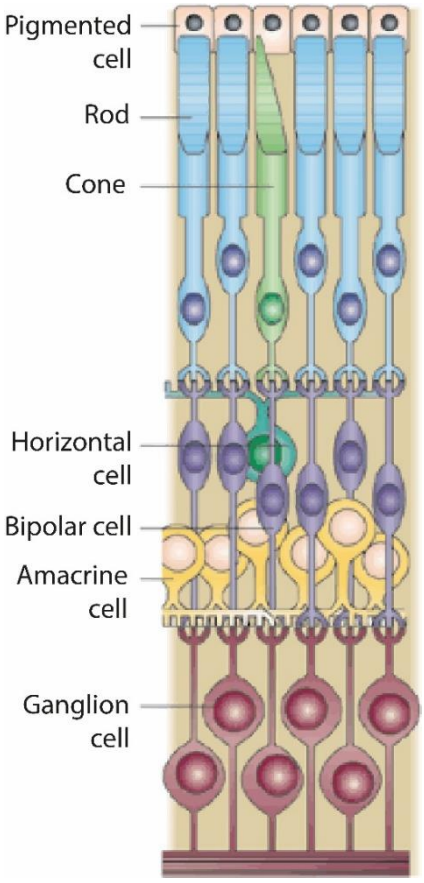

Figure 6: Structural organization of the fish retina; after Dyer and Cepko (2001)

The optic primordia arise from the retinal field, a forebrain region specified at the end of gastrulation (8 hpf). At an early somitic stage (15 hpf), the two optic primordia are completely differentiated and the first cells differentiate (36 hpf; Schmitt and Dowling (1994)). At 72 hpf, all cell types are present, correctly arranged, and the retina becomes completely functional. Sensory motor response starts soon after, as evidenced by visually evoked startle response at 68–79 hpf and tracking eye movements at 73–80 hpf (Easter and Nicola 1996). Since the retinal layers develop at 50 hpf (Schmitt and Dowling 1994), histopathological effects in retinal layer arrangement can be recorded from this point on.

In ecotoxicological research, effects on retinal morphology has frequently been used (Huang et al. 2013, Kim et al. 2013, Mecklenburg and Schraermeyer 2007, Mela et al. 2012, Triebskorn et al. 1994, Wang et al. 2012), wereby modes of action may be various. For instance, effects on the retina have been shown by evaluation of simple histopathology. Estrogens have been shown to influence eye pigmentation in a transgenic medaka (*Oryzias latipes*) model (Lee et al. 2012). Chlorpyrifos induced histopathological changes in the optic tectum of the snakehead fish (*Channa punctatus*) (Mishra and Devi 2014). Additionally, PCBs and methyl mercury induced changes in the retinal photoreceptor layer at a ultrastructural level (Wang et al. 2012). As a consequence, histopathological changes have been shown to be a useful model to screen for chemical harm (Mela et al. 2012). However, underlying mechanisms mostly remained unknown. Therefore, molecular alterations may be added to morphological changes to elucidate

mechanisms and possible adverse outcome pathways. For example, Huang et al. (2014) linked histopathological alterations in the retina caused by benzo(a)pyrene indicated by cellular density and altered phototactic response, to microarrays of 15 vision-related genes. By this link, Huang suggests a defects in visual system development, which derived by perturbation of specific vision-related genes. Since substance-dependent alterations in the retina, observed by histological methods have frequently been used in scientific research, the present study uses a histopathological approach to detect neurotoxic effects in the retina.

Aims of the present study

Although, neurotoxicity have frequently been a central topic in ecotoxicology, there is a lack of suitable test batteries proposed for regulatory purposes. Taking into consideration that neurodegenerative diseases have been attributed to pharmaceuticals and chemicals in drinking water, scientific evaluation of usable test batteries is urgently needed. Therefore, the present study uses a multi-level approach to characterize usable effect mechanisms on multiple organizational levels and systems. This approach was conducted in three individual and consecutive steps:

1. establishment of new and enhancement of present methods;
2. evaluation of methods in single substance tests;
3. comparison of risks and benefits in a battery based neurotoxicity test.

In the first step, the main focus lies on the establishment of new methods and the evaluation and enhancements of existing methods, as well as the reduction of the number of individuals required for testing according to the 3R principle.

Test systems selected were: (A) the olfactory system, (B) the lateral line system and the (C) visual system, representing sensory systems and (D) the acetylcholinesterase system as a representative of a basic functional level transmitter system (Fig. 7).

In the second step, methods were be tested with different substances, namely 2,4-dichlorophenol, 2,4-dinitrotoluene , 4-nonylphenole, caffeine, cypermethrin, dichlorvos, amidotrizoic acid, perfluorooctanoic acid and perfluorooctanesulfonic acid which were selected within the scope of the ToxBox Project (Grummt et al. (2013)).

Prior to neurotoxicity tests, each substance was tested in a fish embryo toxicity test to define the EC_{10} value, this procedure was expected to reduce unspecific systemic effects on neurotoxicity and to provide physiological background data for comprehensive effect evaluation.

In the third step, methods and results were compared with organizational levels and sensitivities to evaluate the risks and benefits in a battery-based neurotoxicity testing scheme.

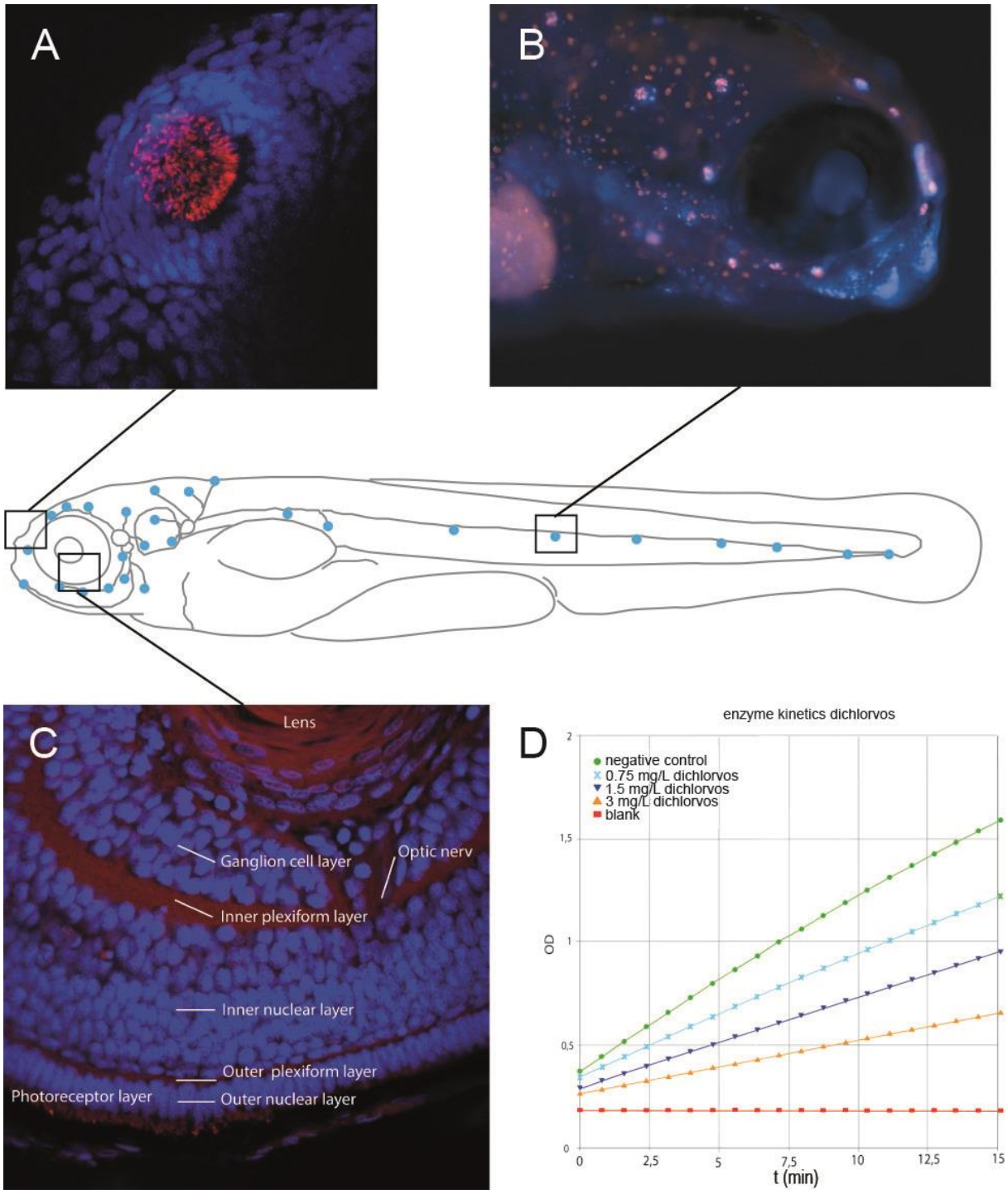

Figure 7: Methodical approach for the establishment and evaluation of a test battery for neurotoxicity tests. A: structural and molecular characterization of effects in the olfactory system; B: Effects on hair cells in neuromasts of the later line; C: structural and histopathological alteration in the retina; D: inhibition of the acetylcholinesterase as evaluated with classical enzyme kinetics. Prior to neurotoxicity tests, acute embryo toxicity was determined *via* embryo toxicity tests according to OECD TG 236.

Chapter I

1. Acetylcholinesterase in zebrafish embryos as a tool to identify neurotoxic effects in sediments

1.1 Abstract

In order to clarify the suitability of zebrafish (*Danio rerio*) embryos for the detection of neurotoxic compounds, the acetylcholinesterase assay was adapted and validated with a series of priority pollutants listed as relevant for the European water policy (Aroclor 1254, 2,3-benzofuran, bisphenol A, chlorpyrifos, paraoxon-methyl, quinoline, and methyl mercury chloride) as well as acetonic extracts from three sediments of known contamination. The acute toxicities of the model substances and the sediment extracts were determined by means of the fish embryo test as specified in OECD TG 236, and concentrations as low as the effective concentration at 10 % inhibition (EC10) were used as the highest test concentration in the acetylcholinesterase test in order to avoid nonspecific systemic effects mimicking neurotoxicity. Among the model compounds, only the known acetylcholinesterase inhibitors paraoxon-methyl and chlorpyrifos produced a strong inhibition to about 20 and 33 %, respectively, of the negative controls. For the sediment extracts, a reduction of acetylcholinesterase activity to about 60 % could only be shown for the Vering Canal sediment extracts; this could be correlated to high contents of acetylcholinesterase-inhibiting polycyclic aromatic hydrocarbons (PAHs) as identified by chemical analyses. Co-incubation of the Vering Canal sediment extracts with chlorpyrifos at EC10 concentrations each did not significantly increase the inhibitory effect of chlorpyrifos, indicating that the mode of action of acetylcholinesterase inhibition by the sediment-borne PAHs is different to that of the typical acetylcholinesterase blocker chlorpyrifos. Overall, the study documents that zebrafish embryos represent a suitable model not only to reveal acetylcholinesterase inhibition, but also to investigate various modes of neurotoxic action.

1.2 Introduction

The European Water Framework Directive 200/60/EC (EU 2001) aims for a good ecological and chemical status of ground and surface waters by 2015. In daughter directives such as Directive 2008/105/EC (EU 2008) environmental quality standards for priority substances and certain other pollutants are defined and priority substances in the field of water policy are listed. An important aspect of ecotoxicological monitoring of aquatic environments within the context of the European Water Framework Directive is the analysis of sediments (Förstner 2008, Hollert et al. 2007, Netzband et al. 2007). Waterborne substances with low water solubility tend to adsorb to free floating particulates, which then might be deposited in the sediments of freshwater and marine ecosystems (Wölz et al. 2010, 2011). Sediments may thus host a bulk of different chemicals including priority pollutants such as polycyclic hydrocarbons (PAHs), PCBs and dioxins. Given that these sediment-borne contaminants may serve as a secondary source of pollutants (Hollert et al. 2007), sediment toxicity has become a major aspect of the European Water Framework Directive.

Most studies into sediment toxicity used various protocols for extraction of the pollutants from the particulate matrix. In contrast to native sediment samples, the effects of which are based on the natural bioavailability of substances, sediment extracts represent tools to identify the full *integrated toxic potential* of sediment-borne chemicals in a worst-case scenario. Using Soxhlet extraction techniques, various screening programs in southern Germany have monitored the toxicological potential in extracts of sediments from, e.g., the rivers Rhine, Neckar, and Danube (Braunbeck et al. 2009, Hollert et al. 2009, Keiter et al. 2009).

In an attempt to further characterize and to more specifically analyze polluted sediments, specific modes of action have received growing attention (Chen and White 2004). DanTox, a joint project funded by the German Ministry for Education and Research, has focused on the establishment of a battery of such methods for specific modes of action in embryonic and larval zebrafish (*Danio rerio*) that could be applied for ecotoxicological screening of sediments (Keiter et al. 2010a, Keiter et al. 2010b). The methods considered suitable for this battery were validated with a set of individual chemical substances selected on the basis of their relevance for the European Water Framework Directive (2,3-benzofuran, Aroclor 1254, bisphenol A, chlorpyrifos, paraoxon-methyl, quinoline) and a set of sediments from locations with a known ecotoxicological background (Altrip and Ehrenbreitstein on the Rhine river and the Vering Canal in Hamburg harbor). Based on routine chemical analyses by the German Federal Institute of Hydrology (Koblenz, Germany), these environmental sampling sites represent a gradient with respect to their load with chemical contaminants: Altrip < Ehrenbreitstein < Vering Canal.

Data for neurotoxicity, one of the more specific endpoints considered within the DanTox project, are still scarce for aquatic ecosystems. One specific mode of neurotoxic action, however, has been fairly well documented in fish: the inhibition of the enzyme acetylcholinesterase (EC 3.1.1.7), which is classically regarded fairly specific for organophosphate- and carbamate-type pesticides. The competitive binding of the agonists to the active center of acetylcholinesterase leads to the accumulation of acetylcholine within synaptic clefts and, thus, to an inactivation of signal transduction across cholinergic synapses (Fulton and Key 2001; Russometal. 2014). The inhibition of the acetylcholinesterase has been widely used as a biomarker of neurotoxicity by both pure substances and environmental samples in different model organisms and tissues

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The inhibition of the acetylcholine esterase has been widely used as a biomarker of neurotoxicity by both pure substances and environmental samples in different model organisms and tissues (De Domenico et al. 2013, Jung et al. 2012, Kopecka-Pilarczyk and Correia 2011, Oropesa et al. 2007, Rodrigues et al. 2013, Xu et al. 2013) and zebrafish proved to be a reliable tool to detect organophosphates and carbamates *in vivo* (Küster 2005, Küster and Altenburger 2006, 2007).

Apart from carbamates and organophosphates, chemicals form other substance classes have been shown to reduce acetylcholine esterase activity by possibly enzyme inhibition, e.g. certain hydrocarbons (PAHs, Kang and Fang (1997)) or dioxins (Xu et al. 2013). However, acetylcholine esterase enzymatic activity may also be altered following degradation of cholinergic neurons by, e.g., bisphenol a (Miyagawa et al. (2007)). Inhibition of acetylcholine esterase activity can be modified by synergistic (Chen et al. 2014a), additive (Mwila et al. 2013) or antagonistic mechanisms (Perez et al. 2013). As a consequence, it may be assumed that the broad spectrum of chemicals found in sediments may lead to an unpredictable combination of inhibitory and stimulatory effects, which makes experimental determination inevitable.

Most methods used for the determination of acetylcholine esterase inhibition are based on the original protocol established by Ellman et al. (1961), which was adapted for use in microtiter plates by Guilhermino et al. (1996). Given that zebrafish has become a most important model organism in (eco) toxicology, Küster (2005) modified the method to fit zebrafish embryos, and (Perez et al. 2013) proposed a reduction in the number of individuals per experimental group. However, given the considerable inherent biological variability within a group of developing zebrafish embryos (especially of wild type strains; Schmidt and Starck (2004)), there is a need for a science-based compromise is need between animal welfare-driven attempts to reduce the number of experimental animals and the purpose of the experiment, i.e. the precise identification of, e.g., lowest observed effect concentration for acetylcholine esterase inhibition.

As for any form of organ- and cell-specific toxicity, neurotoxicity assays should be designed to exclusively reveal primary neurotoxic effects. Although repeatedly tested in various studies, concentrations as high as half maximal effective concentration (EC_{50}) values are likely to also cover, at least to some extent, nonspecific secondary effects caused by systemic, generalized (acute and sublethal) cell and organ toxicity. For example, biotransformation products of some PCBs have been shown to indirectly affect AChE activity (Durou et al. 2007), which, of course, can also be considered as a useful tool for “stress screening”. However, with respect to terminology, such effects should not be termed specifically “neurotoxic”. In an attempt to exclude secondary effects in neuronal systems, the present study was designed to further optimize the

protocol for the measurement of acetylcholinesterase inhibition in early developmental stages of fish by significantly reducing the test concentrations below effective concentration at 10 % inhibition (EC_{10}) values. As model substances, Aroclor 1254, 2,3-benzofuran, bisphenol A, chlorpyrifos, paraoxon-methyl, quinoline, and methyl mercury chloride were used, and the resulting protocol was validated in experiments with three extracts of sediments collected in major German waterways.

1.3 Materials and methods

Chemicals

The substrate for the enzyme kinetics, acetylthiocholine iodide (CAS: 1866-15-5), bovine serum albumin (BSA, CAS: 9048-46-8), Ellmans reagent, 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB, CAS: 69-78-3) and the positive control for acetylcholine esterase inhibition, paraoxon-methyl (PO, CAS: 950-35-6) were purchased from Sigma-Aldrich (Deisenhofen, Germany). All stock solutions and test concentrations were prepared in artificial water according to OECD TG 236 (OECD 2013).

In detail, the model toxicants were prepared as follows: 4 mg/L 3,4-Dichloroaniline (CAS: 95-75-1) and 30 µg/L methyl mercury chloride (CAS: 115-08-3) were prepared from 10 mg/L stock solutions. Chlorpyrifos (CAS: 2921-88-2) and Aroclor 1254 (CAS: 11097-69-1) were dissolved in 100 % dimethyl sulfoxide (DMSO) and diluted with artificial water to final concentrations of 600 µg/L and 6 mg/L in DMSO (final concentration 0.1 %). The two heterocyclic compounds 2,3-benzofuran (CAS: 271-89-6) and quinolone (CAS: 91-22-5) were prepared from 500 mg/L stock solutions, and bisphenol A (CAS: 80-05-7) was first dissolved in 7.14 mM sodium hydroxide (pH 10 – 11) adjusted to pH 7.7 prior to further dilution to test concentrations of 4 mg/L.

All sediment extracts were dissolved in 100 % DMSO at 20 g sediment equivalents per milliliter (SEQ/ml) and diluted with artificial water to the following final concentrations: Altrip 5 mg SEQ/ml, Ehrenbreitstein 5 mg/SEQ/ml and Vering Canal 30 µg SEQ/mL in a final solvent concentration of 0.25 % DMSO.

Protein determination

For protein measurements, a commercial kit, DC-Protein Assay (BioRad, München, Germany), was used. Triton X-100 (CAS: 9002-93-1) was obtained from Merck (Darmstadt, Germany).

Preparation of sediment extracts

The sediment extracts and according extraction process controls were produced as detailed by Hollert et al. (2000) and modified by Keiter et al. (2006). In brief, sediment samples were shock-frozen at -30 °C and freeze-dried (Christ Alpha 1 - 4 freeze dryer, Osterode, FRG). Two replicates of 20 g freeze-dried sediment and SPM samples were separately extracted with 300 ml acetone (Fluka, Buchs, CH) for 12 h using standard reflux (Soxhlet) extractors at approximately 7 cycles per hour. The extracts were allowed to cool and reduced in volume to approximately 5 ml using a rotary evaporator (WB 2001; Heidolph, Kelheim, FRG; 400 mbar, 36 – 38 °C)

before being reduced close to dryness under a nitrogen stream.

Table 1: EC_{10} and EC_{50} values determined in the zebrafish embryo toxicity test n -3)

	EC_{10}	EC_{50}	Solvent (%)	
Monosubstances (mg/L)				
Aroclor 1254	3.86 ± 1.21	6.77 ± 1.51	DMSO	0.2
Bisphenol A	3.84 ± 0.58	5.91 ± 0.66	AW	
Chlorpyrifos	0.62 ± 0.13	1.11 ± 0.15	DMSO	0.1
Methyl mercury chloride	0.0319± 5.21	0.0369 ± 3.35	AW	
2,3 benzofuran	13.98 ± 2.9	18.62± 1.94	AW	
Quinoline	9.5 ± 2.11	13.57± 2.61	AW	
Sediment extracts (mg SEQ/ml)				
Altrip	3.33 ± 0.7	11.46± 1.26	DMSO	0.25
Ehrenbreitstein	2.06 ± 0.22	9.18 ± 8.36	DMSO	0.25
Vering canal	0.033 ± 0.0063	0.07± 0.007	DMSO	0.25

AW: artificial water; SEQ: sediment equivalent

Residues from individual sample replicates were redissolved in 2 ml DMSO for bioassays. Extracts were stored at − 20 °C until testing. As extraction process controls, empty extraction thimbles were subjected to extraction and processed in two parallel experiments. All sediments were extracted by Sabrina Schiwy (RWTH Aachen). As a negative control, pure artificial water was used.

Chemical analysis of sediments

Sediment analytics were kindly provided by Wiebke Meyer and Christine Achten (Dept. of Geology and Palaeontology, Applied Geology, University of Münster, Germany) in cooperation with Maria Larson und Magnus Engwall (Man-Technology-Environment Research Centre (MTM), Department of Natural Science, University of Örebro, Sweden) within the scope of DanTox (Hafner et al. 2015).

Fish

For the neurotoxicity tests, wild-type zebrafish (*Danio rerio*) of the Westaquarium strain from the facilities of the Aquatic ecology and Toxicology group at Heidelberg University were used Details of fish maintenance, egg production and embryo rearing were reviewed recently by Lammer et al. (2009b).

Zebrafish embryo toxicity tests (FET)

The fish embryo tests (FETs) were performed in 24-well plates (TPP, Renner, Dannstadt, Germany), which had been preincubated with 2 ml/well of the test solutions 24 h prior to the test start. In order to prevent evaporation or cross-contamination between the wells, the plates were sealed with self-adhesive foil (Nunc, Nalge Nunc, Wiesbaden, Germany). Zebrafish embryos were exposed to single substances and sediment extracts from 0 to 96 h post-fertilization (hpf) according to OECD TG 236 (OECD 2013). Particular care was taken to expose the embryos at

latest from 1 hpf, when 20 fertilized eggs were individually transferred into the cavities of the 24-well plates. The plates were kept in an incubator at 26±1 °C with a 10:14-h dark:light regime. The test was conducted semistatic; i.e., the medium was changed every 24 h subsequent to the daily inspection of the embryos.

Every 24 h, the embryos were controlled for lethal effects according to OECD TG 236 and for sublethal changes following Bachmann (2002), Schulte and Nagel (1994) as well as Nagel (2002). Both lethal and sublethal effects were used for the determination of EC values with ToxRat® (Ver. 2.10.03; ToxRat Solutions, Alsdorf, Germany).

Acetylcholinesterase inhibition test

EC_{10} concentrations as revealed by the FETs were used as the maximum exposure concentration for acetylcholinesterase inhibition (Table 1). In the acetylcholinesterase inhibition tests, paraoxon-methyl served as a positive control at a concentration of 2 mg/L (EC_{10}, as determined in previous FETs with 1, 2, and 4 mg/L paraoxon-methyl; details not shown). Acetylcholinesterase inhibition tests were run in triplicate with five embryos each. For determination of acetylcholinesterase inhibition, 96-h-old embryos were rinsed three times in artificial water, anesthetized by rapid cooling on ice, blotted dry on filter paper, and euthanized by shock freezing in liquid nitrogen for subsequent storage at − 20 °C. For homogenization, five embryos per concentration were thawed on ice, and 400 µl ice-cold 0.1 M phosphate buffer in 1 % Triton X-100 were added prior to homogenization with a TissueLyzer® at 30 Hz for 1 min (TissueLyzer II, Qiagen, Hilden). Homogenates were centrifuged at 10,000 g for 15 min (4 °C), and supernatants were used for enzyme kinetics and protein determination on a GENios® plate reader (Tecan, Crailsheim, Germany) as described by Küster (2005) and measured on a GENios plate reader (Tecan, Crailsheim, Germany). All enzyme measurements were conducted in quadruplicate for 3 x 5 pooled embryos per concentration. Enzyme activity was calculated as change in optic density (OD)/min/x mg protein content according to Ellman et al. (1961).

Spiking of sediment extracts

To elucidate possible masking effects of AChE-inhibitory substances by the sediment extracts, extracts of Vering Canal were spiked with an EC_{10} concentration (600 µg/L) of chlorpyrifos by adding chlorpyrifos from the stock solution to Vering Canal sediment extract (33 µg/L, Table 1)

Statistics

Statistical analysis for embryo toxicity was accomplished with ToxRat® (Ver. 2.10.03; ToxRat Solutions, Alsdorf, Germany). For enzyme kinetics statistical analysis carried out with Sigma plot 12.3 (Systat-Jandel, Erkrath, Germany) using One-way ANOVA, Dunn´s test.

1.4 Results

Embryo toxicity test

As range finders for subsequent acetylcholinesterase inhibition tests, zebrafish embryo toxicity tests (FETs) were conducted according to OECD TG 236. EC_{10} and EC_{50} values are shown in Table 1. For subsequent acetylcholinesterase inhibition test, the EC_{10} value was selected as the maximum test concentration to exclude unspecific secondary effects of acute toxicity on acetylcholinesterase activities.

Acetylcholinesterase inhibition by paraoxon-methyl (positive control)

In order to confirm that acetylcholinesterase can specifically be measured in 4-day-old zebrafish embryos as documented Bertrand et al. (2001) and Behra et al. (2004) and to define a positive control for acetylcholinesterase inhibition tests, a dose-response relationship for acute toxicity of paraoxon-methyl was established in the FET (Fig. 8). At all concentrations of paraoxon-methyl tested, zebrafish embryos showed convulsions in a dose-dependent manner. Interestingly, chlorpyrifos, Aroclor 1254, and methyl mercury chloride showed similar convulsions in a dose-dependent manner as a sublethal effect. At its EC_{10} concentration of 2 mg/L, paraoxon-methyl showed an inhibition of acetylcholinesterase activity to about 20 % of the negative control.

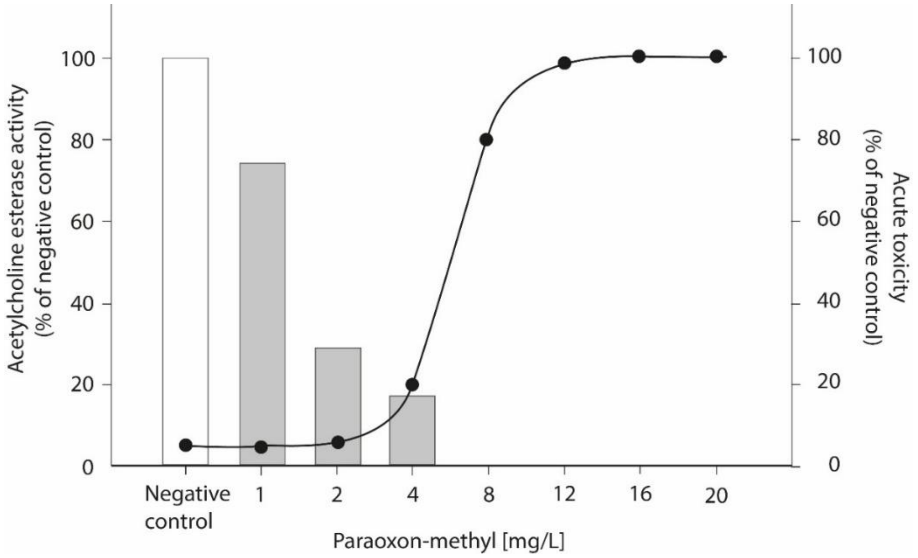

Figure 8: Specific acetylcholine esterase activities and lethal and sublethal effects on embryos in the embryo toxicity test exposed to paraoxon-methyl for 96 hours. Inhibition is expressed as percent of the negative control (100 %, left axis, open column), combined lethal and sublethal effects are expressed as percent of introduced embryos (n = 20, right axis, line graph fitted with ToxRat®).

Acetylcholine esterase inhibition by selected model substances

Embryos were exposed to EC_{10} concentrations (Table 1) of six selected model compounds. None of the solvent controls DMSO and NaOH had any influence on acetylcholine esterase. Likewise, except for chlorpyrifos, none of the test compounds showed effects. Chlorpyrifos, however, produced a statistically significant reduction of acetylcholinesterase activities at concentrations well below the EC_{10} value of 62 mg/L. Acetylcholinesterase activities in embryos exposed to 0.62 mg/L chlorpyrifos averaged 30 % of the activity in the negative control and thus expressed an inhibitory effect corresponding to 87 % of the positive control (2 mg/L paraoxon-methyl, EC_{10}; Fig. 9)

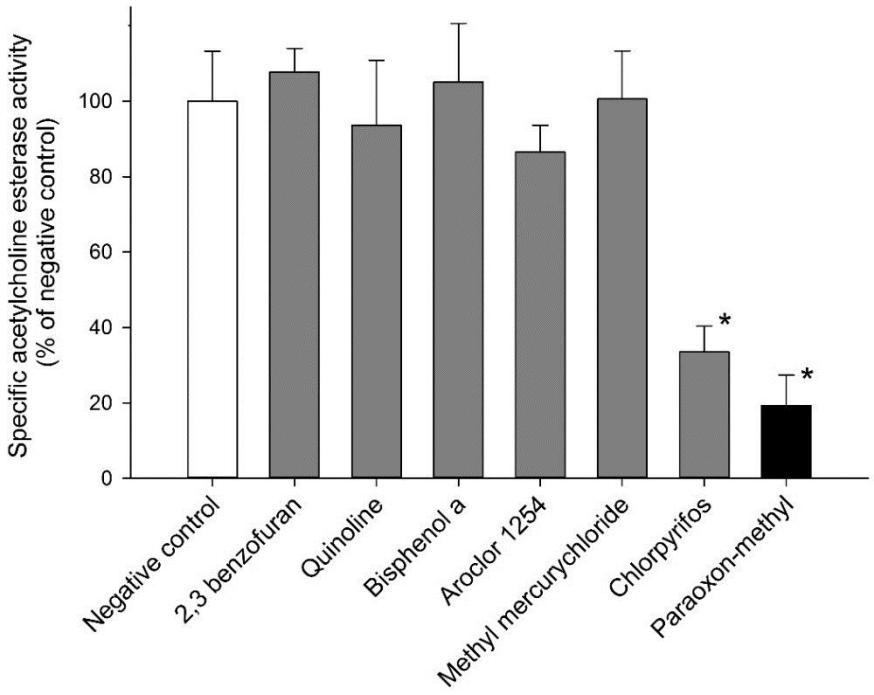

Figure 9: Specific acetylcholine esterase activities in pools of five 4 days old zebrafish embryos following exposure to 2,3-benzofuran, quinoline, bisphenol a, Aroclor 1254, methyl mercury chloride and chlorpyrifos for 96 h. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. * $p < 0.05$ (one-way ANOVA, Dunn´s test).

Embryo exposure to sediment extracts

Following exposure to EC_{10} concentrations of sediment extracts and appropriate solvent and process controls, extracts derived from sediments sampled at Ehrenbreitstein and Altrip did not show any significant effect on acetylcholine esterase activities (Fig. 10). In contrast, sediment extracts from the Vering Canal showed a reduction of acetylcholine esterase activity by approx. 62 %, if compared to the negative control. In fact, dilutions of the Vering Canal extract as low as 20 µg SEQ/ml produced statistically significant reduction of acetylcholinesterase activities (Fig. 10). Solvent and process controls showed no significant change compared to untreated negative controls and to corresponding sediment extracts (details not shown).

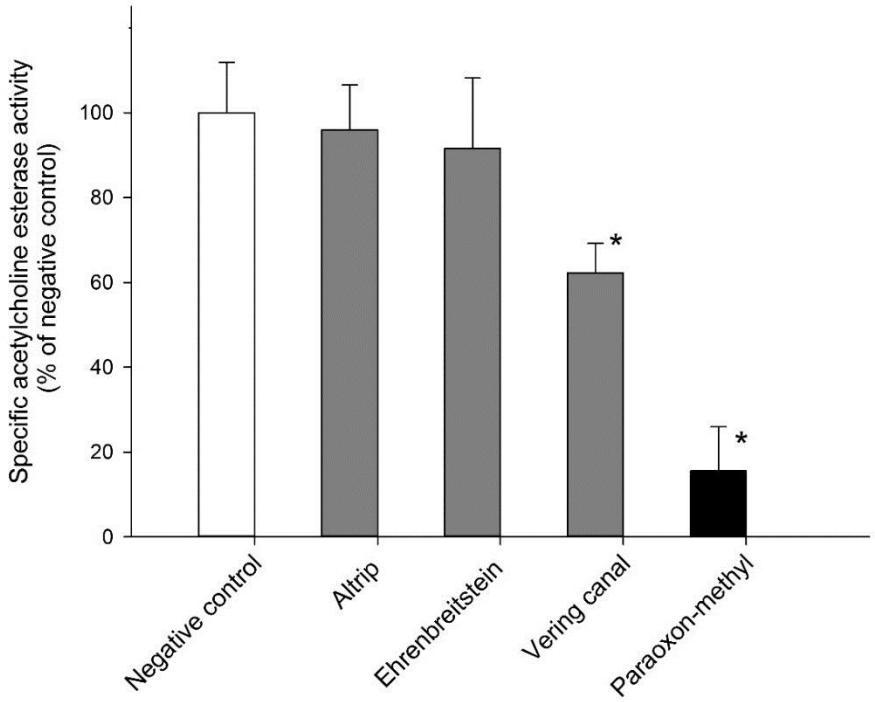

Figure 10: Specific acetylcholine esterase activities in pools of five 4 days old zebrafish embryos following exposure to sediment extracts from Altrip, Ehrenbreitstein and Vering Canal for 96 hours. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. * $p < 0.05$ (one-way ANOVA, Dunn´s test).

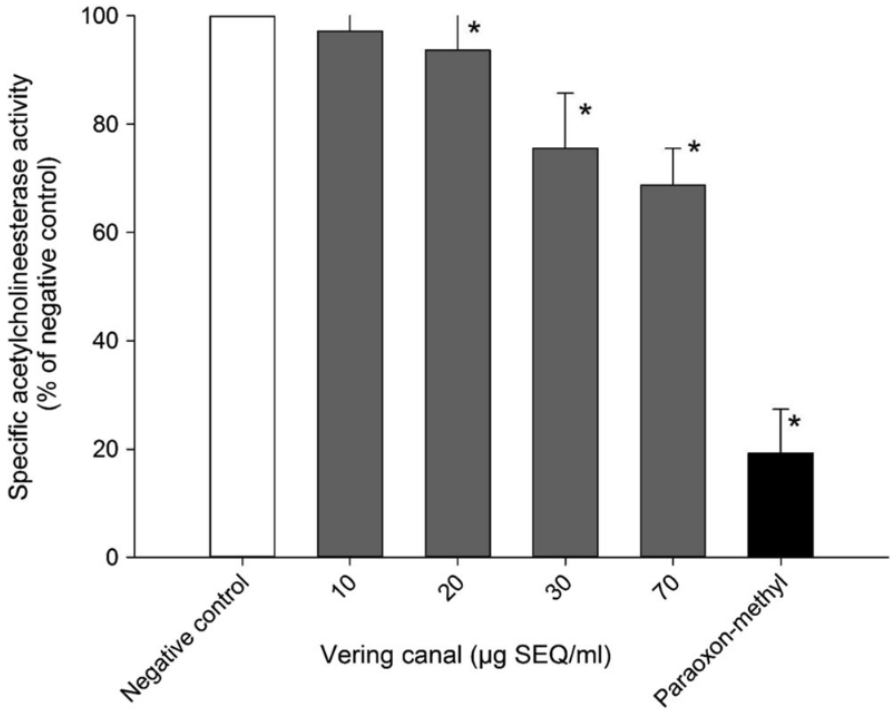

Figure 11 : Specific acetylcholinesterase activities in pools of five 4-day-old zebrafish embryos following 96-h exposure to various dilutions of the sediment extract from the Vering Canal. Inhibition is expressed as percent of the negative control (100 %; *open column*). The positive control (2 mg/L paraoxon-methyl) is given as the *black column*; $n = 3$ pools of five embryos. $*p < 0.05$, statistically different from negative controls (one-way ANOVA, Dunn's test)

Spiking of sediment extracts

In order to determine whether an inhibition of acetylcholinesterase would be detectable given the background of an exposure to the Vering Canal sediment extracts and to learn as to what extent responses of acetylcholinesterase activities would be modulated, zebrafish embryos were exposed to the EC_{10} concentration of the Vering Canal spiked with the EC_{10} concentration of chlorpyrifos (Fig. 12). This combined exposure was carried out in parallel to exposures to corresponding isolated Vering Canal sediment extract and chlorpyrifos at their EC_{10} concentrations. In fact, the sediment extract spiked with chlorpyrifos showed a significant change in acetylcholinesterase activity, if compared to isolated Vering Canal sediment extract exposure. In contrast, there was no significant change to isolated chlorpyrifos exposure. The mean acetylcholinesterase activity values of either exposure were approx. 18 % of the activity in the negative control.

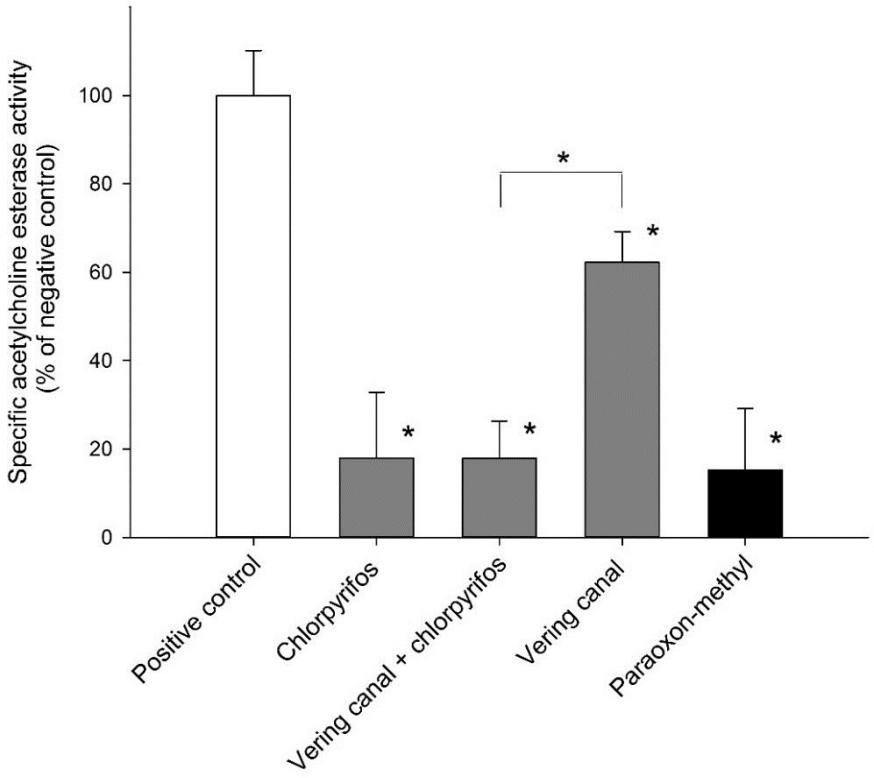

Figure 12: Specific acetylcholine esterase activities in pools of five 4 days old zebrafish embryos following exposure to chlorpyrifos, sediment extract from Vering Canal spiked with an EC_{10} concentration of chlorpyrifos and an unspiked sediment extract from Vering Canal for 96 hours. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. * $p < 0.05$ (One-way ANOVA, Dunn´s test).

1.5 Discussion

EC_{10} values as test concentrations for the determination of specific toxic modes of action

In toxicological risk assessment and chemical regulation, LC_{50} values and, to a smaller extent, EC_{50} data have for long been used to compare the toxic potential of chemicals (OECD 2013, Scholz et al. 2008, Scholz et al. 2013). In addition, acute and subacute toxicity data have been used to select concentrations for subsequent more-in-depth testing and higher-tier testing. However, screening for specific modes of action at such high concentrations is debatable, since secondary (indirect) effects on the specific endpoints are likely to occur independent of the specific mode of action. Endocrine systems, especially those related to general stress response systems, will almost inevitably respond, if only test concentrations are high enough (Kloas et al. 2009, Scholz et al. 2013, Soffker and Tyler 2012, Tyler et al. 1998, US EPA 2007).

Alternatively, inappropriately high test concentrations have been shown to mask specific adaptive responses such as the induction of various forms of cytochrome P450 (Heinrich et al. 2014). With respect to acetylcholinesterase activities, e.g., unspecific toxicants may modulate enzyme activities not only by direct interaction with the enzyme, but also by manipulating acetylcholinesterase expression and biosynthesis or neuronal cell degeneration. As a consequence, given that the search for responses of specific toxicological endpoints is driven by the attempt to reveal sublethal modes of action, the highest test concentration for such screening exercises

should at maximum be within the range of lowest observed effect concentrations or EC_{10} concentrations. In the present study, EC_{10} concentrations were picked, since at this concentration only limited adverse impact on general physiology may be expected, but based on experience from previous experiments EC_{10} concentrations still represent fairly high concentrations for both adaptive reactions and induction of biochemical responses (Braunbeck 1992, 1994, 1998). As illustrated in figure 8, at the EC_{10} concentration of paraoxon-methyl, only moderate physiological effects, i.e. minor convulsions and slight bends of the spine, could be observed as phenotypes typical of hypertonic paralysis induced by acetylcholine esterase inhibition (see, e.g., Behra et al. (2002)). In contrast to macroscopically detectable endpoints, acetylcholinesterase activity already displayed a very marked reduction (cf. Fig. 8). Thus, in more general terms, for further toxicological analyses of specific modes of action, we strongly recommend not to interpret reactions above EC_{10} concentrations as specific toxicological effects.

Modification of acetylcholinesterase activity by the model compound chlorpyrifos

From the list of the priority substances in the field of water policy given in the daughter directive (EU 2008) to the European Water Framework Directive (EU 2001), 2,3-benzofuran, Aroclor 1254, bisphenol A, chlorpyrifos, paraoxon-methyl, quinoline were picked as model substances for the joint research project DanTox (Keiter et al. 2010a, Keiter et al. 2010b). Out of these, only chlorpyrifos gave a clear reduction of acetylcholine esterase activity. The inhibitory potential of chlorpyrifos in zebrafish embryos in the present study differed slightly from that reported in previous studies: In contrast to (Yen et al. 2011), who reported zebrafish embryos exposed to chlorpyrifos from 2 - 5 days post fertilization (dpf) to show an inhibition of about 80 % at 105 µg/L chlorpyrifos, inhibition was only by a factor of 66 % at 620 µg/L in this study. This might be due to an earlier onset of exposure (1 hpf at latest) in the present study. The major active metabolite of chlorpyrifos, chlorpyrifos-oxon, is created via metabolization from chlorpyrifos by cytochrome P4501A (Straus et al. 2000). In early embryo stages, cytochrome P4501A is primarily localized in the liver, which can be assumed to become functional after vascularization of the liver at 56 hpf (Korzh et al. 2008). However, experience from previous studies suggest, that exposure of embryos at an early life-stage may disrupt or alter liver development leading to a decreased sensitivity of liver related endpoints (OECD 2013). The metabolization of chlorpyrifos to chlorpyrifos-oxon induces a metabolite-specific feedback inhibition of the CYP-system (Fukuto 1990, Moralev and Rozengart 2001, Tang et al. 2002), which regulates the production rate of the oxon metabolite. The example of early developmental toxicity of chlorpyrifos highlights the importance of an early start of exposure in fish embryo tests (cf., e.g., OECD 2013).

So far, to the best of our knowledge, 2,3-benzofuran and quinoline have not been studied with respect to acetylcholinesterase inhibition. For methyl mercury chloride, the lack of effects on acetylcholinesterase confirms previous studies with *Hyalella Azteca* , which had been tested at similar concentrations (Steevens and Benson 1999).

In contrast to our results, Aroclor 1254 induced a reduction in brain acetylcholine esterase activity of albino rats after 30 days of exposure, suggesting the possibility of cellular damage by PCB-generated toxic oxygen radicals (Muthuvel et al. 2006). Aroclor 1254 is a highly lipophilic technical mixture of PCBs, which may consist of ≥ 80 different PCB congeners in a variable composition. Different Aroclor 1254 lots may, therefore, lead to considerable variation in effects. In fact, first embryotoxic effects in zebrafish were only seen at 72 hpf, i.e., upon hatching. Aroclor 1254 may thus be assumed to adhere to, but not to readily pass, the chorion due to its strong lipophilic character. As a consequence, accumulation of Aroclor 1254 within the embryo may have been limited to 24 h from hatch

In zebrafish, bisphenol A has not yet been tested for *in vivo* effects on acetylcholine esterase. In rats, mice and the Arctic spider crab *Hyasaraneus*, bisphenol A showed an inhibition of hippocampal acetylcholine esterase after 21 and 35 day exposures, respectively (Fan et al. 2013, Minier et al. 2008). Thus, effects of bisphenol A on cholinergic neurons seem to depend on long-term exposure (Miyagawa et al. 2007). In contrast, following short-term exposure, bisphenol A does apparently not directly affect the acetylcholine esterase.

Manipulation of acetylcholine esterase activity by sediment extracts

Extracts of sediment samples represent complex mixtures of a broad range of chemicals, thus giving rise to an unpredictable variety of additive, synergistic, antagonistic, and/or even potentiating effects. Within the DanTox project, a total of 33 PAHs were detected by chemical analysis in the three sediment samples; among these, 6 PAHs were found at elevated concentrations between 70 and 300 mg/kg in the Vering Canal sediment (Table 2; Hafner et al. 2014). Thus, the Vering Canal showed by far the highest load with chemicals among the three sediment samples.

Table 1: Selection of PAHs identified by chemical analysis within the DanTox project, for some are known to reduce acetylcholine esterase (see references)

PAHs in mg/kg sediment	Altrip	Ehrenbreitstein	Vering Canal	Reference
Phenanthrene	0.26	0.27	217.89	Oropesa et al 2007
Anthracene	0.14	0.34	78.95	Jett et al 1999
Fluoranthene	0.58	0.71	208.42	Jett et al 1999
Pyrene	0.66	1.27	138.95	Jett et al 1999
Chrysene	0.43	0.42	104.21	Kang and Fang 1997

To date, only few studies investigated acetylcholinesterase inhibition following exposure to sediment extracts or native sediments (De Domenico et al. 2013, Gorokhova et al. 2010, Jung et al. 2012, Schvezov and Amin 2011). Although difficult to compare due to variable protocols and the use of different model organisms, all studies detected acetylcholinesterase inhibition in sediment-associated test systems. Jung et al. (2012) reported chemical analyses for 12 chemical classes, including PAHs and three individual substances, however, without giving concentration factors during extraction. Gorokhova et al. (2010) as well as Schvezov and Amin (2011)

exposed the amphipod *Monoporeia affinis* to native sediments, while De Domenico et al. (2013) exposed sea bass (*Dicentrarchus labrax*) to native sediments, which, however, were only analyzed for metals, and these PAHs account for the major part of substances in the sediment extracts tested (Table 2) Taking this into consideration, it is likely that the reduction in acetylcholinesterase activity in the Vering Canal extract might at least partly be due to the elevated concentrations of PAHs. Most recent studies documented a reduction in acetylcholinesterase activity after PAH exposure in a variety of model organisms (Jett et al. 1999, Kang and Fang 1997, Oropesa et al. 2007, Rodrigues et al. 2013). In contrast, Kopecka-Pilarczyk and Correia (2011) as well as Tang et al. (2003) failed to show an inhibition of acetylcholine esterase activity by phenanthrene, pyrene and fluorine in gilthead seabream and benzo[a]pyrene, chrysene, anthracene and pentachlorophenol in C6 and SY5Y cell lines, respectively. It is still not known how PAHs interact with acetylcholinesterase, e.g., by enzyme interaction, by reduced gene expression, or by affecting cholinergic neurons. In summary, acetylcholinesterase-inhibitory substances or mixtures are detectable in various sediments and extracts. Future research should be conducted on additional mechanisms that decrease acetylcholinesterase activity.

Spiking of sediment extracts

Various components in complex environmental samples may readily mask or modulate effects by individual substances. Assuming identical modes of action of the neurotoxic components of the Vering Canal sediment extract and chlorpyrifos, co-incubation should most likely lead to additive effects. However, the similarity in the acetylcholinesterase reduction rates by chlorpyrifos and chlorpyrifos-spiked Vering Canal sediments indicates that the chemical mixture in the Vering Canal sediment extract did not directly add up to the inhibitory potential of chlorpyrifos. Assuming inhibitory effects by PAHs already present in the sediment extract tested (cf. Table 2), these results differ from those by Jett et al. (1999), who showed an increase in the *in vitro* inhibitory potential of the chlorpyrifos-oxon in the presence of pyrene, benzo(a)pyrene, anthracene and fluoranthene. Taking this into consideration, it does not seem likely to assume that PAHs represent the sole acetylcholinesterase-inhibiting component within the Vering Canal extract. Comparing the inhibition values of Chlorpyrifos and the Vering Canal extract, the Vering Canal extract differs by 36 % from chlorpyrifos at EC_{10} concentrations. It may rather be assumed that the high contents of different chemicals in sediments, which lead to a higher toxicity, partially mask synergistic effects caused by inhibitory chemicals at lower concentrations.

1.6 Conclusions

The acetylcholinesterase inhibition assay with zebrafish embryos proved to be a suitable tool for the detection of neurotoxic potentials not only of individual substances, but also of complex mixtures in early developmental stages. Whereas inhibition of acetylcholinesterase activities by organophosphates and carbamates has widely been accepted, the present study provides evidence that PAHs may at least contribute to acetylcholinesterase inhibition in complex mixtures such as sediment extracts. However, since co-incubation of the Vering Canal sediment extracts and chlorpyrifos at EC_{10} concentrations each did not result in additive inhibitory effects, the

mode of action of acetylcholinesterase inhibition by the sediment-borne PAHs was apparently different to that of the typical acetylcholinesterase blocker chlorpyrifos.

Chapter II

2. Acetylcholinesterase in zebrafish embryos as a tool to identify emerging neurotoxic compounds

2.1 Abstract

In order to clarify the suitability of zebrafish (*Danio rerio*) embryos for the detection of neurotoxic compounds, the acetylcholinesterase assay was adapted and validated with a series of priority pollutants listed as relevant for the European water policy (amidotrizoic acid, caffeine, cypermethrin, 2,4-dichlorphenol, dichlorvos, 2,4-dinitrotoluene, 4-nonylphenol, perfluoroctanoic acid and perfluoroctansulfonic acid). The acute toxicities of the model substances were determined by means of the fish embryo test as specified in OECD TG 236, and the EC_{10} concentrations were used as the highest test concentration in the acetylcholinesterase test. Among the model compounds, only the known acetylcholinesterase inhibitor dichlorvos produced a strong inhibition to about 45 % of the negative controls at EC_{10}. In contrast, cypermethrin induced a slight increase in acetylcholinesterase activity to 107 %. In co-exposures, dichlorvos eliminated acetylcholinesterase activation by cypermethrin *in vivo*. Measurements of acetylcholinesterase activity *in vitro* with cypermethrin exposure indicated an influence on acetylcholinesterase activity by regulation beyond a direct interaction with the enzyme underlining the possibility of an adverse outcome pathway for cypermethrin on a low concentration level. Overall, the study documents that zebrafish embryos represent a suitable model not only to reveal acetylcholinesterase inhibition, but also to investigate different modes of neurotoxic action.

2.2 Introduction

Challenges of a connected and urbanized world are various: rising population density and following rising urbanization force governments to change habits in regulation, allocation and preservation of natural resources. Within this context, water plays a central role in human life, in which the allocation of high-quality drinking water plays a major challenge role for economy and science. Since anthropogenic influences are a major risk for water quality and since a wide range of chemicals and pharmaceuticals reach drinking water in various ways, public concern about harmful emerging compounds in drinking water are increasing (Pal et al. 2014, Postigo and Barceló 2015). These compounds with anthropogenic origin are identified in water bodies, following regulatory measures such as European chemical policy REACH (EU 2006b) and the EU Water Framework Directive (EU 2008). Since a wide range of possibly groundwater-reaching substances are thought to have neurodevelopmental effects (Grandjean and Landrigan 2006), evidence is rising that chronic exposure to neurotoxicants, may cause neurodevelopmental disorders (e.g. autism, attention-deficit hyperactivity disorder, dyslexia or others; Fox et al. (2012), Grandjean and Landrigan (2014)). Due to the increasing number of emerging compounds in drinking water, research programs have to directly aim for a hazard-based risk as-

sessment, and, therefore, the development of acute and mechanism-specific bioassays to identify putative damage potentials.

Within this context, a joint research measure has been established by the Federal Ministry of Education and Research (BMBF), "Risk Management of Emerging Compounds and Pathogens in the Water Cycle (RiSKWa)" (Huckele and Track 2013a). Within this measure, one branch aims for the design of a harmonized testing strategy for an exposure- and hazard-based risk management of anthropogenic trace substances written down in a guidance document. This project (Tox-Box: Securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany, Grummt et al. (2013)) deals with the establishment of standardized methods, combined in a test battery, concerning endocrine effects, neurotoxicity, genotoxicity and germ cell damage as additional endpoints. This test battery is supposed to provide information on substances with scarce toxicological date, to evaluate these substances according to the Health-Related Indicator Value (HRIV) approach developed by the German Federal Environment Agency (UBA). The present study is concerned with neurotoxicity endpoints and aims to verify a commonly used biomarker for neurotoxicity of anthropogenic, water-soluble trace substances *in vivo* and *in vitro*, the cholinergic transmitter system of zebrafish (*Danio rerio*) embryos.

The inhibition of the AChE has widely been used as a biomarker for the neurotoxic potential of pure substances and environmental samples in different model organisms and tissues (De Domenico et al. 2013, Jung et al. 2012, Kais et al. 2015b, Kopecka-Pilarczyk and Correia 2011, Oropesa et al. 2007, Rodrigues et al. 2013, Xu et al. 2013) and has been proven to be a reliable tool to detect organophosphates and carbamates *in vivo* (cf. introduction, Küster (2005), Küster and Altenburger (2006, 2007)).

The method applied in this study has been basically established by Ellman et al. (1961) and adopted to microplate by Guilhermino et al. (1996). Given that zebrafish (*Danio rerio*) has become an important model organism in toxicology, Küster (2005) adapted this setup for the use with zebrafish embryos. Perez et al. (2013) applied a reduction of the necessary number of individuals. This enables insight into possible fluctuations caused by biological variability, which is a basal phenomenon in biotic systems and most important in toxicological effect assessment. Additionally, in scope of a reduction of necessary individuals, biological variability might change sensitivities in an ecotoxicological risk assessment. Since high variations in results are commonly reduced by an increase in test specimens per replicate, the lack of data for individual behavior of model organisms, requires a detailed analysis of the AChE-activity test in order to evaluate the test as a potential biomarker in environmental toxicity assessment.

2.3 Material and methods

Chemicals

The substrate for the enzyme kinetics, acetylthiocholine iodide (ATCh, CAS: 1866-15-5), bovine serum albumin (BSA, CAS: 9048-46-8), Ellman´s reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, CAS: 69-78-3), the positive control for acetylcholinesterase inhibition and paraoxon-methyl (PO, CAS: 950-35-6) were purchased from Sigma-Aldrich (Deisenhofen, Germany). All chemicals for exposure experiments were purchased from Sigma-Aldrich at the highest purity available, unless stated otherwise, namely amidotrizoic acid, caffeine, cypermethrin, 3,4-dichloraniline, dichlorvos, 2,4-dichlorophenol, 2,4-dinitrotoluene, 4-nonylphenol, perfluorooctanoic acid and perfluorooctanesulfonic acid. For test concentrations in the embryo test and acetylcholinesterase (AChE) Assay see Table 2. All toxicant stock solutions and test concentrations were prepared in artificial water according to OECD TG 236 (OECD 2013).

Protein determination

For protein measurements, the commercial DC-Protein Assay kit (BioRad, München, Germany) was used. Triton X-100 (CAS: 9002-93-1) and sodium phosphate (CAS: 7601-54-9) to prepare a 0.1 M phosphate buffer in 1 % Triton X-100, were obtained from Merck (Darmstadt, Germany).

Fish

All adult zebrafish used for breeding were wild-type descendants of the "West-aquarium" strain and obtained from the Aquatic Ecology and Toxicology breeding facilities at the University of Heidelberg (licensed under no. 35-9185.64/BH). Details of fish maintenance, egg production and embryo rearing have repeatedly been described in detail (Baldwin et al. 2003, Kimmel et al. 1995a, Kimmel et al. 1988, Nagel 2002, Spence and Smith 2005, Wixon 2000a) and have been updated for the purpose of the zebrafish embryo toxicity test by Lammer et al. (2009) and Sessa et al. (2008).

Zebrafish embryo toxicity tests (FET)

In order to avoid indirect effects by systemic acute toxicity potentially mimicking neurotoxic effects, the highest test concentrations in the neurotoxicity tests were set at the EC_{10} for any kind of effect in zebrafish embryos. For this end, fish embryo tests were performed according to the protocol specified by OECD TG 236 (OECD 2013). In brief, embryos were exposed at latest from 1 hpf (hour(s) post-fertilization) in glass vessels, which had been pre-incubated (saturated) for at least 24 h, to a series of dilutions of the respective toxicants. After control of the fertilization success, embryos were transferred to 24-well plates (TPP Renner, Dannstadt, Germany), which had been pre-incubated with 2 ml of the test solutions per well for 24 hours prior to the test start, and kept in an incubator at 26.0 ± 1.0 °C with a 10:14 h light regime. In order to prevent evaporation or cross-contamination between the wells, the plates were sealed with self-adhesive foil (Nunc, Wiesbaden, Germany). Embryo tests were classified as valid, if the mortality in the negative control was ≤ 10 %, and the positive control (3,4-dichloroaniline) showed mortalities between 20 and 80 % (Lammer et al. 2009b). All fish embryo tests were run

in in three independent replicates. Zebrafish embryos were exposed to the test substances until 96 hours post-fertilization (hpf) in a semi-static fashion, i.e. the medium was changed every 24 hours subsequent to the daily inspection of the embryos. Every 24 hours, the embryos were controlled for lethal effects according to OECD TG 236 as well as for sublethal changes using the endpoint specified by Bachmann (2002), Schulte and Nagel (1994) as well as Nagel (2002). Both lethal and sublethal effects were used for the determination of EC values.

Acetylcholinesterase inhibition test

All acetylcholinesterase inhibition assays were performed in triplicate in 24-well plates with five embryos per test concentration until 72 hpf (Fig. 13). The acetylcholinesterase inhibition assay was first optimized for paraoxon-methyl, which was used as a positive control (2 mg/L, EC_{10}, as determined in previous FETs with 1, 2 and 4 mg/L paraoxon-methyl; Kais et al. (2015b)) in all subsequent tests. As a negative control, un-treated embryos were used. Controls and test solutions were replaced every 24 h upon daily inspections of embryos.

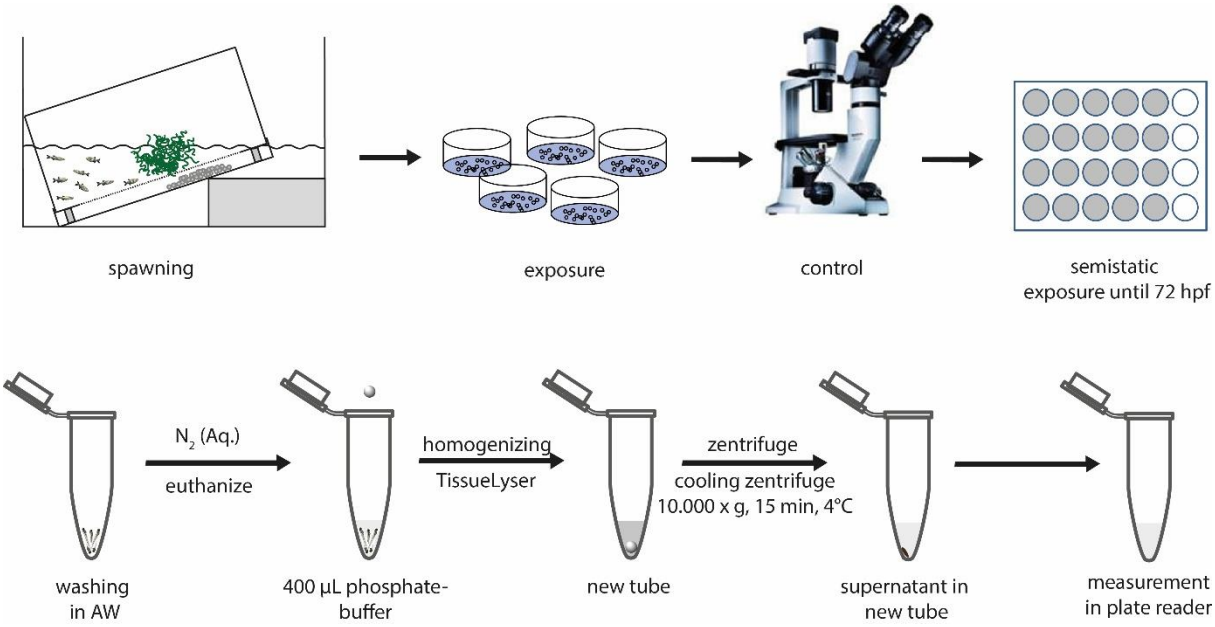

Figure 13: Acetylcholinesterase inhibition test; upper: spawning after Sessa et al (2008) and exposure regime according to OECD TG 236; lower: processing of embryos for homogenation with subsequent enzyme kinetics.

For the test concentrations in the acetylcholinesterase inhibition assay see Table 2. Acetylcholinesterase inhibition tests were run in three independent replicates with five embryos each. For determination of acetylcholinesterase inhibition, 72 h old embryos were rinsed three times in artificial water, anesthetized by rapid cooling on ice (Wilson et al. 2009) prior to complete removal of residual water and euthanized by shockfreezing in liquid nitrogen for subsequent storage at -20 °C. For homogenization, five embryos per concentration were thawed on ice, and 400 µL ice-cold 0.1 M phosphate buffer in 1 % Triton X-100 were added prior to homogenization with a TissueLyzer® at 30 Hz for 1 min (TissueLizer II, Quiagen, Hilden). Homogenates were centrifuged at 10,000 g for 15 min (4 °C), and supernatants were used for enzyme kinetics

and protein determination on a GENios® plate reader (Tecan, Crailsheim, Germany) as described by Küster (2005) and Perez et al. (2013). All enzyme measurements were conducted in four parallel reads per replicate and three independent replicates per concentration with five pooled embryos each (Perez et al. 2013). Enzyme activities were calculated as specific enzyme activity (U, Units) per protein content (mg), according to Ellman et al. (1961).

$$A_{sp} = \frac{(\Delta E \cdot test\ volume \cdot 10^6\ [\mu mol/mol]\ )}{min \cdot (\varepsilon_{ATC} \cdot sample\ volume \cdot protein\ amount)}$$

$mOD = mean\ OD$

$A_{sp} = specific\ enzyme\ activity$

$\Delta E = change\ in\ \ extinction\ \frac{OD}{min}$

$\varepsilon = molar\ absorbtivity\ of\ substrate\ \frac{1}{mol \times cm}$

Protein content was measured as optic density (OD) for 8 parallel reads per replicate and three replicates per concentration. Protein content in mg/ml was calculated with a BSA-standard curve analyzed with a 2nd order polynomial regression. For normalization of the enzyme kinetics, the mean value of protein content was used after excluding outliers higher or lower than two times the standard deviation.

In vitro AChE activity

For measurements of acetylcholinesterase activity *in vitro*, untreated controls in three independent replicates with five embryos each were homogenated as detailed above. After transfer of homogenates to the plates, each well was incubated for 15 min at RT with 50 µl cypermethrin (instead of 50 µL phosphate buffer) and additional DMSO to adjust DMSO content to 0.2 % in four different concentrations (1, 5, 10 and 20 ng/L) in phosphate buffer, leading to a final 0.1 % DMSO exposure concentration if combined with 50 µL supernatant (Fig. 14). Afterwards, the procedure was followed as shown above (acetylcholinesterase inhibition test).

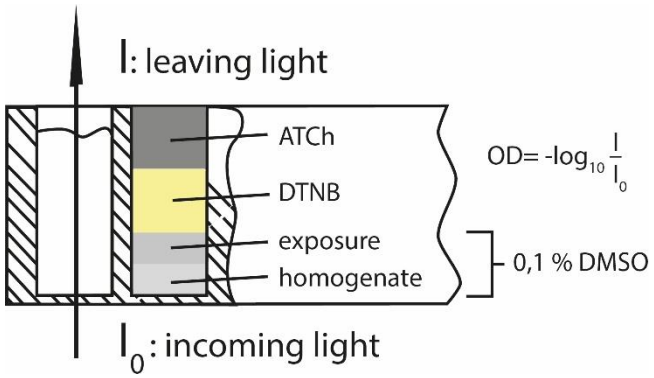

Figure 14. Cross section of a 96-well plate. Colors indicate: full body homogenate of five zebrafish embryos in 1 x PBS, exposure concentration of 1, 5, 10 and 20 ng/L cypermethrin or 0.75, 1.5 and 3 mg/L dichlorvos respectively, chromoagent DNTB and 100 µl of acetylthiocholine jodite,

Combination test

To elucidate a possible synergistic inhibitory effect of the two effective substances dichlorvos and cypermethrin, dichlorvos at its NOEC (0.18 mg/L) was spiked with three concentrations of cypermethrin (1, 5, 10 and 20 ng/L). Combination tests were run in three independent replicates with five embryos per concentration.

Data analysis

Statistical analysis for embryo toxicity was accomplished with ToxRat® (vers. 2.10.03; ToxRat Solutions, Alsdorf, Germany). For enzyme kinetics, statistical analysis was carried out with Sigma plot 12.3 (Systat software Inc., Erkrath, Germany) using One-way ANOVA in combination with Dunn´s test.

Table 2: Preparation of toxicant test solutions and test concentrations in the acetylcholine-esterase inhibition assays

Substance in [mg/L]	CAS	Test concentrations		
		Stock	FET	AChE-Assay
Amidotrizoic acid	117-96-4	8.9	0.56 - 8.9	2.25 - 8.9
Caffeine	58-08-2		19.4 -1000	6.05 - 24.2
Cypermethrin	52315-07-8	1*	31.3 - 1000 ng/L	0.1 - 20 ng/L
3,4-Dichloroaniline	95-75-1	10	4	not in use
Dichlorvos	62-73-7	800	1.75 - 20	0.048 - 3
2,4-Dichlorophenol	120-83-2	1000	0.1 - 12	0.4 - 1.6
2,4-Dinitrotoluene	121-14-2	100	4.9 - 25	1 - 4
4-Nonylphenol	84852-15-3	6	0.094 - 1.5	0.047 - 0.189
Perfluorooctanoic acid	335-67-1	1000	400 - 1000	112.5 - 550
Perfluorooctansulfonic acid	1763-23-1	~40 %[#]	3.125 - 50	1.66 - 6.6

*stock solution solved in DMSO, final test concentration with 0.1 % DMSO; [#]factory-provided

2.4 Results

Embryo toxicity tests

As range-finders for subsequent acetylcholinesterase inhibition tests, zebrafish embryo toxicity tests (FETs) were conducted according to OECD TG 236. Cypermethrin (0.1 % DMSO) and amidotrizoic acid showed neither lethal nor sublethal effects at the solubility limit and below. For perfluorooctanoic acid, effects were exclusively coagulation of early embryonic stages observed at each concentration; therefore, sublethal effects could not be determined. For EC_{10},

EC_{50} and LC_{50} values, see Table 3. To exclude unspecific secondary effects on acetylcholinesterase activities, induced by acute toxicity, subsequent acetylcholinesterase inhibition test were conducted with the EC_{10} value as maximum test concentration.

Table 3: EC and LC values (mg/L) of amidotrizoic acid, caffeine, cypermethrin, dichlorvos, 2,4-dichlorophenol, 2,4-dinitrotoluene, 4-nonylphenol, perfluorooctanoic acid and perfluorooctansulfonic acid as determined in the fish embryo test with zebrafish (*Danio rerio*)

Test substance	LC_{50}	EC_{10}	EC_{50}
Amidotrizoic acid	n.d.	n.d.	n.d.
Caffeine	514.89 ± 0.0	24.2 ± 1.86	45.9 ± 5.16
Cypermethrin	n.d.	n.d.	n.d.
Dichlorvos	8.1 ± 0.49	2.83 ± 0.53	4.65 ± 0.54
2,4-Dichlorophenol	7.1 ± 0.28	1.6 ± 0.48	2.3 ± 0.06
2,4-Dinitrotoluene	n.d.	3.9 ± 0.08	6.9 ± 0.07
4-Nonylphenol	1.505 ± 0.39	0.144 ± 0.06	0.276 ± 0.09
Perfluorooctanoic acid	759 ± 116	n.d.	n.d.
Perfluorooctansulfonic acid	34.2 ± 1.03	6.65 ± 1.19	21.39 ± 1.05

Acetylcholinesterase inhibition by selected model substances

Embryos were exposed to the EC_{10} concentrations (Table 3) of nine selected model substances, as determined in range-finding FETs. As a negative control for cypermethrin, DMSO showed no significant influence on acetylcholinesterase activity. Likewise, except for cypermethrin and dichlorvos, none of the test compounds induced significant alterations of acetylcholinesterase activity. Acetylcholinesterase activities in embryos exposed to dichlorvos ranged between 45 % of the activity in the negative control and 73 % of the inhibitory effects of the positive control paraoxon-methyl, whereas acetylcholinesterase activities in embryos exposed to cypermethrin showed a slight but significant increase in activity of 107 % over the negative control (Fig. 15).

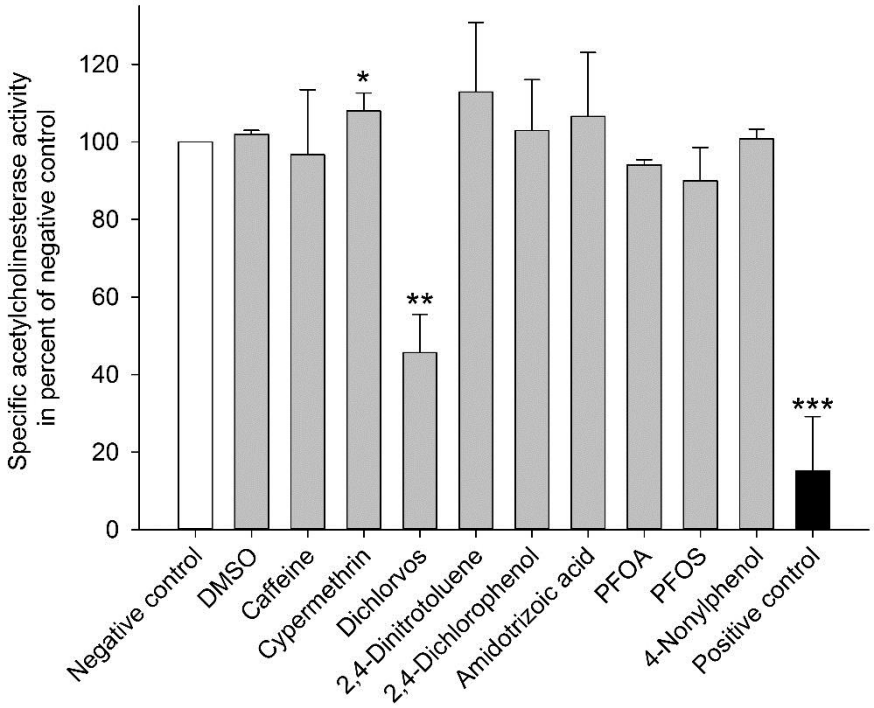

Figure 15: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following exposure to DMSO, amidotrizoic acid, caffeine, cypermethrin, dichlorvos, 2,4-dinitrotoluene, 2,4-dichlorophenol, 4-Nonylphenol, PFOA and PFOS for 96 h. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistical difference from negative controls: *p < 0.05, **p < 0.01, ***p < 0.001 (one-way ANOVA, Dunn´s test).

Dose-response relationships

After determination of acetylcholinesterase activity of substances exposed to EC_{10} concentrations, a graded concentration series was used for dichlorvos and cypermethrin with EC_{10} as the highest concentration (Table 3) to exclude non-specific secondary effects. Both substances showed a dose response relationship in either inhibition for dichlorvos (Fig. 16) or activation for cypermethrin (Fig. 17) with a No Observed Effect Concentration (NOEC) for dichlorvos at 0.18 mg/L and cypermethrin at 5 ng/L.

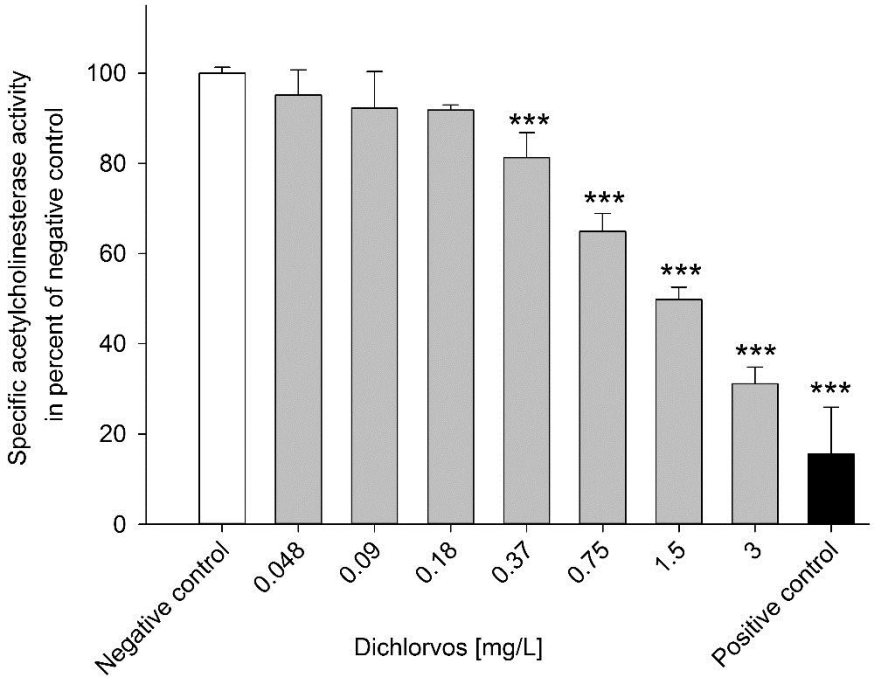

Figure 16: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following exposure to dichlorvos for 96 h. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistically difference from negative controls: ***p < 0.001 (one-way ANOVA, Dunn´s test).

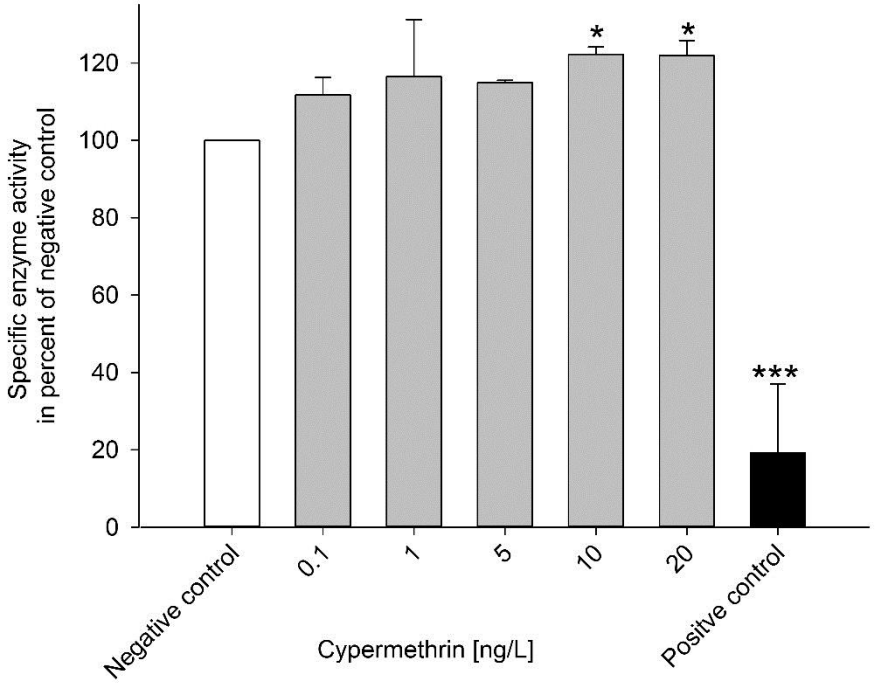

Figure 17: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following exposure to cypermethrin for 96 h. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistically difference from negative controls: *p<0.05, ***p < 0.001 (one-way ANOVA, Dunn´s test).

Combination exposure to dichlorvos and cypermethrin

Dichlorvos is known to directly interact with the catalytic center of the acetylcholinesterase, but mechanisms for cypermethrin are still unknown. To elucidate if mechanisms of cypermethrin-related acetylcholinesterase activation interferes with or partially overrides dichlorvos related inhibition, a co-exposure scenario was used. Dichlorvos NOEC (0.18 mg/L) was spiked with three different concentrations of cypermethrin (1, 10 and 20 ng/L) that had already shown activation of acetylcholinesterase. Activity of acetylcholinesterase showed no previously observed dose-response relationship after the combined dichlorvos-NOEC and cypermethrin exposure. Even more, the previously overserved activation of acetylcholinesterase was eliminated (Fig. 18).

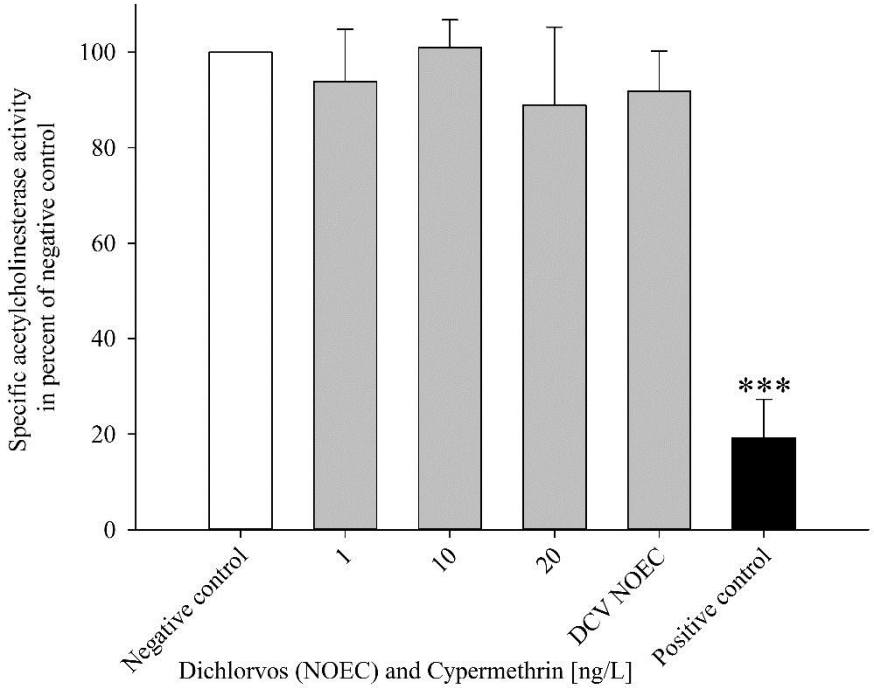

Figure 18: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following exposure to dichlorvos NOEC and dichlorvos NOEC with a cypermethrin concentration series for 96 h. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistically difference from negative controls: ***$p < 0.001$ (one-way ANOVA, Dunn´s test).

In vitro effect determination

Since, effects of cypermethrin were eliminated by dichlorvos, we excluded the possibility of a regulation on DNA level, e.g. an increase in acetylcholinesterase gene expression, by exposure of homogenized and centrifuged pooled embryos to a cypermethrin concentration equal to that used *in vivo*. Embryo samples showed no dose-response relationship or any significant increase in acetylcholinesterase activity induced by cypermethrin (Fig. 19). Dichlorvos was used in addition to analyze if chosen concentrations were effective *in vitro* (Fig. 20). Dichlorvos showed

the same dose-response relationship as revealed in *in vivo* exposure; however, inhibition was even more effective *in vitro* than *in vivo*.

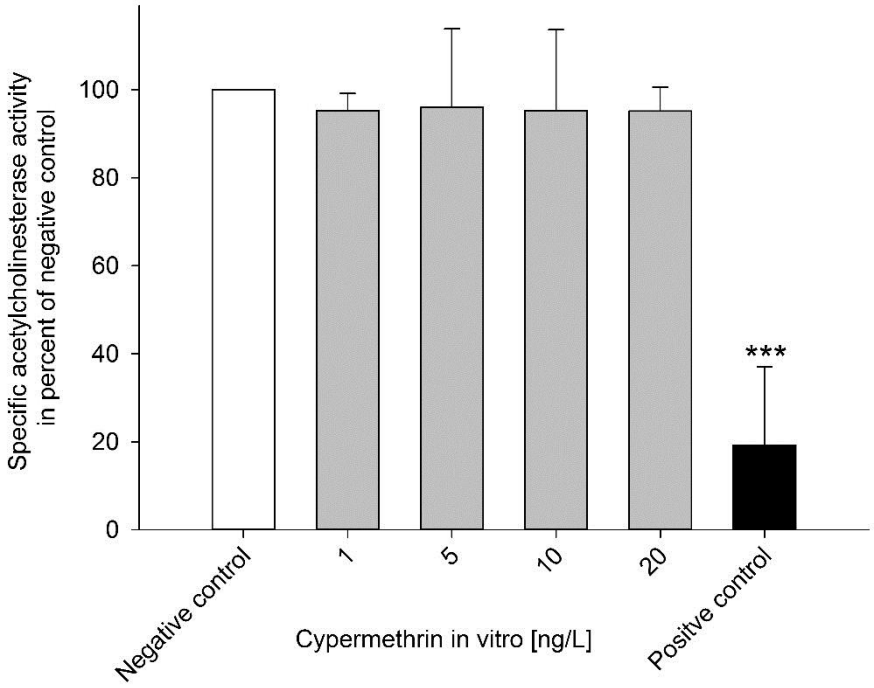

Figure 19: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following *in vitro* exposure to cypermethrin for 15 min. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistically difference from negative controls: *** $p < 0.001$ (one-way ANOVA, Dunn´s test).

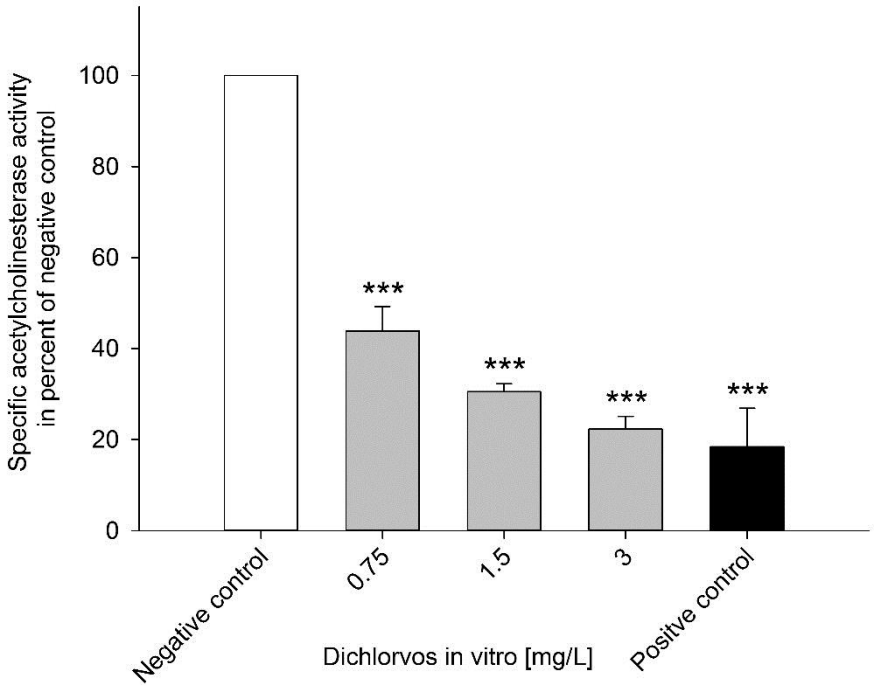

Figure 20: Specific acetylcholinesterase activities in pools of five 4-d old zebrafish embryos following

in vitro exposure to dichlorvos for 15 min. Inhibition is expressed as percent of the negative control (100 %; open column). The positive control (2 mg/L paraoxon-methyl) is given as the black column. N = 3. Statistically difference from negative controls: ***p<0.001 (one-way ANOVA, Dunn´s test).

2.5 Discussion

From the nine chosen reference substances, which had been selected for their possible groundwater impact and neurotoxicity, only two showed a significant influence on acetylcholinesterase activity in zebrafish embryos. So far and to the best of our knowledge, 2,4-dinitrotoluene, 2,4 dichlorophenol and amidotrizoic acid have not been tested with regard to acetylcholinesterase activity alteration.

In the present study, 4-nonylphenol showed no effect on AChE activity at 189 mg/L on zebrafish embryos. In recent years, 4-nonylphenol has been used as a model with endocrine disrupting characteristics (Chandrasekar et al. 2011b, Puy-Azurmendi et al. 2014), but studies on AChE activity are limited or even absent in fish embryos. Nevertheless, results provide recent findings of Maltais and Roy (2014) who revealed a lack of effect on AChE in muscle of the immature freshwater fish copper redhorse (*Moxostoma hubbsi*) after exposure to 1, 10, 50 µg/L nonylphenol for 21 days. In contrast, Li (2008) reported a decrease in acetylcholinesterase activity in muscle of adult male guppies (*Poecilia reticulata*) after exposure to 60 and 150 µg/L nonylphenol for seven days but not in brain AChE activity. Other endocrine disrupting chemicals (i.e. ethinylestradiol) have shown to increase AChE activity in muscle of Atlantic salmon (Greco et al. 2007). Considering possible differences in sensibility of brain and muscle AChE to different substances, a smaller amount of enzyme due to the use of 5 embryos, and a mixture of all tissues by using full-body homogenates may potentially conceal effects in divers' tissues.

Caffeine, which is one of the most commonly used psychoactive drugs, showed a lack of effect on AChE activity in zebrafish embryos after exposure to 24.2 mg/L. In contrast, Teixido et al. (2013) showed a 50 % inhibition of AChE activity of zebrafish embryos at a concentration of 2.5 mM (~480 mg/L) while defining an EC_{50} at 0.8 mM (~153 mg/L). Concentrations at EC_{50} reach the range of teratogenic effects were strong malformation appear. These malformations possibly interfere with AChE activity. As we used the EC_{10} concentration of 24.2 mg/L to exclude this interference, it seems likely that possibly occurring inhibitory effects were below the detection limit in our test system. However, Karadsheh et al. (1991) revealed 50 % inhibition of electric eel (*Electrophorus electricus*) AChE *in vitro* at a concentration of 87 µM (~16.9 mg/L) and Li et al. (2012) a significant inhibition of brain AChE activity after four days exposure of adult goldfish (*Carassius auratus*) to ≥ 0.08 mg/L caffeine. Additionally, caffeine has shown to be a non-competitive inhibitor in human AChE (Pohanka and Dobes 2013). Electric eel AChE has shown to be more susceptible to activity inhibition by various substances, if compared to AChE of other fish (Assis et al. 2007, Assis et al. 2012, Silva et al. 2013). Therefore, we conclude differences in AChE and concentrations leading to the fact that caffeine had no observable influence in AChE activity in our model system.

PFOS and PFOA are perfluorinated organic compounds with scarce or no data on specific neurotoxicity available. In our work, PFOS and PFOA showed no effect on AChE activity of zebrafish embryos at concentrations of 6.6 mg/L and 550 mg/L. These results support findings of Kim et al. (2010), who tested concentrations from 50 µg/L to 50 mg/L of PFOS and PFOA on AChE of adult common carp (*Cyprinus carpio*).

Dichlorvos is a synthetic AChE-inhibiting organophosphate pesticide. In contrast to other organophosphates (e.g. chlorpyrifos or parathion) dichlorvos is a direct AChE inhibitor, where no bio-activation by an oxon production is needed (Safety/INCHEM. 1989). In the present work, dichlorvos has shown an expected effect on AChE activity in zebrafish embryos. Activity of the AChE decreased to 45 % if compared to the negative control *in vivo* at the EC_{10} concentration of 3 mg/L. In dose-response tests, a significant decrease in AChE was observed at ≥0.37 mg/L, with a NOEC at 0.18 mg/L. In fact, the inhibitory potential of dichlorvos in zebrafish embryos in the present study differed slightly from that reported in previous studies. Varo et al. (2008) observed a decreased in AChE activity to 85.7 % in head and 69.4 % in muscle of Spanish toothcarp (*Aphanius iberus*) after exposure to 2 mg/L dichlorvos. Silva et al. (2013) showed a 50 % inhibition of AChE activity at 5.52 µM (~1.2 mg/L) in *Cichla ocellaris* and at 0.04 µM (~0.009 mg/L) in *Colossoma macropomum*. Zebrafish and Spanish toothcarp seem to react less sensitive to dichlorvos inhibition. Thus, *in vivo* experiments are dependent on multiple parameters (i.e. exposure scenario, pharmacokinetics, bioelimination, a. o.), substance uptake is likely to be hindered by these factors, if compared to *in vitro* experiments as used by Silva et al. (2013). As shown in Figure 20, dichlorvos showed a higher inhibition of AChE activity *in vitro* (by a factor of two) if compared to *in vivo* exposure (Fig. 16) underlining differential bioavailability *in vivo*. Nevertheless, differences in species-specific susceptibility to inhibitors *in vitro* have been shown for a wide range of aquatic fishes (Assis et al. 2007, Assis et al. 2012, Silva et al. 2013). For dichlorvos, susceptibility differs by a factor of ~17,000 in different studies (Assis et al. 2007, Assis et al. 2012, Silva et al. 2013), which impedes a direct comparision of zebrafish specific results on AChE inhibition.

Cypermethrin is a pyrethroid insecticide, which is mainly used for its characteristics as a repellent. Its primary targets in insects are voltage gated sodium channels (Casida et al. 1983). In contrast to dichlorvos and recent works, cypermethrin caused a significant increase in AChE activity of zebrafish embryos at concentration of 10 and 20 ng/L. In recent studies, a decrease in AChE activity was shown for brain AChE of C*atla catla* fingerlings after exposure of to 0.443 µg/L cypemethrin for 60 days (Vani et al. 2012), for liver and muscle tissues of *Clarias gariepinus* after exposure to 20 µg/L cypermethrin for five days (Olalekan 2014) and in liver and muscle tissue of fingerlings of *Labeo rohita* exposed to 0.082 µg/L cypermethrin (Tiwari et al. 2012). Indeed, our results differ from recent works, but were conducted at lower concentrations and a different model, leading to different results. For instance, differences in tissues-specific AChE activity after exposure to zinc and copper. Copper increased AChE activity in brain and muscle, zinc only in muscle. (Gioda et al. 2013). Assis et al. (2012) revealed species

specific differences in AChE activity. Additionally, AChE gene expression increased after exposure to glyphosate (Prevot-D'Alvise et al. 2013) resulting in an increased AChE activity (Samanta et al. 2014). Pyrethroids are able to increase AChE activity as well, as shown for honey bees (*Apis melifera*) after deltamethrin exposure (Badiou and Belzunces 2008) and for Common carp (*Cyprinus carpio*) (Szegletes et al. 1995). Besides classical drug targets of pyrethroids that have been already shown, pyrethroids are possibly able to act by binding to the acetylcholine receptor (Soderlund et al. 2002). It is not clear, if cypermethrin acts via direct AChE interaction or by regulation of AChE expression, therefore, we conducted a combinatory study to elucidate possible interactions of dichlorvos and cypermethrin at the same possible mode of action.

In the combination exposure, AChE activity revealed a lack of AChE activation with any tested cypermethrin concentration. It may be concluded, that dichlorvos inhibits AChE activation by AChE inhibition below the detection limit of the concentrations used. This would lead to a masked effect of cypermethrin either on a direct or regulatory level. To exclude influences by gene regulation, which might lead to a higher AChE activation and therefore to a higher activity in the co-exposure scenario, we conducted a single substance *in vitro* exposure. In the *in vitro* experiment cypermethrin showed no inhibitory effect if compared to dichlorvos *in vitro* inhibition or the negative control. Therefore, we conclude a possible regulatory mechanism of cypermethrin according to Soderlund et al. (2002), which is masked by the inhibitory potential of dichlorvos in combinatory exposure.

2.6 Conclusion

The acetylcholinesterase inhibition assay with zebrafish embryos proved to be a suitable tool to detect AChE inhibitors and activators *in vivo*. Whereas inhibition of AChE by organophosphates has widely been shown and information about adverse outcome pathways of additional substances, formerly known to be AChE inhibitory, is still limited, the present study provides evidence that pyrethroid insecticides may influence additional mechanism than they were designed for. However, since co-exposure of dichlorvos and cypermethrin had no synergistic effect, the mode of action of AChE activation by cypermethrin was apparently different to that of the typical acetylcholinesterase inhibitor dichlorvos as shown *in vitro*. After all and in dependence on recent work on AChE inhibition in multiple species, we strongly support a standardization for fish based protocols on AChE inhibition in risk assessment of emerging contaminants with a combination of *in vitro* and *in vivo* methods.

Chapter III

3. An optimized method to assess neurotoxic effects in the lateral line of zebrafish (*Danio rerio*) embryos

3.1 Abstract

In order to clarify the suitability of the lateral line of zebrafish (Danio rerio) embryos as a model for the screening of neurotoxic effects, existing neuromast assays were adapted, improved and validated with a series of chemicals known or unknown for their ototoxic potential (caffeine copper sulfate, dichlorvos, 2,4-dinitrotoluene, neomycin, 4-nonylphenol, perfluorooctanesulfonic acid). Present methods were improved by (1) the introduction of a 4-step scoring system, (2) the selection of neuromasts from both the anterior and posterior lateral line systems, (3) a combined DASPEI/DAPI staining applied after both a permanent and pulse exposure scenario, and (4) an additional screening for nuclear fragmentation. Acute toxicities of the model substances were determined by means of the fish embryo test as specified in OECD TG 236, and EC10 concentrations were used as the highest test concentration in the neuromast assay. The enhanced neuromast assay identified known ototoxic substances such as neomycin and copper sulfate as neurotoxic at sensitivities similar to those of established methods, with pulse exposure leading to stronger effects than permanent exposure. Except for caffeine, all substances tested (dichlorvos, 2,4-dinitrotoluene, 4-nonylphenol, perfluorooctanesulfonic acid) produced significant toxic effects in neuromasts at EC_{10} concentrations. Depending on the test substances and their location along the lateral line, specific neuromasts differed in sensitivity. Generally, neuromasts proved more sensitive in the pulse exposure scenario. Whereas for neomycin and copper sulfate neuromasts located along the anterior lateral line were more sensitive, posterior lateral line neuromasts proved more sensitive for the other test substances. Nuclear fragmentation could not only be associated with all test substances, but, albeit at lower frequencies, also with negative controls, and could, therefore, not be assigned specifically to chemical damage. The study thus documented that for a comprehensive evaluation of lateral line damage both neuromasts from the anterior and the posterior lateral line have to be considered. Given the apparently rapid regeneration of hair cells, pulse exposure seems more appropriate for the identification of lateral line neurotoxicity than permanent exposure.

3.2 Introduction

Water plays a central role in human life, and the allocation of high-quality drinking water is a major challenge. Given the multitude of anthropogenic compounds found in surface and ground waters, anthropogenic impact may be a major risk for water quality. An increasing number of anthropogenic pollutants has been identified in water bodies, and since only a small number of priority pollutants are being considered by the EU Water Framework Directive, research programs have been initiated to develop acute and mechanism-specific bioassays to identify the putative damage potential within a hazard-based risk assessment. For this end, a joint research program has been established by the German Federal Ministry of Education and Research

(BMBF): "Risk management of emerging compounds and pathogens in the water cycle (RiSKWa)"(Huckele and Track 2013b). One of the RiSKWa-funded projects, ToxBox, aims at the design of a harmonized testing strategy for an exposure- and hazard-based risk management of anthropogenic trace substances ("Securing drops of life – an enhanced health-related approach for risk assessment of drinking water in Germany", (Grummt et al. 2013) and deals with the establishment of standardized methods concerning endocrine effects, neurotoxicity, genotoxicity and germ cell damage as additional endpoints for the assessment of the health-related indicator value (HRIV) approach originally developed by the German Federal Environment Agency (UBA). Among the endpoints covered within ToxBox, neurotoxicity is, by far, the least far developed, and the present study was designed to optimize existing methods for the identification of neurotoxicity in aquatic vertebrates. Together with other major sensory organs, the lateral line was investigated in zebrafish (*Danio rerio*) as the major model for aquatic toxicity assessment.

In aquatic vertebrates, the lateral line system is a sensory system consisting of interconnected neuromasts, which detect changes of mechanical pressure in the surrounding medium. Morphologically, it is divided into the anterior lateral line system (ALL) primarily located around the head and the posterior lateral line system (PLL) mostly spanning the trunk (Bleckmann and Zelick 2009, Ghysen and Dambly-Chaudiere 2004, Metcalfe et al. 1985, Raible and Kruse 2000)(Fig. 22). Due to the presence of primary sensory neurons, the so called hair cells, which are homologous to neurons in the inner ear of humans (Fritsch et al. 2013, Hayashi et al. 2015, Ton and Parng 2005) and are located very close to the epidermal surface of the fish, the lateral line system is of particular interest for the determination of neurotoxic effects *in vivo* (Froehlicher et al. 2009b). As a consequence, it has not only been used in neurotoxicology (Linney et al. 2004), but also in ecotoxicology (Hill et al. 2005, Scholz et al. 2008, Segner 2009). Although, however, the homology of hair cells in the lateral line and in the human inner ear makes the former a potential model for human neurotoxicology (Fritzsch et al. 2007) data on the neurotoxicity of specific substances are still scarce, so far. Likewise, despite the direct contact of hair cells with the surrounding medium, which makes an early response to neurotoxic chemicals highly likely, there is only a limited number of ecotoxicological studies using the lateral line (Coffin et al. 2010, Coffin et al. 2009, Froehlicher et al. 2009b, Harris et al. 2003, Linbo et al. 2006).

So far, comparability between different protocols for the determination of neuromast damage by chemical substances is limited due to differences in (1) exposure time, (2) the number and localization of neuromasts analyzed, (3) the staining methods applied, and (4) the actual endpoints recorded. The number of neuromasts studied range from one single (Linbo et al. 2009) to all neuromasts present at a specific age (Harris et al. 2003). Whereas (Linbo et al. 2009) exclusively looked at head neuromasts, the study by (Montalbano et al. 2014) is restricted to trunk neuromasts. Neurotoxic effects in hair cells are visualized via staining intensity with fluorescent dyes (Buck et al. 2012, Murakami et al. 2003, Owens et al. 2009), hair cell counts

(Linbo et al. 2009, Linbo et al. 2006, Owens et al. 2008) or both (Harris et al. 2003). Most studies used DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide), a styryl dye staining mitochondria (Bereiter-Hahn 1976, Bereiter-Hahn and Voth 1994), and the intensity of fluorescence is usually quantified via a 3-step scoring scheme by Harris et al. (2003).

Most importantly, the lateral line system has been shown to be able to quickly regenerate after chemical-induced hair cell loss (Harris et al. 2003, Hernandez et al. 2007, Lush and Piotrowski 2014). As a consequence, depending on the substance-specific mode of action, short-term exposure may lead to stronger effects than long-term exposure. Since there is also increasing evidence of differential susceptibility of single neuromasts to chemical injury (Harris et al. 2003, Murakami et al. 2003), the establishment of a standardized protocol for a neuromast assay seems overdue.

Therefore, the present study was designed to make a contribution to the optimization and standardization of a neuromast assay (1) by increasing the sensitivity of the test by the use of selected multiple neuromasts, (2) by reducing the risk of false positive results via inclusion of additional staining techniques, (3) by optimizing the exposure period (permanent versus pulse exposure), and (4) by extending existing scoring schemes for quantification of fluorescence intensities. In addition to known ototoxic compounds (neomycin, copper sulfate), caffeine, dichlorvos, 2,4-dinitrotoluene, 4-nonylphenol and perfluorooctanesulfonic acid (PFOS), were used as model compounds.

3.3 Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany) at the highest purity available, unless stated otherwise. For exposure experiments, caffeine, copper sulfate (Merck, Darmstadt), dichlorvos, 2,4-dinitrotoluene, neomycin, 4-nonylphenol, perfluorooctanesulfonic acid were used. For test concentrations in the fish embryo tests and neuromast assays, see Table 5. All toxicant stock solutions were prepared in artificial water according to OECD TG 236 (OECD 2013).

Fish

All adult zebrafish used for breeding were wild-type descendants of the "West-aquarium" strain and obtained from the Aquatic Ecology and Toxicology breeding facilities at the University of Heidelberg (licensed under no. 35-9185.64/BH). Details of fish maintenance, egg production and embryo rearing have repeatedly been described in detail (Kimmel et al. 1995a, Kimmel et al. 1988, Nagel 2002, Spence and Smith 2005, Wixon 2000a) and have been updated for the purpose of the zebrafish embryo toxicity by Lammer et al. (2009b) and Sessa et al. (2008).

Zebrafish embryo toxicity tests (FET)

In order to avoid indirect effects by systemic acute toxicity potentially mimicking neurotoxic effects, the highest test concentrations in the neurotoxicity tests were set at the EC_{10} for any

kind of effect in zebrafish embryos.

For determination of acute toxicity in fish embryos (LC data), fish embryo tests were performed according to the protocol specified by OECD TG 236 (OECD (2013). In brief, embryos were exposed at latest from 1 HPF (hour(s) post-fertilization) in glass vessels, which had been pre-incubated (saturated) for at least 24 h, to a series of dilutions of the respective toxicants. After control of the fertilization success, embryos were transferred to 24-well plates (TPP Renner, Dannstadt, Germany), which had been pre-incubated with 2 ml of the test solutions per well for 24 hours prior to the test start, and kept in an incubator at 26.0 ± 1.0 °C un-der a 10:14 h light regime. In order to prevent evaporation or cross-contamination between the wells, the plates were sealed with self-adhesive foil (Nunc, Wiesbaden, Germany). Embryo tests were classified as valid, if the mortality in the negative control was ≤ 10 %, and the positive control (3,4-dichloroaniline) showed mortalities between 20 and 80 % (Lammer et al. 2009). All fish embryo tests were run in in three independent replicates.

Zebrafish embryos were exposed to the test substances until 96 hours post-fertilization (hpf) in a semi-static fashion, i.e. the medium was changed every 24 hours subsequent to the daily inspection of the embryos. Every 24 hours, the embryos were controlled for lethal effects according to OECD TG 236 (coagulation, non-formation of somites, non-detachment of the tail and lack of heart beat) as well as for sublethal changes using the endpoints specified by Bachmann (2002), Schulte and Nagel (1994) as well as Nagel (2002). Both lethal and sublethal effects were used for the determination of EC values.

Neuromast assay

All neuromast assays were performed in triplicate in 24-well plates with four embryos per test concentration until 96 hpf. The neuromast assay was first optimized for copper sulfate, which was used as a positive control (1.6 mg/L) in all subsequent tests. As negative controls, untreated embryos incubated in artificial water according to OECD TG 236 (OECD 2013) were used. Controls and test solutions were replaced every 24 h upon daily inspections of embryos.

For test concentrations in the neuromast assay, see Table 5. Neomycin and copper sulfate were tested in the following two scenarios: (1) 96 h permanent exposure according to the protocol for the fish embryo test (OECD 2013), and (2) pulse exposure for 30 min from 96 hpf. All other compounds were only tested following the permanent exposure scenario.

For in vivo staining of neuromasts, DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide) and DAPI (4′,6-diamidino-2-phenylindole) were used: The 0.05 % DASPEI stock solution in 5 % DMSO was diluted with artificial water to a final strength of 0.005 % DASPEI (50 µg/ml) in 0.5 % DMSO. The 1 mg/ml DAPI stock solution was prepared in artificial water and diluted to 1 ng/ml. DASPEI primarily stains mitochondria (Bereiter-Hahn and Voth 1994). With a staining time as short as 30 min, mitochondria-rich hair cells within vital neuromasts were stained with only marginal background (Fig. 23). As a DNA intercalation marker, DAPI is commonly used to stain nuclei (Fig. 23). In the neuromast assay, DAPI serves (1) as an internal control to prevent false positive results due to unspecific loss of DASPEI staining and

(2) for an enhanced structure resolution of the neuromasts. During and subsequent to the staining procedure, embryos were protected from light to prevent bleaching and phototoxic effects of the dyes.

Following exposure, embryos were stained with 50 µg/ml DASPEI (0.005 %) for 30 min. After 3 × 10 min rinsing in artificial water, embryos were stained with 1 ng/ml DAPI for 30 min. After another 3 × 10 min rinse in artificial water, embryos were anesthetized by 5 min incubation in 0.016 % MS 222 (tricaine, ethyl-3-aminobenzoate) and mounted laterally in low melting (29 °C) agarose on glass bottom culture dishes (MatTek Corp., Ashland, USA).

For the neuromast assays, the following neuromasts were selected for the anterior and posterior lateral line systems (Fig. 22; terminology according to Harris et al. (2003), Metcalfe et al. (1985), Raible and Kruse (2000): M2, O2, IO2 and IO3 for the anterior lateral line system; MI1, MI2, P1, P2 and P3 for the posterior lateral line system. Fluorescence intensity of the selected neuromasts was recorded photographically for each individual embryo by imaging with a Nikon Eclipse 90i epifluorescence microscope (Nikon, Tokyo, Japan) equipped with a Nikon Fluor 60×/1,00W submerged objective and a Nikon DASPEI (ex461 / em589) and DAPI (ex405 / em500) filter set.

Scoring

Based on the 3-step scoring scheme by Harris et al. (2003), the quantification of neuromast fluorescence intensity was enhanced to a 4-grade system for the scoring of neuromast damage: Each neuromast was scored from grade 0 (unchanged control: all hair cells well stained) over grade 1 (minor damage: reduced mitochondrial staining intensity and/or less hair cells) and grade 2 (strong damage: mitochondria only faintly stained and /or strongly reduced number of hair cells) to grade 3 (very strong damage: mitochondria and hair cells barely detectable).

For data presentation, the overall score of an embryo was determined by forming the average of the scores for single neuromasts of the respective embryo. For each replicate, the average score for all 4 embryos per replicate was computed, and for each treatment, the average score of the 3 replicates was calculated. For differential sensitivity of the neuromasts of the anterior and the posterior lateral line systems, the neuromasts of either system were calculated separately.

Table 4: Combined DASPEI- / DAPI-Score

Score	**staining quality**
0	hair cells are present, excellent staining
1	reduced staining and/or less hair cells
2	staining least present and/or barely any hair cells remaining
3	absent staining

Table 5 Preparation of toxicant test solutions and test concentrations in the neuromast assays

Toxicant	CAS no.	Stock solution (mg/L)	Test concentrations (mg/L)	
			Fish embryo tests	Neuromast asays[2]
Caffeine	58-08-2	1000	19.4, 42.7, 93.9, 206.6, 454.5, 1000	6.05, 12.1, 24.2
Copper sulfate[1]	7758-98-7	8	0.03, 0.3, 0.6, 1, 1.6	0.03, 0.3, 0.6, 1, 1.6
Dichlorvos	62-73-7	800	1.75, 2.63, 3.95, 5.93, 8.82, 13.3, 20	0.75, 1.5, 3
2,4-Dinitrotoluene	121-14-2	50	4.9, 7.4, 11.1, 16.6, 25	1, 2, 4
Neomycin	81405-10-3	30 g/L	2.3, 4.6, 9.2, 18.4 and 36.8	2.3, 4.6, 9.2, 18.4, 36.8
4-Nonylphenol	84852-15-3	6	0.18, 0.37, 0.75, 1.5, 3.0	0.144, 0.72, 0.36
Perfluorooctanesulfonic acid	1763-23-1	400	3.125, 6.25, 12.5, 25, 50	1.66, 3.33, 6.65

[1] Copper sulfate at a concentration of 1.6 mg/l was used as a positive control for neuromast assays with all other toxicants.

[2] EC_{10}, EC_5 and $EC_{2.5}$ except for copper sulfate and neomycin, which were tested in a full dose-response test (cf. Figs. 4 and 5).

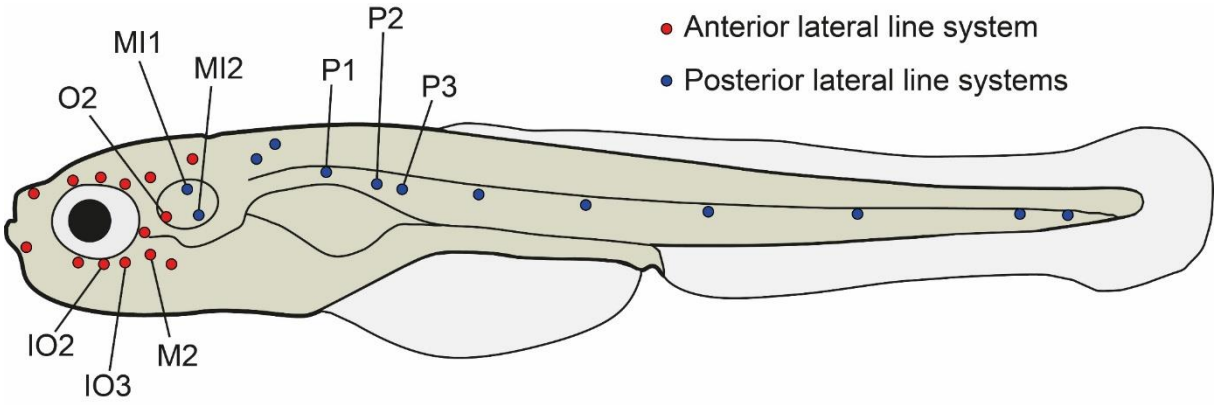

Figure 21: Localization of neuromasts in a 96 h zebrafish (Danio rerio) embryo according to the terminologies by Metcalfe et al. (1985) and Raible and Kruse (2000). As representatives of the anterior lateral line system (red dots), the neuromasts IO2, IO3, M2 and O2 were analyzed (red dots); as representatives of the posterior lateral system (blue dots), the neuromasts MI1, MI2, P1, P2 and P3 were selected.

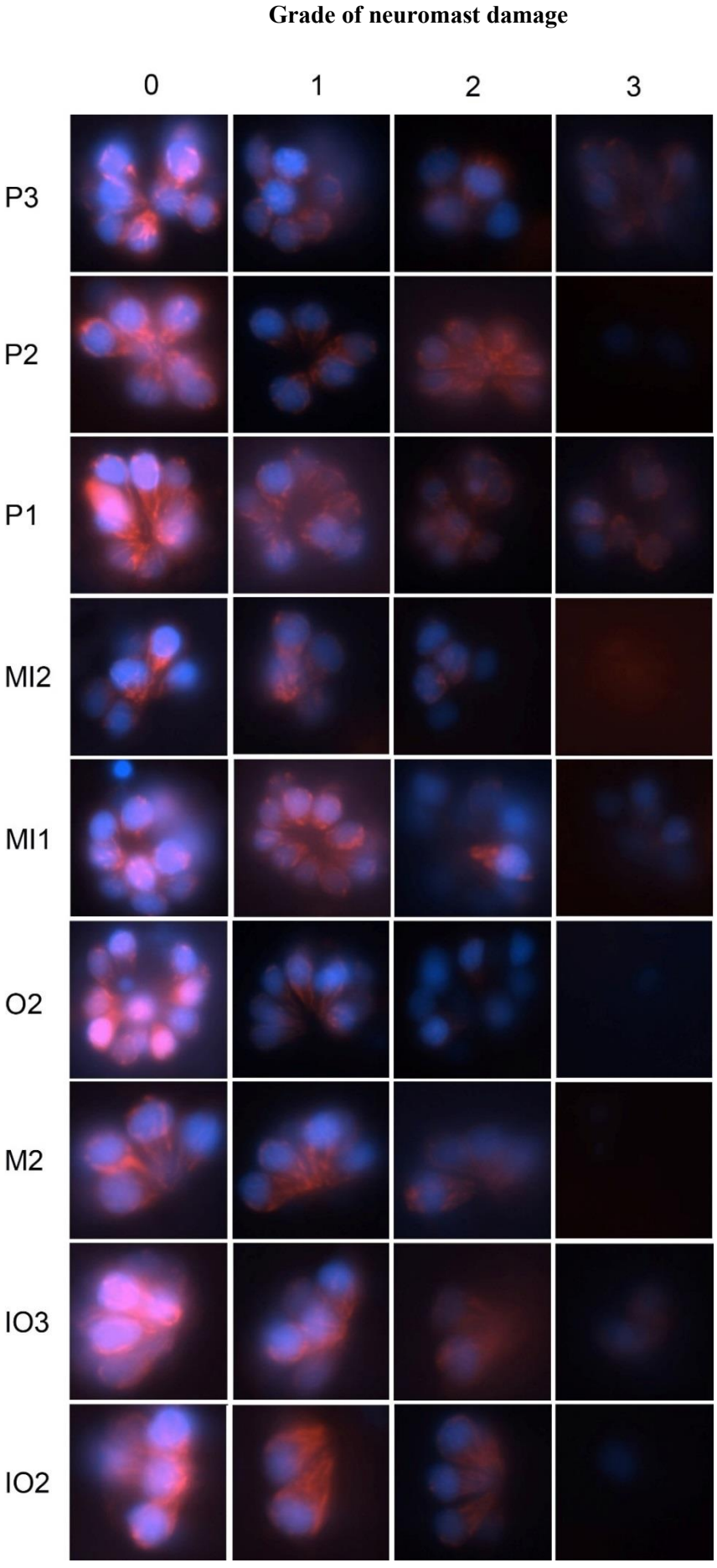

Figure 22: Scoring of damage in selected zebrafish (*Danio rerio*) neuromasts (from left to right): 0 – unchanged control: all hair cells well stained; 1 – minor damage: reduced mitochondrial staining intensity and/or less hair cells; 2 – strong damage: mitochondria only faintly stained and /or strongly reduced number of hair cells; 3 – very strong damage: mitochondria and hair cells barely detectable.

Statistics

Statistical analysis for embryo toxicity was accomplished with ToxRat® 2.10.03 (ToxRat Solutions, Alsdorf, Germany). For neurotoxicity, statistical analyses were carried out with Sigma Plot 12.3 (Systat-Jandel, Erkrath, Germany) using One-way ANOVA in combination with Dunn´s test by comparing replicates for significant differences and combining the mean scores per fish of 3 replicates of one treatment (n = 12).

3.4 Results

Embryo toxicity tests

Prior to the neuromast assays, zebrafish embryo toxicity tests according to OECD TG 236 were conducted to screen for ranges of acute toxicity of selected substances and to determine EC_{10} values (Table 6). For subsequent neuromast assays, EC_{10} values were selected as the maximum test concentrations to exclude unspecific secondary effects on hair cells due to acute toxicity.

Table 6: EC and LC values (mg/L) of caffeine, dichlorvos, 2,4,-dinitrotoluene, 4-nonylphenol and perfluorooctansulfonic acid as determined in the fish embryo test with zebrafish (Danio rerio)

Test substance	EC_{10} (mg/L)	EC_{50} (mg/L)	LC_{50} (mg/L)
Caffeine	24.2 ± 1.86	45.9 ± 5.16	514.89 ± 0.0
Dichlorvos	2.83 ± 0.53	4.65 ± 0.54	8.1 ± 0.49
2,4-Dinitrotoluene	3.9 ± 0.08	6.9 ± 0.07	n.d.
4-Nonylphenol	0.144 ± 0.06	0.276 ± 0.09	1.505 ± 0.39
Perfluorooctansulfonic acid	6.65 ± 1.19	21.39 ± 1.05	34.2 ± 1.03

n.d. – not determined

DASPEI / DAPI staining

For all test substances and the negative control, DAPI and DASPEI showed distinct fluorescence signals in either nuclei (DAPI; Fig 23: blue) or mitochondria (DASPEI; Fig 23: red) and a similar reduction of fluorescence for either staining following chemical exposure (Fig. 22).

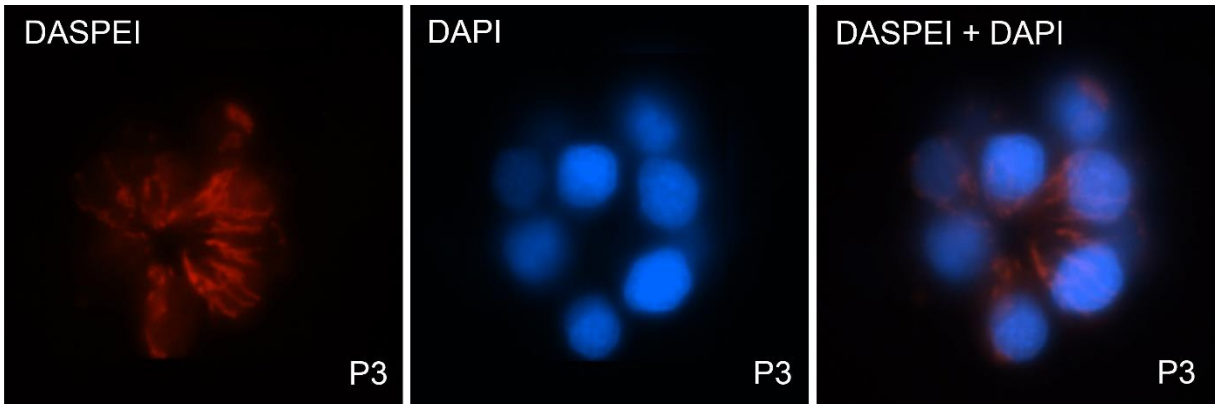

Figure 23: Neuromast P3 in 96 h old untreated control zebrafish (*Danio rerio*) larva: after 30 min staining with DASPEI (mitochondria: red) and DAPI (nuclei: blue), neuromasts in zebrafish (*Danio rerio*) embryos can be visualized with almost no background signal.

Neuromast degeneration by neomycin and copper sulfate (positive controls)

In most studies, neuromast damage has been quantified using the 3-step scoring scheme by Harris et al. (2003). In order to compare the new enhanced 4-step scoring system with previous studies, zebrafish embryos were exposed to the known ototoxic reference compounds neomycin (Fig. 24) and copper sulfate (Fig. 25). After 96 h permanent exposure, neomycin induced a highly significant reduction in fluorescence of hair cells from a concentration of 9.2 mg/L (Fig. 24); at higher concentrations of 2.3 and 4.6 mg/L, the fluorescence score did not increase. In contrast, copper sulfate showed a monotonous dose-related increase in damage, i.e. a concentration-dependent decrease in fluorescence intensity from 0.8 mg/L copper sulfate (Fig. 25).

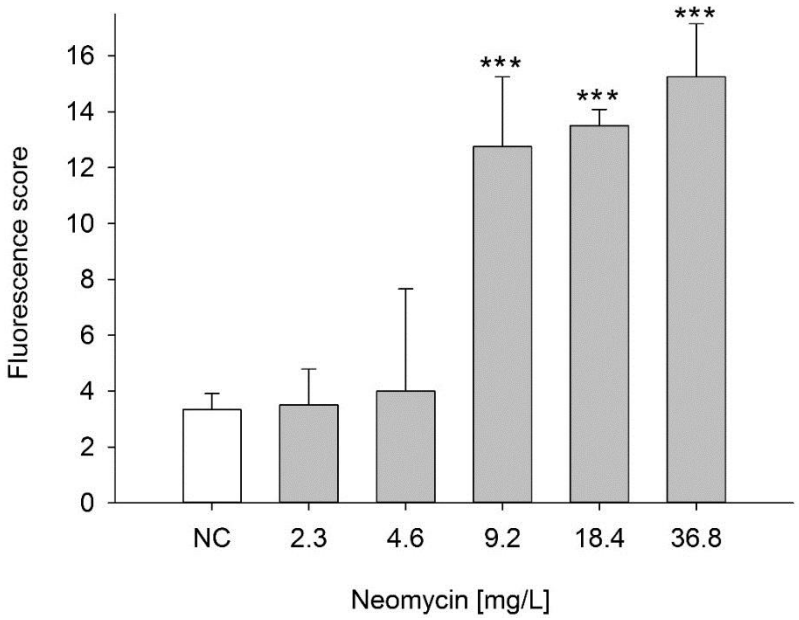

Figure 24: Fluorescence score of 9 neuromasts of 4 zebrafish (Danio rerio) embryos in 3 replicates following permanent exposure to neomycin for 96 hpf. Fluorescence score value is expressed as mean score per embryo (n=12). Statistically difference from negative controls: *** $p < 0.001$ (One-way ANOVA, Dunn´s test)

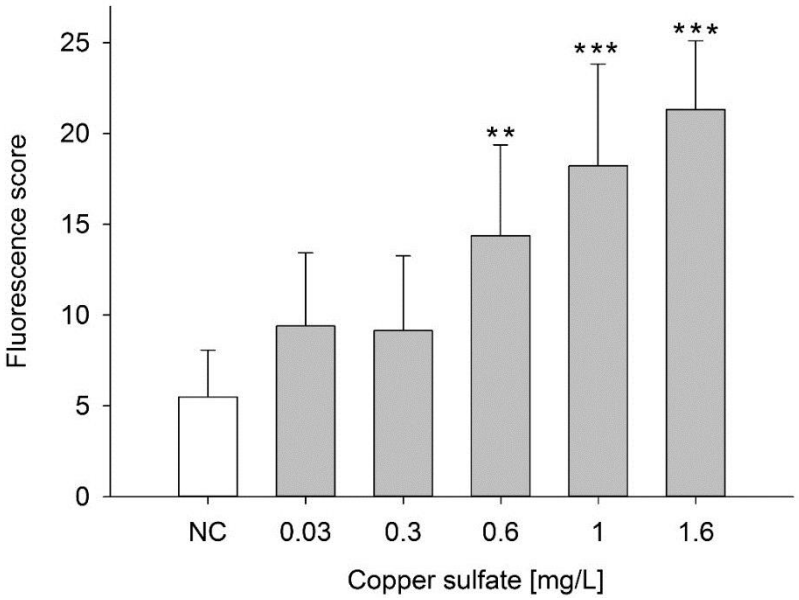

Figure 25: Fluorescence score of 9 neuromasts of 4 zebrafish (*Danio rerio*) embryos in 3 replicates following permanent exposure to copper sulfate for 96 hpf. Fluorescence score value is expressed as mean score per embryo (n=12). Statistically difference from negative controls: ** p<0.01, *** p < 0.001 (One-way ANOVA, Dunn´s test)

Neuromast damage in relation to exposure time

For neomycin, pulse exposure for 30 min usually resulted in a stronger reduction of hair cell fluorescence than 96 h permanent exposure (Fig. 26). Pulse exposure thus also led to a statistically significantly lower detection limit for neurotoxic damage by neomycin (4.6 mg/L), if compared to permanent exposure (9.2 mg/L; Fig. 26). In contrast, for copper sulfate, there was no clear trend between 96 h permanent and 30 min pulse exposures (Fig. 27).

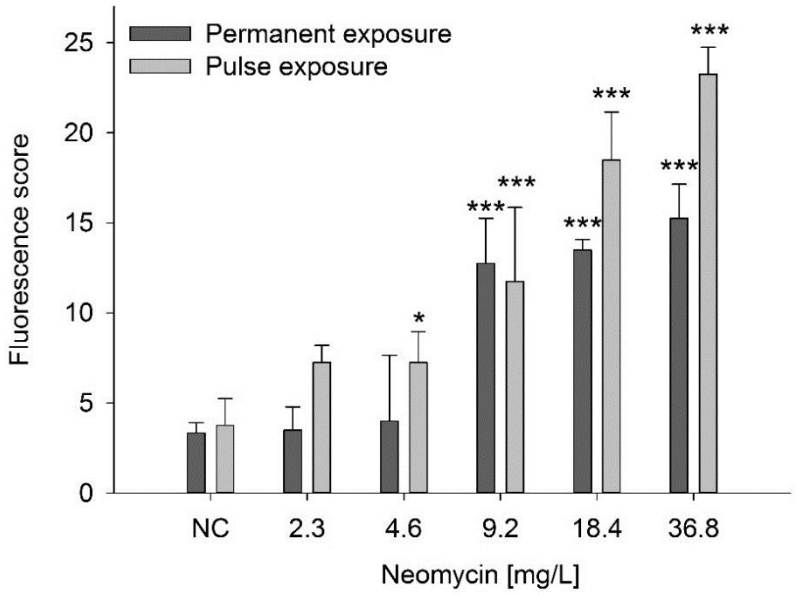

Figure 26: Fluorescence score of 9 neuromasts of 4 embryos in 3 replicates following exposure to Neomycin for 96 hpf in permanent (dark grey column) and pulse exposure (light grey column) for 30 min. Fluorescence score value is expressed as mean score per embryo (n=12), asterisks: statistically different from negative controls: * p < 0.05, ** p<0.01, *** p < 0.001 (One-way ANOVA, Dunn´s test)

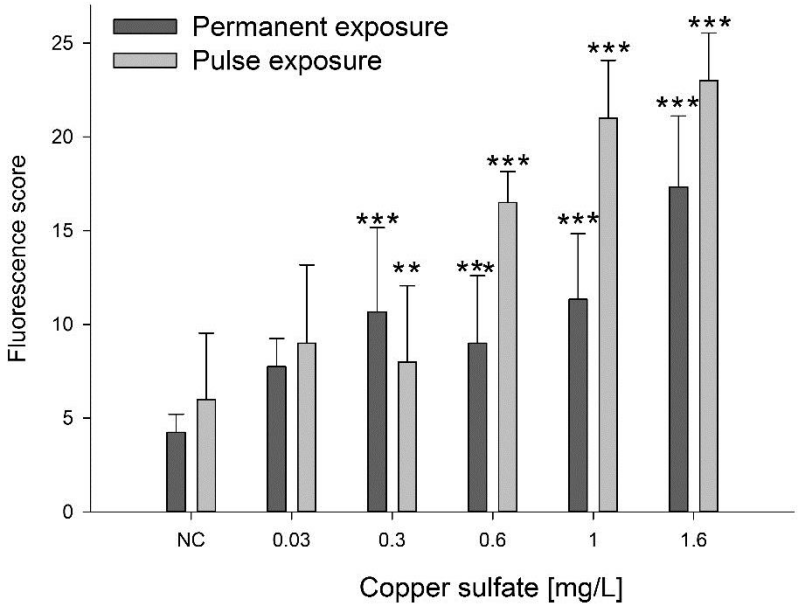

Figure 27: Fluorescence score of 9 neuromasts of 4 embryos in 3 replicates following exposure to Copper sulfate for 96 hpf in permanent (dark grey column) and pulse exposure (light grey column) for 30 min. Fluorescence score value is expressed as mean score per embryo (n=12), asterisks: statistically different from negative controls: ** $p<0.01$, *** $p < 0.001$ (One-way ANOVA, Dunn´s test)

Neuromast degeneration by selected model substances

Following 96 h permanent exposure scenarios, only 2,4-dinitrotoluene followed the trend of neomycin and copper sulfate with neuromasts of the anterior lateral line being more sensitive than those of the posterior lateral line, albeit statistically not significant (Fig. 31). In contrast, 96 h permanent exposure to dichlorvos, 4-nonylphenol and PFOS produced stronger damage in neuromasts of the posterior lateral line (Fig. 31).

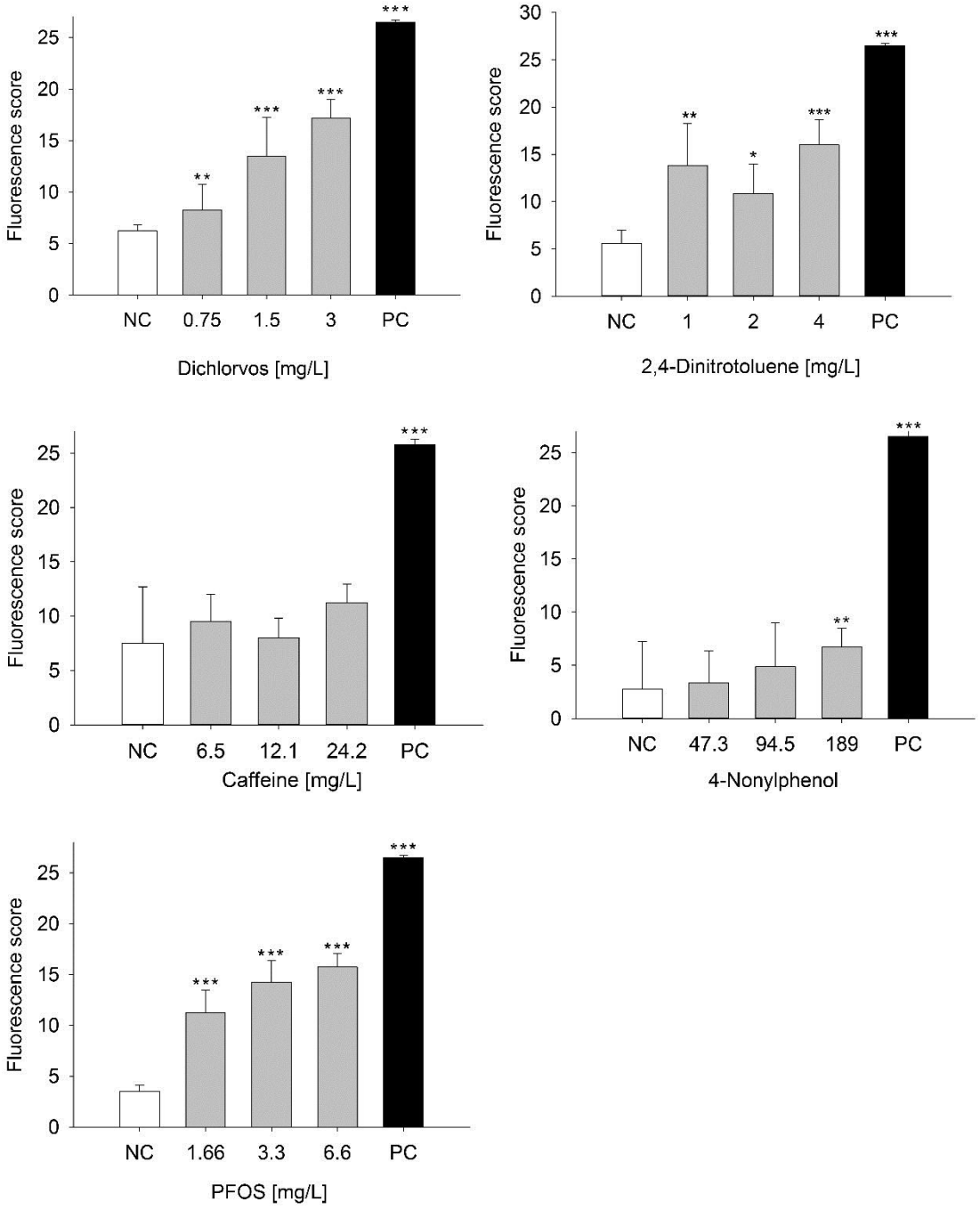

Figure 28: Fluorescence score of 9 neuromasts of 4 zebrafish (Danio rerio) embryos in 3 replicates following exposure to EC10, EC5 and EC2.5 of dichlorvos, 2,4-dinitrotoluene, caffeine, 4-nonylphenol and perfluorooctansulfonic acid (PFOS), with an additional copper sulfate concentration of 10 µM as a positive control (PC; ■) for 96 hpf. NC = negative control (□). Fluorescence score value is expressed as mean score per embryo (n = 12). Statistical difference from negative controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA, Dunn´s test).

Neuromast damage by neomycin and copper sulfate in the anterior versus the posterior lateral line by selected model substances

Given that the lateral line system of 96 hours old zebrafish embryos is composed of two line systems (Fig. 22), neuromasts of both lateral line systems were analyzed separately for effects after permanent and pulse exposure to neomycin and copper sulfate. Whereas for neomycin the lower detection limits were similar for either exposure scenario (Fig. 29), 96 h permanent exposure to neomycin consistently produced stronger damage in neuromasts of the anterior lateral line showed than in those of the posterior lateral line. In contrast, 30 min short-term exposure to neomycin gave stronger results in the neuromasts of the posterior lateral line (Fig. 29).

For copper sulfate, the lower detection limit for neuromast damage was clearly lower (0.03 mg/L) for 96 h permanent exposure than for 30 min pulse exposure (0.6 mg/L; Fig. 30). 96 h permanent exposure to copper sulfate usually resulted in similar effects in neuromasts of the anterior and the posterior lateral line systems, however, with a statistically non-significant trend to higher sensitivity in the anterior lateral line system, whereas 30 min pulse exposure consistently resulted in stronger effects in the posterior lateral line (Fig. 30).

Thus, for both neomycin and copper sulfate, anterior lateral line neuromasts show stronger effects after 96 h permanent exposure, whereas for 30 min pulse exposure posterior lateral line neuromasts proved more sensitive.

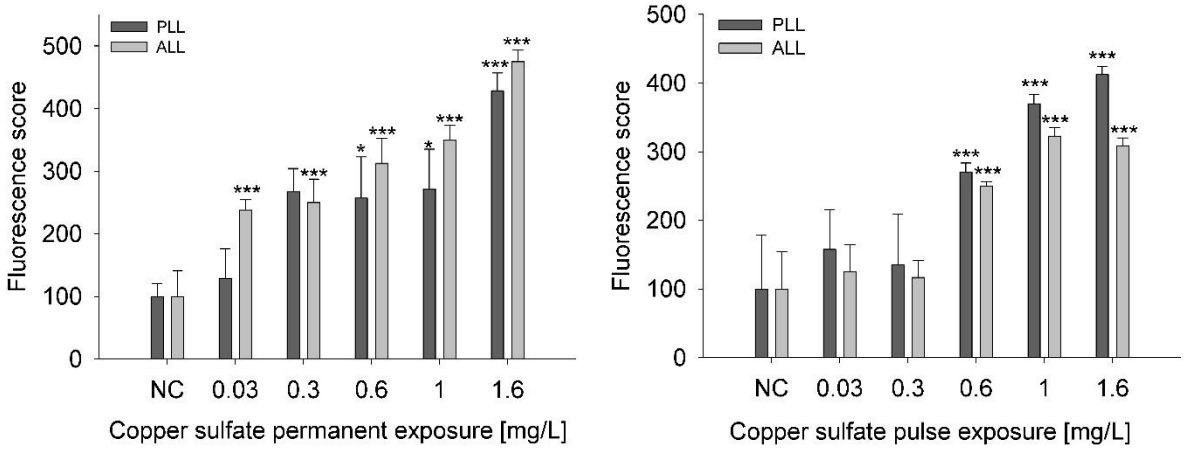

Figure 29: Left permanent right pulse; Fluorescence score of PLL (dark grey column) and ALL (light grey column) neuromasts of 4 embryos in 3 replicates following exposure to copper sulfate for 96 hpf. Fluorescence score value is expressed as mean score per embryo (n=12) and percent of negative control; * statistically different from negative controls: * $p < 0.05$ ** $p < 0{,}005$ *** $p < 0{,}001$ (One-way ANOVA, Dunn´s test)

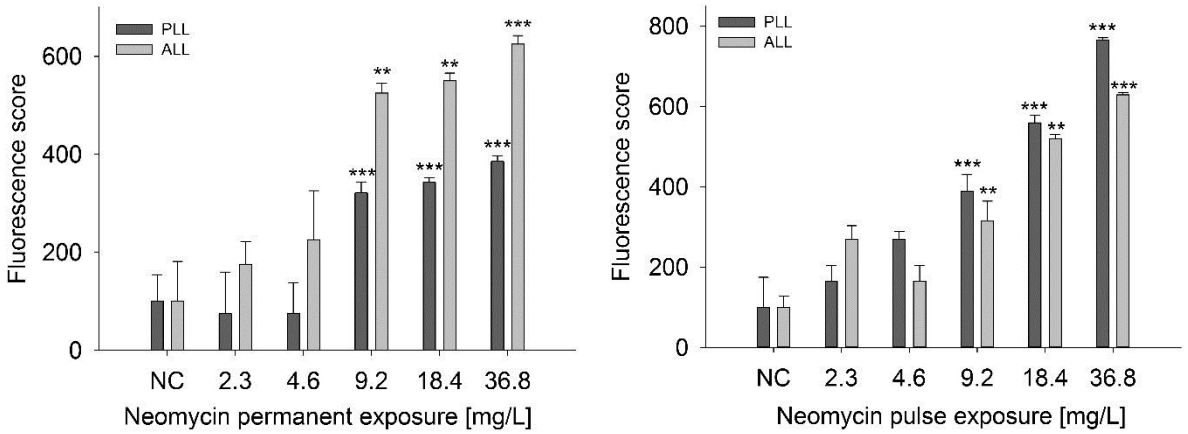

Figure 30: left permanent, right pulse; Fluorescence score of PLL (dark grey column) and ALL (light grey column) neuromasts of 4 embryos in 3 replicates following exposure to neomycin for 96 hpf. Fluorescence score value is expressed as mean score per embryo (n=12) and percent of negative control; * statistically different from negative controls: ** $p < 0{,}005$ *** $p < 0{,}001$ (One-way ANOVA, Dunn´s test)

Neuromast damage by neomycin and copper sulfate in the anterior versus the posterior lateral line by selected model substances

Following 96 h permanent exposure scenarios, only 2,4-dinitrotoluene followed the trend of neomycin and copper sulfate with neuromasts of the anterior lateral line being more sensitive than those of the posterior lateral line, albeit statistically not significant (Fig. 31). In contrast, 96 h permanent exposure to dichlorvos, 4-nonylphenol and PFOS produced stronger damage in neuromasts of the posterior lateral line (Fig. 31).

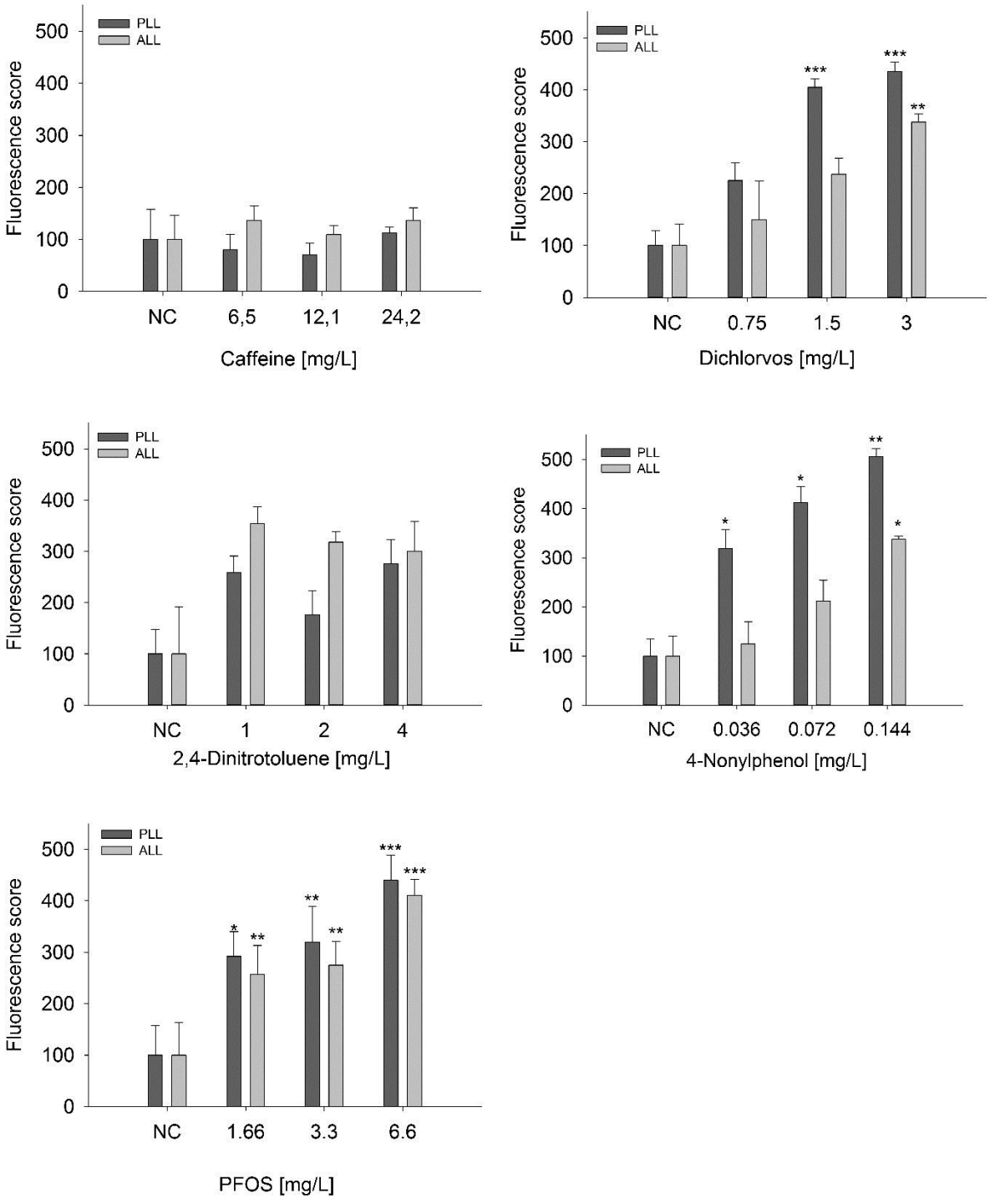

Figure 31: left permanent right pulse; Fluorescence score of PLL (dark grey column) and ALL (light grey column) neuromasts of 4 embryos in 3 replicates following exposure to EC10, EC10½ and EC10¼ of dichlorvos, PFOS, 4-NP, DNT and caffeine with an additional copper sulfate concentration of 10 µM for 96 hpf. Fluorescence score value is expressed as mean score per embryo (n=12) and percent of negative control; * statistically different from negative controls: * $p < 0.05$ ** $p < 0{,}005$ *** $p < 0{,}001$ (One-way ANOVA, Dunn´s test)

Nuclear fragmentation

Some neurotoxicants, e.g. neomycin, have been shown to induce apoptosis in lateral line neuromasts (Kaur et al. 2007, Resende and Adhikari 2009). Since apoptosis usually goes along with nuclear fragmentation, DAPI staining should be capable of imaging apoptotic hair cells. In fact, DAPI-stained fragments with a lack of DASPEI and smaller volume than normal nuclei were found after exposure to caffeine, 2,4-dinitrotoluene and 4-nonylphenol (Fig. 32 E). However, such fragments could also be identified in neuromasts of negative control (Fig. 32, left, A-D).

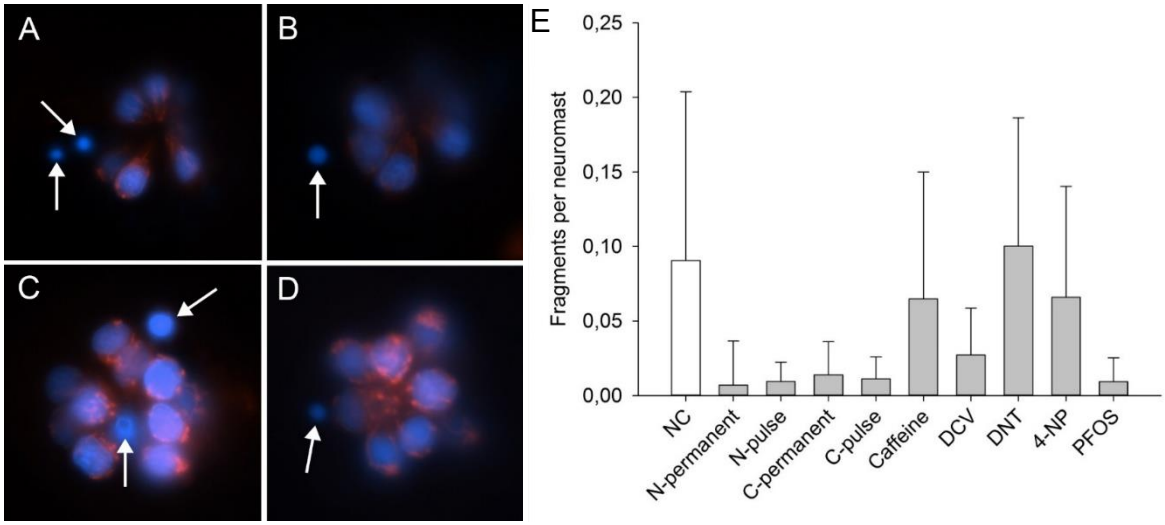

Figure 32: Nuclear fragmentation of a O2 neuromast (left) from unexposed zebrafish embryos (A-D; 96 hpf); determined fragments per neuromasts in tested substances, neomycin (N) and copper sulfate (C) in permanent exposure and pulse exposure, caffeine, dichlorvos (DCV), dinitrotoluene (DNT), 4-nonylphenol (4-NP) and perfluoroctansulfonic acid (PFOS) in permanent exposure.

3.5 Discussion

The neuromast assay as a screening tool for neurotoxicity

The lateral line system has extensively been used to screen for aminoglycoside antibiotics (Coffin et al. 2010, Fan et al. 2015, Forge 1985, Harris et al. 2003, Murakami et al. 2003, Owens et al. 2007, Van Trump et al. 2010, Wu et al. 2015) and other known ototoxic substances (Hernandez et al. 2006, Johnson et al. 2007, Olivari et al. 2008). Even though the lateral line system has already been suggested as a useful tool to screen for ototoxicity in (eco-) toxicological studies (Froehlicher et al. 2009b), a standardized method has not been established. As a consequence, there is considerable variability in in the protocols with respect to (1) the amount and position of neuromasts screened, (2) the actual endpoints recorded and (3) exposure times.

Various authors have shown that the sensitivities of neuromasts differ with maturation stage (Harris et al. 2003, Murakami et al. 2003, Santos et al. 2006) or position along the lateral line (Hernandez et al. 2006, Montalbano et al. 2014). Therefore, in the present study, nine neuromasts were chosen based on their position in the anterior and the posterior lateral line systems, on developmental stage and on the number of hair cells.

In the present study, a combination of two fluorescent dyes, DASPEI and DAPI, was used to assess the degree of hair cell damage. Although, the uptake mechanism of DASPEI into hair cells is still unknown, it has widely been used to specifically stain hair cells (Buck et al. 2012, Coffin et al. 2009, Johnson et al. 2007, Owens et al. 2008, Ton and Parng 2005). Since DASPEI has been documented to be exclusively taken up by intact hair cells, DASPEI fluorescence has been suggested to be proportional to the amount of intact hair cells within a given neuromast (Brown et al. 2011, Van Trump et al. 2010). A decrease in DASPEI fluorescence can directly be traced back to a decrease in the number and viability of hair cells, and a lack of DASPEI fluorescence can be related to a loss of kinocilia and stereocilia (Linbo et al. 2006). In contrast, to the best of our knowledge, DAPI hast only rarely been used to stain DNA in living hair cells (Olivari et al. 2008). Although the reactivity of DAPI in living or dying hair cells is under discussion in literature, DAPI consistently showed clear signal in neuromasts of the negative control in our study, and most interestingly, following exposure to ototoxicants, DAPI fluorescence showed an overall close correlation to the fading of DASPEI fluorescence (cf. Fig. 3).

Neomycin and copper as known ototoxicants

In order to calibrate the extended 4-step grading system with existing work, which usually relies on a 3-step grading (Harris et al. 2003), neomycin and copper sulfate were used as well-known ototoxic substances. In contrast to the study of Owens et al. (2009), where a significant difference between pulse and permanent exposure could not be shown, 30 min pulse exposure to neomycin resulted in a lower detection limit than 96 h permanent exposure (4.6 *versus* 9.2 mg/L). Given the differences in susceptibility between single neuromasts, this discrepancy is most likely to differential choice of neuromasts: Whereas Owens et al. (2009) used eight head neuromasts (SO1, SO2, IO1-4, M2, O2; ALL) and two trunk neuromasts, (MI1, MI2; PLL), our study used neuromasts from the anterior and posterior lateral systems in a ratio of 4 : 5, and anterior lateral line neuromasts were apparently more susceptible to neomycin under permanent exposure conditions, whereas posterior lateral line neuromasts proved more sensitive following pulse exposure (cf. Fig. 9). In fact, heterogeneity in the reaction of neuromast hair cells seems quite common in relation to their maturation stage (Harris et al. 2003, Murakami et al. 2003, Santos et al. 2006) or their localization along the lateral line system (Hernandez et al. 2006, Montalbano et al. 2014), and corresponding variability between hair cells of the inner ear has already been documented for zebrafish (Bang et al. 2001, Chang et al. 1992, Platt 1993).

Apart from differential susceptibility of hair cells, the differences between permanent and pulse exposure scenarios may be a result of ongoing regeneration of the neuromasts. In zebrafish neuromasts, hair cell regeneration is particularly effective (Cruz et al. 2015, Liang et al. 2012). (Harris et al. 2003) showed regeneration of neuromasts after neomycin exposure after 12 – 24 h. Most interestingly, hair cells undergoing maturation seem to have a neomycin-insensitive window (Santos et al. 2006), resulting in a “protected” period in hair cell development. Ongoing hair cell regeneration is likely to lead to a consistent mean number of viable hair cells and, thus, a more consistent fluorescence signal after permanent exposure than after pulse exposure. Alternatively, the selected test concentrations being non-lethal, permanent exposure is more likely

to initiate effective repair or adaptation mechanisms than 30 min pulse exposure.

For the visualization of copper sulfate ototoxicity (McNeil et al. 2014), the number of neuromasts investigated range from one or two (Hernandez et al. 2006, Linbo et al. 2009) to the entire posterior lateral line (Buck et al. 2012), and, as for neomycin, both exposure start and duration vary. In the present study, there was no difference in the lower detection limits for copper sulfate between permanent and 30 min pulse exposures, which is in line with findings by Hernandez et al. (2006) and Buck et al. (2012), but is slightly more sensitive the method used by Linbo et al. (2006). However, given that permanent exposure is more likely to occur under field conditions, the permanent exposure scenario was used in subsequent neuromast assays.

Acute toxicity a tool to assist range-finding for the neuromast assay

The fish embryo test has repeatedly been documented to be a reliable tool for acute and sublethal toxicity assessment (Belanger et al. 2013, Braunbeck et al. 2014, Lammer et al. 2009a). In risk assessment and chemical regulation, LC_{50} and, to a smaller extent, EC_{50} data have for long been used to compare the toxic potentials of chemicals (OECD 2013, Scholz et al. 2008, Scholz et al. 2013), and acute and sublethal toxicity data are used as range-finders for subsequent more-in-depth and higher-tier testing. Screening for specific modes of action at concentrations equal to or close to acutely toxic concentrations is debatable, since secondary (indirect) effects the specific endpoints are likely to react independent of the specific mode of action, be it neurotoxicity (Kais et al. 2015a) or endocrine disruption (Kloas et al. 2009, Scholz et al. 2013, Soffker and Tyler 2012, Tyler et al. 1998). For instance, given that neurons close to the epidermal surface of fish may be affected by epidermal inflammation (d'Alencon et al. 2010), unspecific inflammatory agents may equally affect epidermal and hair cells (Leite et al. 2013), thus mimicking hair cell ototoxicity. As a consequence, in order to avoid interference with effects secondary to acute toxicity, for the screening of potential neuromast toxicants, the highest test concentrations were set at EC_{10} concentrations (Kais et al. 2015a). According to experience from numerous previous studies, EC_{10} concentrations are still high enough to induce both adaptive reactions and sublethal biochemical responses (Braunbeck 1992, 1994, 1998, Kais et al. 2015a).

Neuromast damage by selected model substances

To the best of our knowledge, there are no previous studies associating caffeine, dichlorvos, 4-nonylphenol, 2,4-dinitrotoluene, perfluorooctanesulfonic acid (PFOS) with lateral line damage. Except for caffeine, all model substances produced significant effects in lateral line neuromasts in a dose-dependent manner.

As a phosphate ester pesticide, dichlorvos is a known acetylcholine esterase inhibitor (Assis et al. 2012, Di Tuoro et al. 2011, Holth and Tollefsen 2012, Kais et al. 2015a, Qujeq et al. 2012, Rath and Misra 1981, Varo et al. 2008). In fact, neuromasts have cholinergic inhibitory efferent fibers (Dambly-Chaudiere et al. 2003), which are likely to be affected by acetylcholine esterase inhibitors. Since DASPEI is exclusively taken up by intact hair cells (Brown et al. 2011, Van Trump et al. 2010), prolonged inhibition of cholinergic inhibitory fibers by dichlorvos may

easily lead to decreased hair cell viability and, thus, decreased DASPEI fluorescence.

In addition, after chronic exposure of rats, dichlorvos induced delayed neurotoxicity by impairment of mitochondrial energy metabolism (Kaur et al. 2007) and a decrease in transmembrane potential in mitochondria leading to an increased production of reactive oxygen species (ROS), apoptosis and nuclear condensation (Binukumar et al. 2010, Kaur et al. 2007). Since DASPEI preferentially stains mitochondria, decreased DASPEI fluorescence might easily indicate toxic effects in mitochondria (Chang et al. 2013). Likewise, the decline in DAPI fluorescence observed in zebrafish hair cells may well be due to the nuclear condensation shown by Binukumar et al. (2010).

4-Nonylphenol has not only been shown to function as an endocrine disruptor (Yang et al. 2015), but also to induce a broad range of neurotoxic effects in organisms (Jie et al. 2013, Kajta and Wójtowicz 2013). There is increasing evidence that endocrine disrupting chemicals such as 4-nonylphenol are generally able to induce consistent aberrations in juvenile or adult nervous systems (Frye et al. 2012, Litwa et al. 2014, Masuo and Ishido 2011, Parent et al. 2011) as well as functional disorders (Yücedağ et al. 2014). Froehlicher et al. (2009a) associated disrupted development of neuromasts with the presence of non-functional estrogen receptor (ER) subtype ERβ2-RNA in zebrafish lateral line hair cells, assuming an essential role for estrogen receptors in lateral line hair cell development and maturation. Litwa et al. (2014) documented a loss of mitochondrial membrane potential and ERα- and ERβ-specific immunofluorescence in mouse hippocampal cells by 4-nonylphenol, assuming impairment of estrogen receptor signaling due to nonylphenol exposure. Both processes would trigger alterations in DASPEI and DAPI fluorescence.

There are multiple modes of action of 4-nonylphenol, which might lead to the toxic effects seen in lateral line neuromasts. Nakazawa and Ohno (2001) linked exposure to 4-nonylphenol to an inhibition of acetylcholine-activated currents of recombinant human neuronal nicotinic acetylcholine receptors / channels. Moreover, there is increasing evidence that dopaminergic neurons are affected by 4-nonylphenol (Ishido et al. 2005, Masuo et al. 2004). In fact, both acetylcholine and dopamine receptors have been discovered in zebrafish lateral line neuromasts (Ghysen and Dambly-Chaudiere 2004), and inhibition of neuronal receptors may easily result in a functional loss by inactivation or overstimulation of neurons. The dose-dependent reduction of fluorescence intensity indicating a 4-nonylphenol-related decrease in hair cell viability can thus be directly be related to receptor inhibition, which has been associated with the induction of both neuronal apoptosis and cell proliferation (Resende and Adhikari 2009). However, since 4-nonylphenol has also been shown to induce chronic inflammation in mouse brain (Zhang et al. 2008), more unspecific mechanisms have also to be taken into consideration. Again, such processes would also be recognized as neurotoxic, even if the primary mode of action is inflammation.

Perfluorooctanesulfonic acid (PFOS) is known to induce multiple adverse effects by different modes of action in various organs and systems, e.g. developmental toxicity (Ulhaq et al. 2013a), genotoxicity (Jernbro et al. 2007), endocrine function (Han and Fang 2010, Keiter et al. 2012)

as well as immunotoxicity (Peden-Adams et al. 2008). In neurotoxicity, modes of action for PFOS still appear inconclusive (Mariussen 2012):In larval fish, PFOS disrupts normal behavior (Chen et al. 2013, Spulber et al. 2014, Ulhaq et al. 2013b) indicating effects on neural systems. This effects likely do occur by inducing oxidative stress and interference with the expression of alpha tubulin in zebrafish (Zhang et al. 2011) and mitochondria mediated apoptosis shown in mouse leydig cells (Zhang et al. 2015) and *Caenorhabditis elegans* (Chen et al. 2014b).

PFOS has also been shown to interact with the estradiol receptor in both H295R cells and zebrafish (Du et al. 2013). Benninghoff et al. (2010) suggested similar effects for PFOS as for 4-nonylphenol and Bisphenol A with respect to the activation of estradiol receptor α. Spachmo and Arukwe (2012) showed changes in ERα and ERβ transcripts after PFOS exposure in Atlantic salmon (*Salmo salar*). Finally, as suggested for 4-nonylphenol, inflammation also seems possible for PFOS. Whereas Hoff et al. (2003) failed to detect any inflammatory processes in the liver of common carp (*Cyprinus carpio*) after PFOS exposure, Fang et al. (2013) identified upregulation of genes with inflammatory function after exposure of early life stages of marine medaka (*Oryzias melastigma*).

Information about potential modes of action for 2,4-dinitrotoluene is scarce, even if neurotoxicity has been documented in beagles (Ellis et al. 1979). There is no evidence of processes similar to those described for dichlorvos, 4-nonylphenol and PFOS in the literature. Therefore, the elucidation of the mechanisms underlying the changes in DASPEI fluorescence of neuromast hair cells require further investigation.

In contrast to permanent exposure to neomycin and copper sulfate, where the ALL showed higher effect strength, neuromasts exposed to mono substances (dichlorvos, 4-nonylphenol and PFOS; Fig 11) showed higher effect strength in PLL neuromasts. Beside different exposure times, our results, therefore, indicate substance dependent sensitivities of different neuromasts and confirm the observations made by Harris et al. (2003), Montalbano et al. (2014) and Murakami et al. (2003). Since chemical interaction with neuromasts isn't sufficiently understood, it seems obvious that differences in sensitivities, induced by either exposure time or substance, lead to different results while using different neuromasts.

Nuclear fragmentation

The induction of apoptosis has been shown for copper sulfate (Wang et al. 2015), dichlorvos (Binukumar et al. 2010), neomycin (Park et al. 2014), 4-nonylphenol (Weber et al. 2002) and perfluorooctansulfonic acid (Liu et al. 2007). In order to elucidate if apoptotic processes also play a role in neuromast damage, nuclear fragments were quantified (cf. Fig. 13). Nuclear fragmentation could be observed after incubation with any concentration of the test compounds. However, apoptosis was also seen in negative controls at variable frequencies, which confirms observations by Olivari et al. (2008). Due to the high variability in the occurrence of apoptosis in various treatment groups, statistically significant differences could not be determined.

3.6 Conclusions

Substances may well affect the lateral line, at which knowledge of the specific substance targets is of high interest to interpret observed results in an (eco-)toxicological risk assessment. Some mode of actions seem to induce apoptosis or, at least, decreased cell viability either by direct interaction with mitochondria or by interaction with estrogen receptors. It could be shown, that neuromasts of the lateral line system show different susceptibilities either substance or maturation / location dependent.

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Chapter IV

4. Characterization and visualization of structural alterations in the olfactory system of zebrafish embryos

4.1 Abstract

In order to clarify the suitability of sensory organs of zebrafish embryos (*Danio rerio,* 96 hpf) for the detection of neurotoxic compounds, anti-bodies against molecular markers (Gap 43, G*αS*/olf, G*αq,* ANO2) were characterized in the olfactory epithelium. To detect alterations in marker expression as well as structural disruption in olfactory epithelium, embryos were exposed to EC_{10} concentrations of priority pollutants listed as relevant for the European water policy (2,4-dichlorphenol, 2,4-dinitrotoluene, dichlorvos), as well as to known positive controls (Triton X 100, copper sulfate, zinc sulfate) according to OECD TG 236. Two markers could be detected in the olfactory epithelium (ANO2, G*αS*/olf), whereas only G*αS*/olf could be associated to olfactory neurons. Gap 43 showed a clear signal in the olfactory bulb, whereas the Gq signal was restricted to the vascular system. Whereas G*αS*/olf as a neuronal marker for structural alteration in the olfactory epithelium reacted to zinc sulfate, but not to the mono substances. Overall, the olfactory epithelium proved suitable to visualize structural damage. However, since pure substance test did not lead to observable effects in the OE, additional substances need to be tested.

4.2 Introduction

Since anthropogenic influences are a major risk for water quality and a wide range of chemicals and pharmaceuticals attain in various ways to the drinking and surface water, public concern about harmful emerging compounds in drinking and surface water are increasing (Pal et al. 2014, Postigo and Barceló 2015). Sensory systems in fish, for example vision, olfaction and tactile sense, interact with the surrounding water environment to gather information which is necessary for survival. While a near-surface localization of these senses is advantageous for obtaining such information, this direct exposure to the surrounding, possibly polluted environment leads to an immediate risk for harm. One of these directly exposed sensory organs is the sense of smell (olfaction). Olfaction plays a crucial role in the life of fish. Predator-prey relationships, feeding, mating and social behavior as well as migration require a functional olfactory system (Hara 1975, Spehr et al. 2006). Changes in these essential behaviors are able to alter population–based processes. Therefore, an altered olfactory function can lead to ecological relevance. The use of the olfactory system in ecotoxicology has a long tradition and is based on multiple methods. Tierney et al. (2010) reviewed multiple used methods and tested substance classes as well as environmental relevant and laboratory studies and, thereby, documented the usability of the olfactory system in ecotoxicology.

Given that, the development of alternative methods in terms of the 3R principle Russell and Burch (1959) was a major task in the ToxBox Project, zebrafish embryos at a non-protected stage (96 hpf) were used. Since first olfaction-dependent behaviors can be induced at 96 hpf

(Lindsay and Vogt 2004), suggesting the presence of functionally matured neurons, this age seemed promising to detect effects in the olfactory epithelium. The main odorant-receiving units in the olfactory epithelium, and therefore, the main points of interest are the ORN`s (olfactory receptor neurons), which are divided in ciliary (cORN) and microvillus olfactory receptor neurons (mORN) as well as crypt cells (Byrd and Brunjes 1995, Doving and Lastein 2009). These receptor neurons can be distinguished by either presenting microvilli (mORN), cilia (cORN) or both (crypt cells), by the location of their soma in the olfactory epithelium (Hamdani and Døving 2007) as well as by specific molecular markers. According to Hansen and Zielinski (2005), ciliated ORCs express the G protein subunit G*α*olf/s, whereas microvillus or crypt ORCs express subunits G*α*0 and G*α*q/11. Since, there is a variety of possible molecular markers to detect specific structures or cell types in the olfactory epithelium. In the present study, a small selection of antibodies (Gap 43, G*αS*/olf, G*αq* and ANO2) was chosen after extensive literature review. (*cf. introduction)*

4.3 Material and Methods

Chemicals

For exposure experiments, zinc sulfate, copper sulfate and Triton X-100 were purchased from Merck (Schwalbach, Germany). The primary and secondary antibodies were purchased from Santa Cruz and Invitrogen (see Table 8). Additionally, anti-TMEM 16B (ANO2) was provided by courtesy of Prof. Dr. Stephan Frings from the University of Heidelberg. Stock solutions and test concentrations for exposure experiments were prepared in artificial water according to OECD TG 236 (OECD 2013; cf. Table 7). DAPI was used from a 1 mg/ml aliquot stock and diluted to 1 ng/ml. Antibodies were diluted in 1 x PBS (Table 8).

Table 7: Preparation of toxicant test solutions and test concentrations

Substance	CAS	Concentrations	
		Stock	Exposure [mg/L]
Triton X-100	9002-93-1	99.9 %	0.67 - 48.3
Zinc sulfate	7446-19-7	8 g/L	180 - 15.000
Copper sulfate	7758-98-7	8 g/L	2.49 - 39.4
2,4-Dichlorophenol	120-83-2	1000 mg/L	0.4 - 1.6
2,4-Dinitrotoluene	121-14-2	100 mg/L	1 - 4
Dichlorvos	62-73-7	800 mg/L	0.048 - 3

Fish

All adult zebrafish used for breeding were wild-type descendants of the “West-Aquarium” strain and obtained from the Aquatic Ecology and Toxicology breeding facilities at the University of Heidelberg (licensed under no. 35-9185.64/BH). Details of fish maintenance, egg production and embryo rearing have repeatedly been described in detail (Kimmel 1988, Kimmel et al. 1995b, Nagel 2002, Spence et al. 2008, Wixon 2000b) and have been updated for the purpose of the zebrafish embryo toxicity by Lammer et al. (2009b) and Sessa et al. (2008).

Zebrafish embryo toxicity tests (FET)

In order to avoid indirect effects by systemic acute toxicity, potentially mimicking neurotoxic effects, the highest test concentrations in the neurotoxicity tests were set at the EC_{10} (Chapter II p37, Table 2) for any kind of effect in zebrafish embryos. For this end, fish embryo tests were performed according to the protocol specified by OECD TG 236 (OECD 2013). In brief, embryos were exposed at latest from 1 hpf (hour(s) post-fertilization) in glass vessels, which had been pre-incubated (saturated) for at least 24 h, to a series of dilutions of the respective toxicants. After control of the fertilization success, embryos were transferred to 24-well plates (TPP Renner, Dannstadt, Germany), which had been pre-incubated with 2 ml of the test solutions per well for 24 hours prior to the test start, and kept in an incubator at 26.0 ± 1.0 °C with a 10:14 h light regime. In order to prevent evaporation or cross-contamination between the wells, the plates were sealed with self-adhesive foil (Nunc, Wiesbaden, Germany). Embryo tests were classified as valid, if the mortality in the negative control was ≤ 10 %, and the positive control (3,4-dichloroaniline) showed mortalities between 20 and 80 % Lammer et al. (2009b). Zebrafish embryos were exposed to the test substances until 96 hours post-fertilization (hpf) in a semi-static fashion, i.e. the medium was changed every 24 hours subsequent to the daily inspection of the embryos. Every 24 hours, the embryos were controlled for lethal effects according to OECD TG 236 as well as for sublethal changes using the endpoint specified by Bachmann (2002), Schulte and Nagel (1994) as well as Nagel (2002). Both lethal and sublethal effects were used for the determination of EC values.

Immunohistochemistry

4.3.1.1 Wholemount staining of zebrafish embryos

After 96 hpf exposure to either copper sulfate, zinc sulfate, Triton X-100, 2,4-dichlorophenol, 2,4-dinitrotoluene or dichlorvos, embryos were washed 3 x 10 min with artificial water. Embryos which grew up in artificial water without any treatment were used as negative controls. Embryos were immobilized in 4 °C artificial water for 15 min, according to Wilson et al. (2009) and fixed in 4 % paraformaldehyde in phosphate-buffered saline for 4 h at room temperature. After fixation, embryos were washed 3 x 10 min with phosphate-buffered saline supplemented with 0.1 % Triton X-100 (PBST) prior to permeabilization in ice-cold acetone for 7 min at -20 °C. After permeabilization, embryos were rinsed with distilled water for 5 min and washed 3 x 10 min with PBST. Embryos were incubated in blocking solution for 4 h at room temperature, with agitation, prior to incubation with the primary antibody (Table 8) in blocking solution (2 % bovine serum albumin in PBST) overnight at 4 °C. The day after, embryos were washed 5 x 10 min with PBST and incubated with the secondary antibody in 1 x PBS overnight at 4 °C. From this point on, embryos were kept in dark to prevent photo-bleaching of secondary antibodies. After additional washing, 3 x 10 min with PBST, embryos were incubated with 1 ng/L DAPI in PBS for 30 min at room temperature and washed 3 x 10 min with PBS. For microscopy, embryos were mounted in low gelling agarose at 29 °C on glass bottom culture dishes (MatTek, Ashland, USA).

4.3.1.2 Cryosectioning technique and staining of adult and embryonic zebrafish

Residual substance traces were removed by washing 3 x with artificial water. Afterwards embryos were immobilized in 4 °C artificial water for 15 min, according to Wilson et al. (2009). Adult fish were taken from breeding groups and anesthetized in buffered MS 222 (100 mg/L) for 10 min with following decapitation. Heads of adults and whole embryos were fixed in 4 % PFA in 1 x PBS for 4 h at RT. Afterwards the embryos and heads of adults were washed 3 X 10 min with 1 X PBS and then gradually incubated in a 10 % sucrose solution in 1 x PBS for 3 h at room temperature and in a 30 % sucrose solution overnight at 4°C, to prevent formation of ice crystals in following freezing steps. The embryos and heads of adults were moved into molding cups and embedded with Tissue Freezing Medium (Leica, Nussloch, Germany). Thereafter, the embryos and heads of adults were positioned for coronal sectioning using a forceps, frozen at -20°C with the Leica cryostat CM 3050S and stored in a freezer at -20°C. Coronal cryo sections of 25 µm thickness were made using the Leica cryostat CM 3050S, collected on gelatinized microscope slides, and stored in a freezer at -20°C.

For staining, cryo sections were encircled with Super PAP Pen (Daido Sangyo, Osaka, Japan) as a liquid barrier and dried at room temperature in a moistening chamber for 10 min. Afterwards, the sections were fixed with 4 % PFA for 5 min and subsequently washed 3 x 10 min in 1 x PBS. The sections were blocked with 2 % BSA in PBST for 1 h at room temperature. Thereafter, the sections were incubated for 2 hours with solutions of primary antibodies in appropriate dilutions in PBS (Table 8). The slides were washed 3 x 10 min with 1 x PBS and subsequently incubated with a 1:1000 solution of appropriate secondary antibodies (Table 8) under light-exclusion at 4°C overnight. The next morning the sections were washed 3 x 10 min in 1 x PBS and subsequently incubated with 70 µL of 1 µg/ml DAPI per section for 1 min under light-exclusion. Afterwards, the slides were washed 3 x 5 min in 1 x PBS and lastly, the cover slips were mounted with GelMount, Aqueous Mounting Medium (Sigma Aldrich) onto the slides. The slides were kept light-excluded until microscopy.

Microscopic Analysis

The immunohistochemical stainings were analyzed with a Nikon ECLIPSE 90i microscope and photographed with a Nikon DS-Ri1 camera for cryo-slides and a Nikon C1 camera for confocal laser scanning microscopy of wholemounts. Alexa Fluor 568 was activated by a yellow laser (561 nm) and emits red light (605 nm). Alexa Fluor 594 was activated by green light (519 nm) and emits orange-red light (617 nm). DAPI was excited with ultraviolet light (405 nm), and emits blue light (500 nm). A water immersion objective with a 60x magnification was used for imaging of wholemounts with scan speed set to 1/16, whereas for cryo sections, a plan oil emersion objective at 60x magnification was used.

Table 8: Antibodies and supplemental dyes.

	Concentration	Company	Abbreviation	Dilution
Primary antibodies				
Gα s/olf ; Heterotrimeric IgG (C-18) of rabbit, polyclonal	200 µg/ml	Santa Cruz: sc-383	Gα s/olf	1/200
Gα q (E-17) of rabbit polyclonal IgG	200 µg/ml	Santa Cruz: sc-393	Gα q	1/50
TMEM 16B of guinea pig polyclonal IgG		AG Frings [1]	ANO2	1/100
GAP-43 (B-5) of mouse, monoclonal	200 µg/ml	Santa Cruz: sc-17790	GAP	1/50
Secondary antibodies				
Alexa Fluor 568 donkey anti-rabbit IgG	2 mg/ml	Invitrogen, A10042	dk α rb A568	1/1000
Alexa Fluor 488 goat anti-guinea pig IgG	2 mg/ml	Molecular Probes, A11073	gt α gp A488	1/1000
Alexa Fluor 594 donkey anti-mouse pig IgG	2 mg/ml	Invitrogen, A21203	dk α ms A954	1/1000
Supplemental dye				
4′,6-Diamidin-2-phenylidon (DAPI)	1 mg/ml	Sigma-Aldrich	DAPI	1/1000

[1] provided by AG Sinnesphysiologie, Prof. Dr. Stephan Frings, University of Heidelberg

4.4 Results

Detection of molecular markers within the sensory system of adult zebrafish

4.4.1.1 Gab 43

Staining with dk α ms A954 detecting anti-Gab 43 showed a precise binding to axons of the olfactory bulb of adult zebrafish. Stained axons center in the olfactory tract, reaching towards the telencephalon (Fig. 33 B). In contrast, a lack of signal could be observed in the olfactory epithelium (Fig. 33 A, OE).

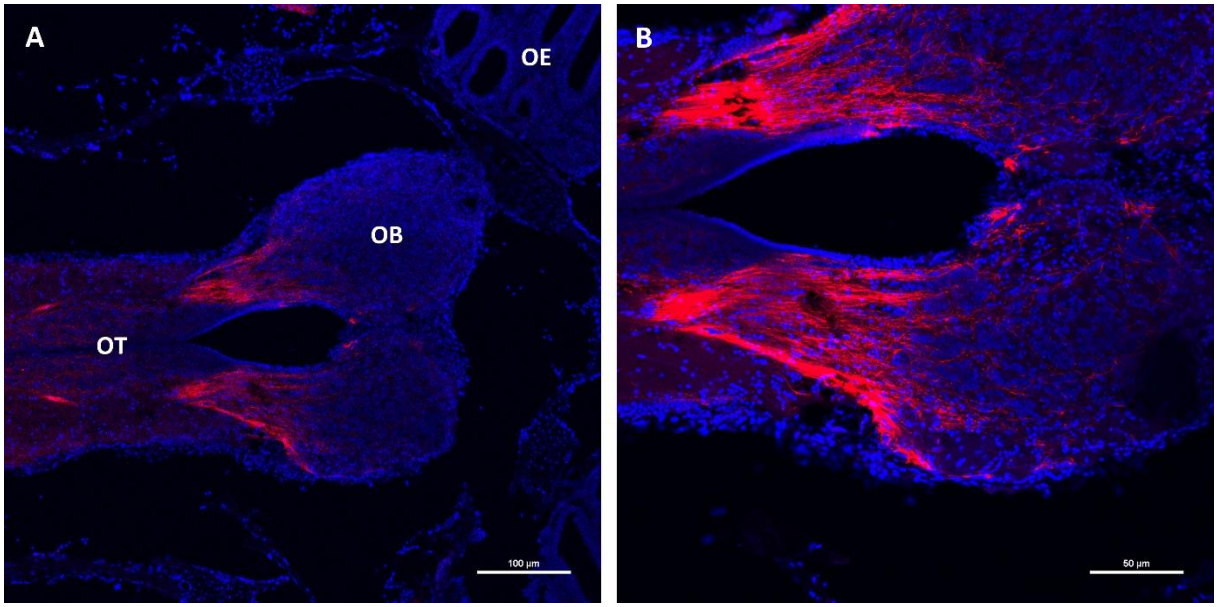

Figure 33: Staining of the olfactory epithelium and bulb of an adult zebrafish with dk α ms A954 detecting anti-Gab 43 (red) and DAPI (blue) as a counter-staining A: Olfactory rosette and bulb; B: higher magnification of the olfactory bulb with brightly stained axons in red; OE: olfactory epithelium, OB: olfactory bulb, OT: olfactory tract; left bar 100 µm, right bar 50 µm.

4.4.1.2 GαSolf

Staining of the adult olfactory epithelium with dk α rb A568 detecting anti-$G_{\alpha Solf}$, revealed a strong fluorescence signal near the center of the olfactory rosette (Fig. 34 A and B). The strongest fluorescence signal was detectable in the apical regions of the olfactory epithelium without stained nuclei, the region of the cillia of the cilliary olfactory receptor neurons (Fig. 34 D, arrow). The signal was limited to the center of the olfactory rosette, sourrounded by unstained cells in the perphery, characterized by nuclei with a elongated shape (Fig. 34 C *).

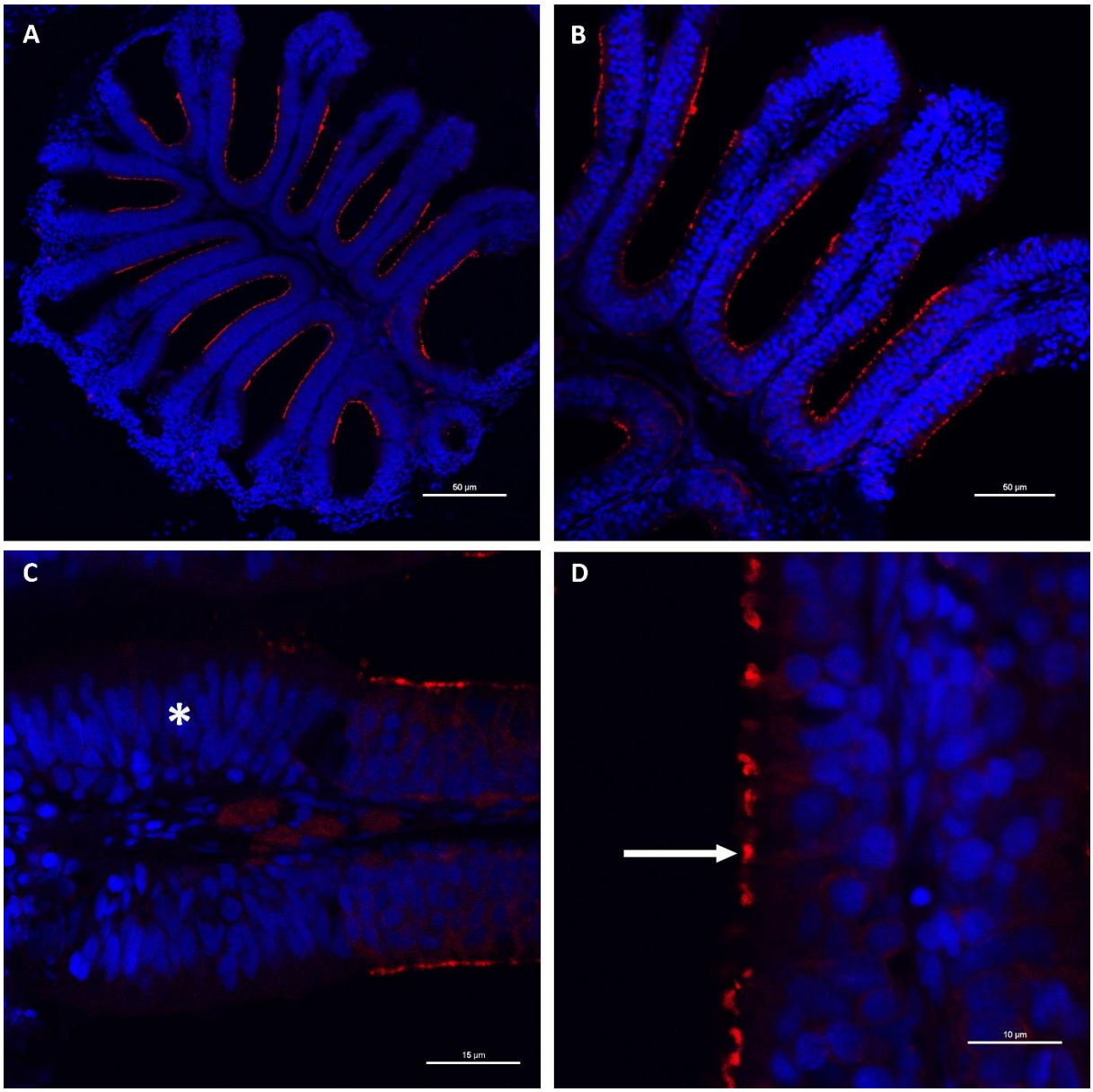

Figure 34: Staining of the olfactory epithelium of an adult zebrafish with dk α rb A568 detecting anti-GαSolf (red) and DAPI (blue) as a counter-stain; A: Olfactory rosette; B: higher magnification of the olfactory lamellae; C: close-up of the olfactory epithelium, white asterisk indicated longitudinal nuclei; D: magnification of the olfactory epithelium, white arrow indicates cilia on the surface of the olfactory epithelium. Bars: A/50 µM, B/50 µM, C/15 µM, D/10 µM.

4.4.1.3 ANO2 / GαSolf Co-stain

Staining with gt α gp A954 detecting anti-ANO2 revealed a fluorescence signal in the periphery of the olfactory rosette comprising nuclei with an elongated shape (Fig. 35 A, B). Additionally, single cells located in the top layer of the olfactory epithelium with different shape in the central part were stained as well (Fig. 35 B, arrow). Interestingly, anti-ANO2 could be detected in the photoreceptor layer of the retina (Fig. 35 C, D) as well.

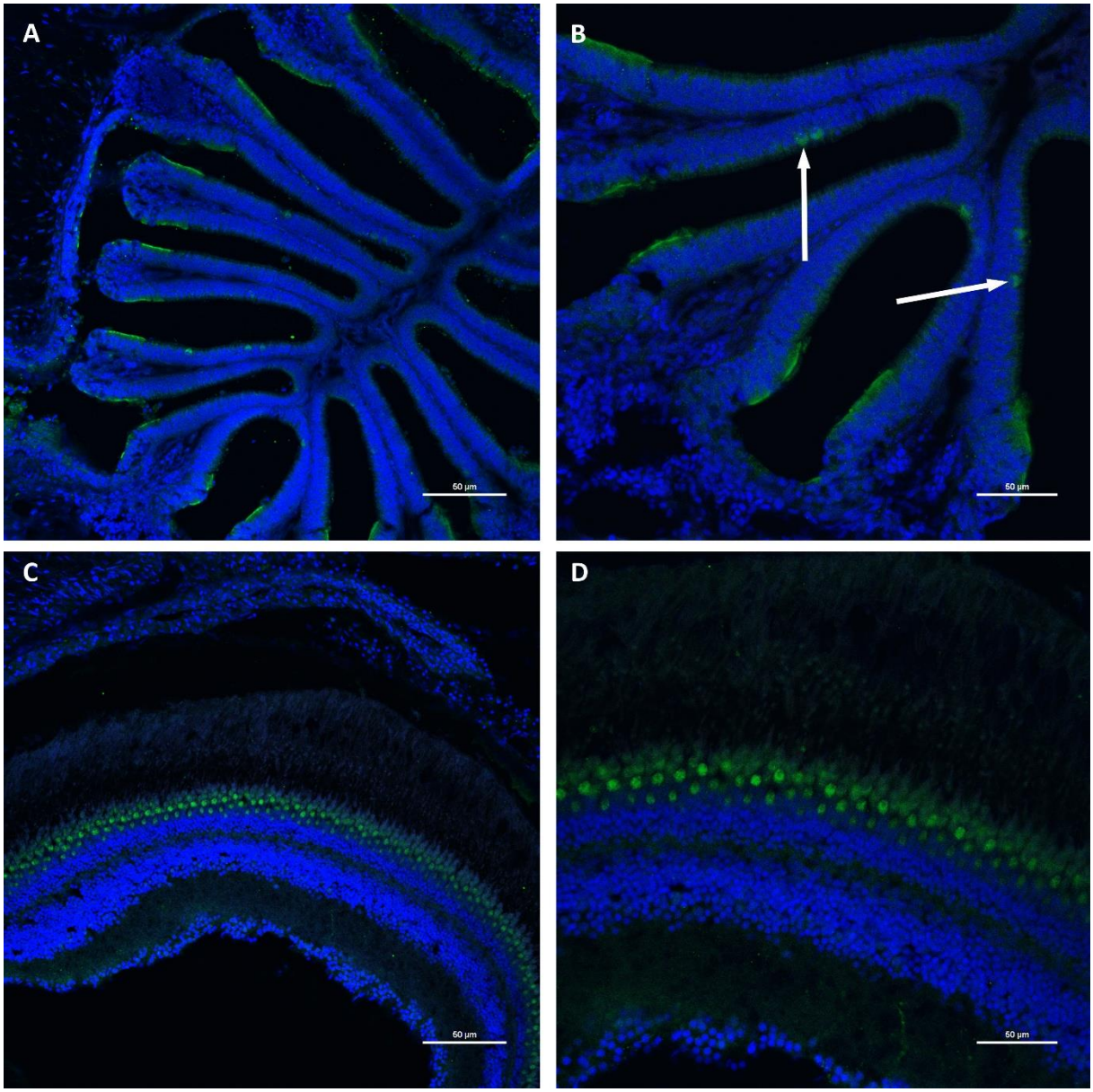

Figure 35: Staining of the olfactory epithelium (A, B) and retina (C, D) of an adult zebrafish with gt α gp A954 detecting anti-ANO2 (in green) and DAPI (in blue) as counter-staining; A, B: olfactory rosette with ANO2 signal in the periphery of the olfactory epithelium; C, D: retina with ANO2 signal in the photoreceptor layer; Bars: A/50 µM, B/50 µM, C/50 µM, D/50 µM.

Thus, ANO2 was detected in the periphery of the olfactory epithelium co-staining with $G_{\alpha Solf}$ and ANO2 should reveal differences in the expression patterns of antigens in the olfactory epithelium. ANO2 and $G_{\alpha Solf}$ showed a lack of co-localization (Fig. 36 A, B) indicating the expression of both markers in different functional regions of the olfactory epithelium.

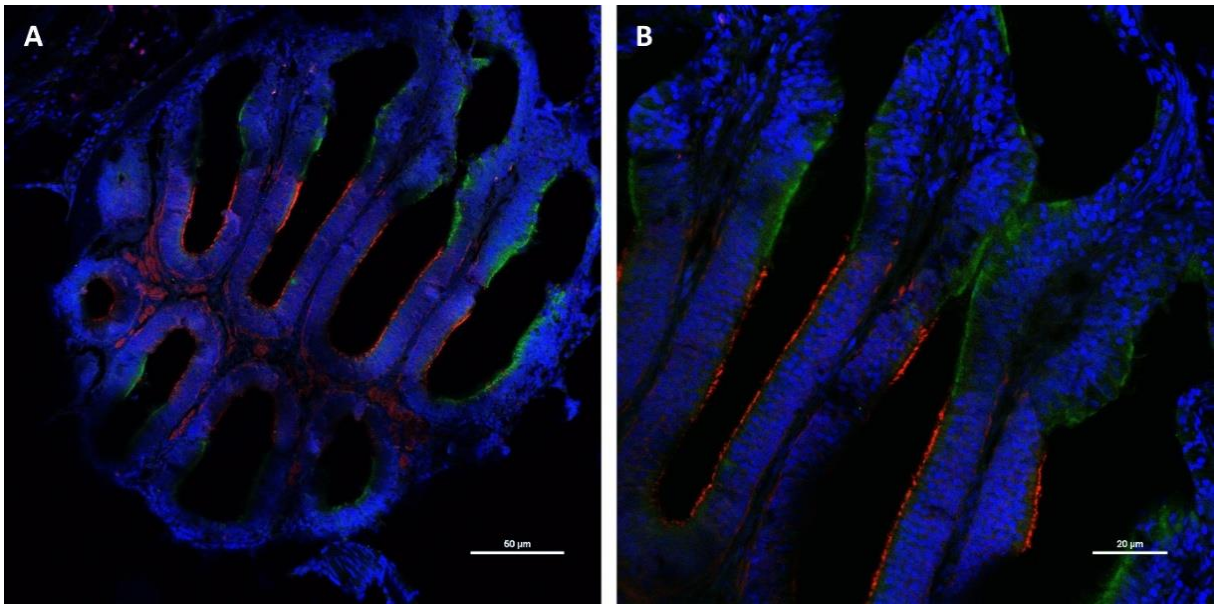

Figure 36: Staining of the olfactory epithelium of an adult zebrafish with gt α gp A954 detecting anti-ANO2 (in green), co-stained with dk α rb A568 detecting anti-GαSolf (in red) and DAPI (in blue) as counter-staining; A, olfactory rosette with ANO2 signal in the periphery of the olfactory epithelium and GαSolf near the midline raphe; B: close-up of the olfactory epithelium; Co-stain with anti-ANO2 and anti-GαSolf of the olfactory rosette showing different localizations of the antigens (A,B); Bars: A/50 µM, B/50 µM.

Application of the revealed markers to the embryo model test system

After screening for signals in the olfactory epithelium of ANO2, Gap 43 and $G_{\alpha Solf}$ and for the transfer of the detected signals of the chosen molecular marker $G_{\alpha Solf}$ to the embryo model system, embryos were stained with dk α rb A568 detecting anti-$G_{\alpha Solf}$ using wholemount and cryo techniques. In wholemount stainings, $G_{\alpha Solf}$ was detected in the cilia of the olfactory receptor neurons (Fig. 37 B, arrow) as previously shown for the adult olfactory epithelium (Fig. 37 A, arrow). However, the basal nuclear epithelium could not be visualized sufficiently with this method. Using cryo techniques, the signal in the cilia could be detected poorly, whereas the basal epithelium showed a clear structure resolution with DAPI staining. However, the cilia seemed attached to the surface of the olfactory epithelium (Fig. 36 A, arrow).

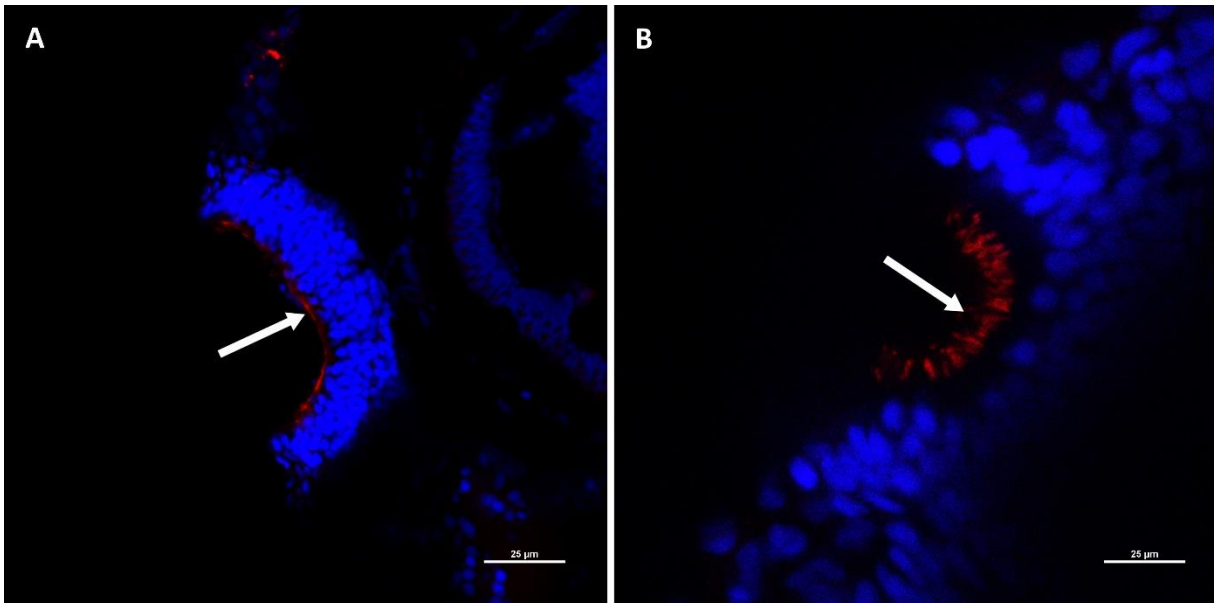

Figure 37: Staining of the olfactory epithelium of zebrafish embryos with dk α rb A568 detecting GαSolf (red) and DAPI (blue) as a counter-staining; A: olfactory epithelium of a zebrafish embryo (96 hpf) in cryo-technique; (B) in wholemount staining; white arrow indicates location of cilia; Bars: A/25 µM, B/25 µM.

Gαq as an additional marker in the olfactory epithelium of zebrafish embryos

To reveal possible effects on microvillus olfactory neurons, embryos were stained with dk α rb A568 detecting anti-$G_{\alpha q}$. Staining of the olfactory epithelium of zebrafish embryos with anti-$G_{\alpha q}$ revealed a lack of signal in the olfactory epithelium, except a slight background (Fig. 38). In contrast, a for the $G_{\alpha q}$ antibody specific binding sequence could be detected in blood vessels of the posterior cardinal veins (PCV), the dorsal aorta (DA) and the intersegmental vessels (ISV) of the blood circulating system (Fig. 39).

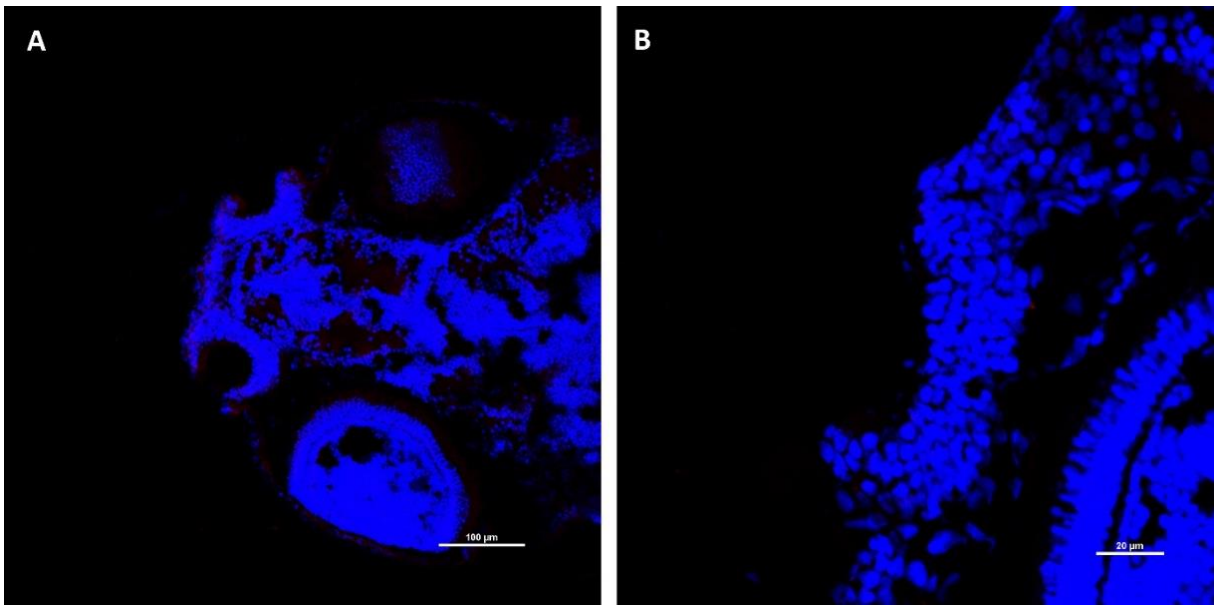

Figure 38: Cryo-staining of the olfactory epithelium of a zebrafish embryo (96 hpf) with dk α rb A568 detecting anti-G αq (in red) and DAPI (in blue) as a counter-staining; A: head of a zebrafish embryo with both olfactory pits; B: higher magnification of the olfactory epithelium; Bars: A/100 µM, B/20 µM.

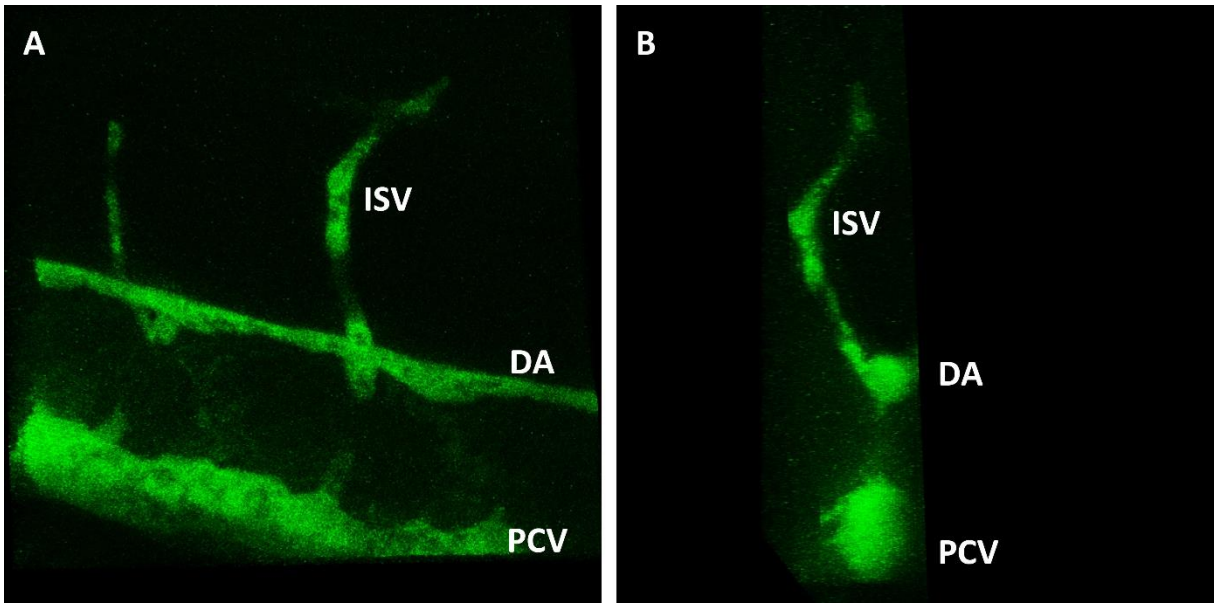

Figure 39: Wholemount staining of the blood circulating system of a zebrafish embryo (96 hpf) with dk α rb A568 detecting anti-G αq (in green) A: lateral view of the blood vessel at the trunk region. B: transversal view of blood vessels of the blood circulating system; DA: dorsal aorta, PCV: posterior cardinal vein, ISV: intersegmental vessel, after Gore (2012); 40x magnification.

Effects by known olfactory toxicants

To screen for positive controls, epithelia of zebrafish embryos exposed to copper sulfate, zinc sulfate and Triton X-100 in five concentrations were wholemount-stained with anti-$G_{\alpha Solf}$ and DAPI. With Triton X-100, the structure of the basal epithelium was disrupted without any evidence of a dose-response relationship in the tested concentrations. The cilia and the marker protein $G_{\alpha Solf}$ did not show any influence by Triton X-100 (Fig. 40).

Zink sulfate showed a dose dependent disruption of the olfactory epithelium (Fig. 41). The number of cilia decreased, whereas the signal of the marker protein $G_{\alpha Solf}$ showed no decrease in fluorescence. At the highest concentration tested (15 g/L), ciliary olfactory neurons were still detectable (Fig. 41).

For copper sulfate, the rising concentrations led to a dose dependent loss in stained cilia, with a lack of observable cilia in the highest concentration whereas the signal of the marker protein $G_{\alpha Solf}$ showed no decrease in fluorescence. The basal olfactory epithelium showed a lack of substance-specific changes in structure preservation (Fig. 42).

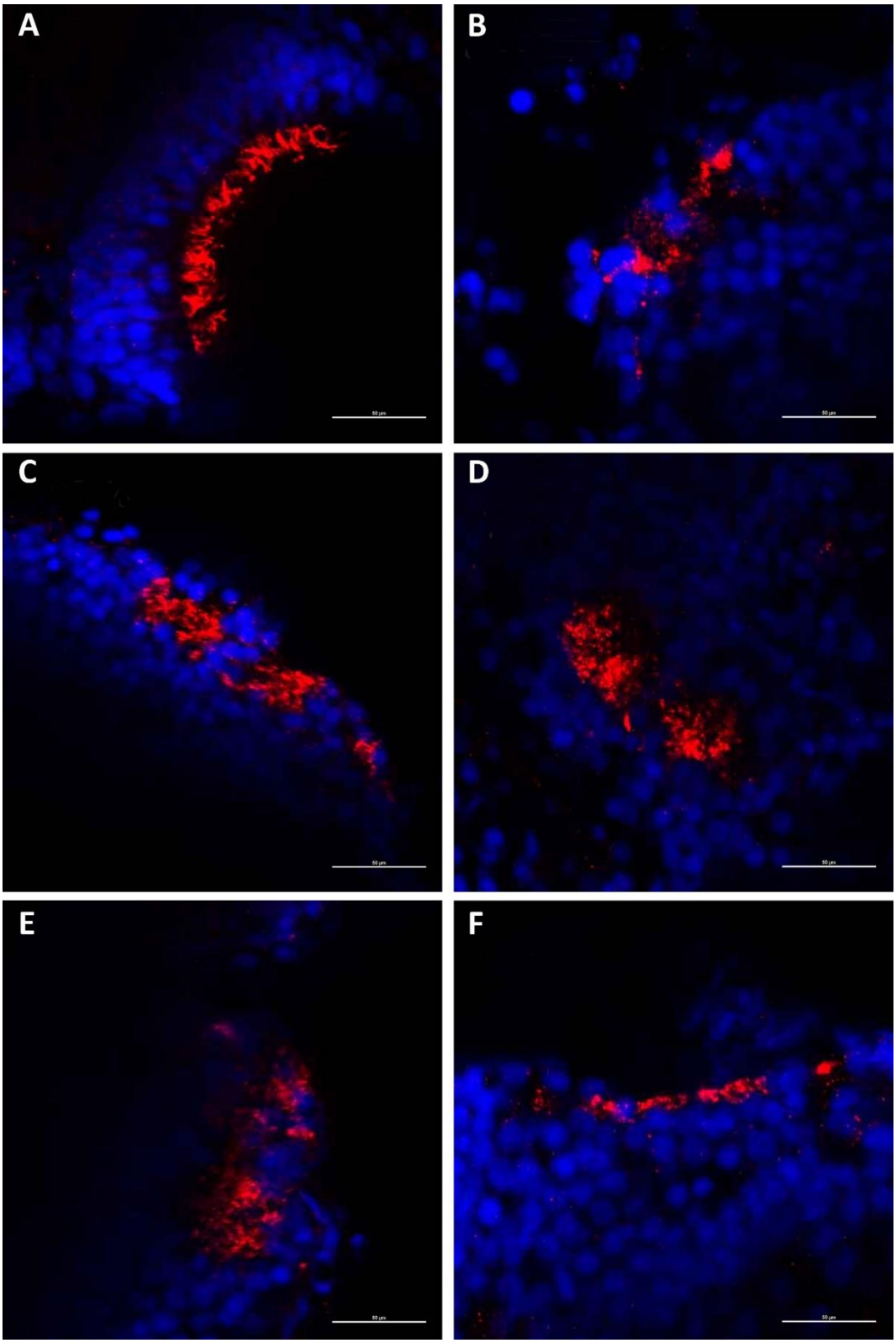

Figure 40: Olfactory epithelium of a zebrafish embryo (96 hpf) exposed to five different concentrations of Triton X-100 for 30 minutes, stained with dk α rb A568 detecting GαSolf (indicating cillia, in red) and DAPI as a counter-staining (indicating nuclear layer, in blue); A: negative control, B: 2.67mg/L, C: 5.35 mg/L, D: 10.7 mg/L, E: 21.4 mg/L and F: 48.3 mg/L Triton X-100; Bars 50 µM

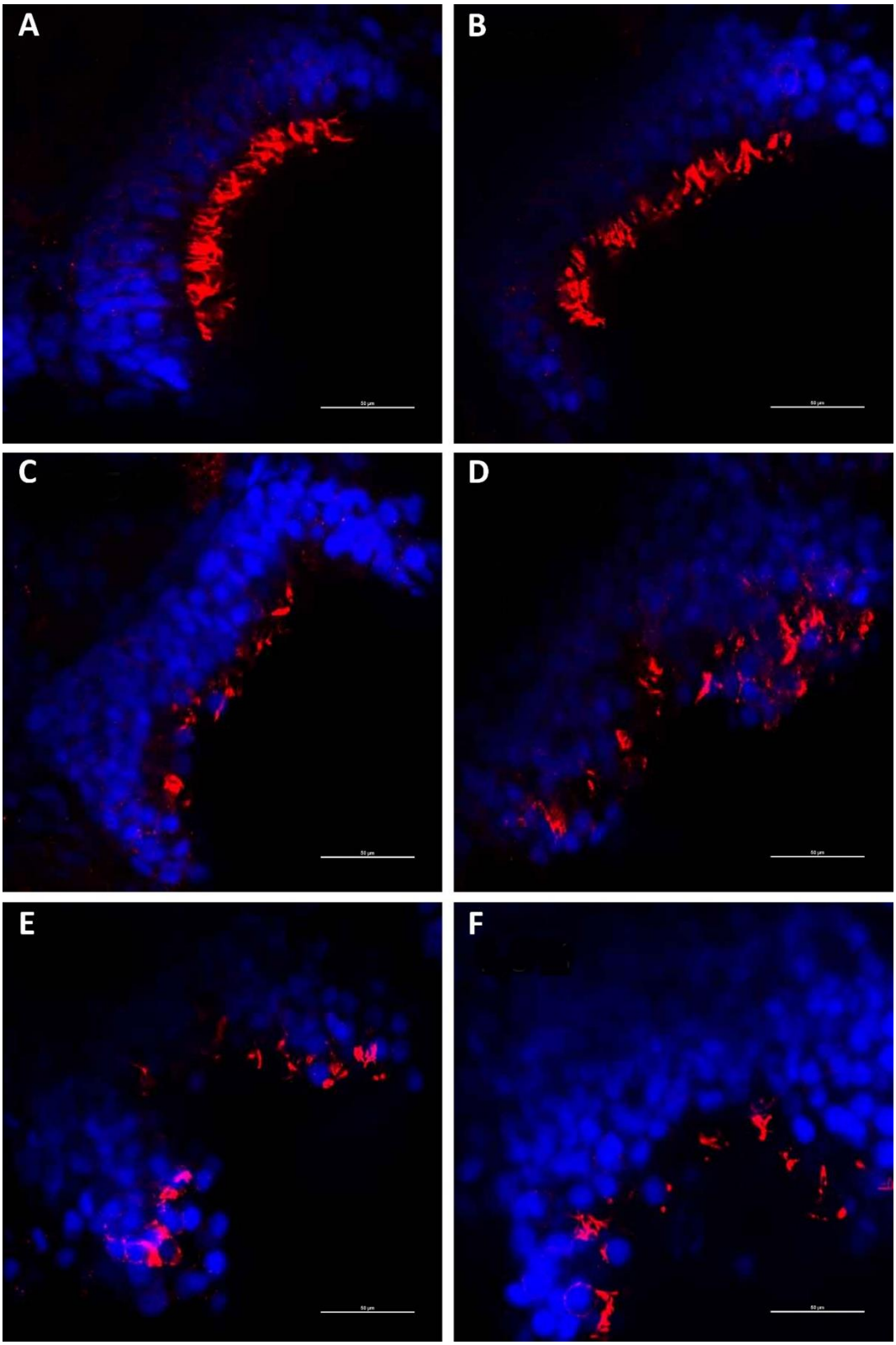

Figure 41: Olfactory epithelium of a zebrafish embryo (96 hpf) exposed to five different concentrations of zinc sulfate for 30 minutes, stained with dk α rb A568 detecting GαSolf (indicating cillia, in red) and DAPI as a counter-staining (indicating nuclear layer, in blue); A: negative control, B: 180 mg/L, C: 550 mg/L, D: 1.67 g/L, E: 5 g/L and F: 15 g/L zinc sulfate; Bars 50 µM.

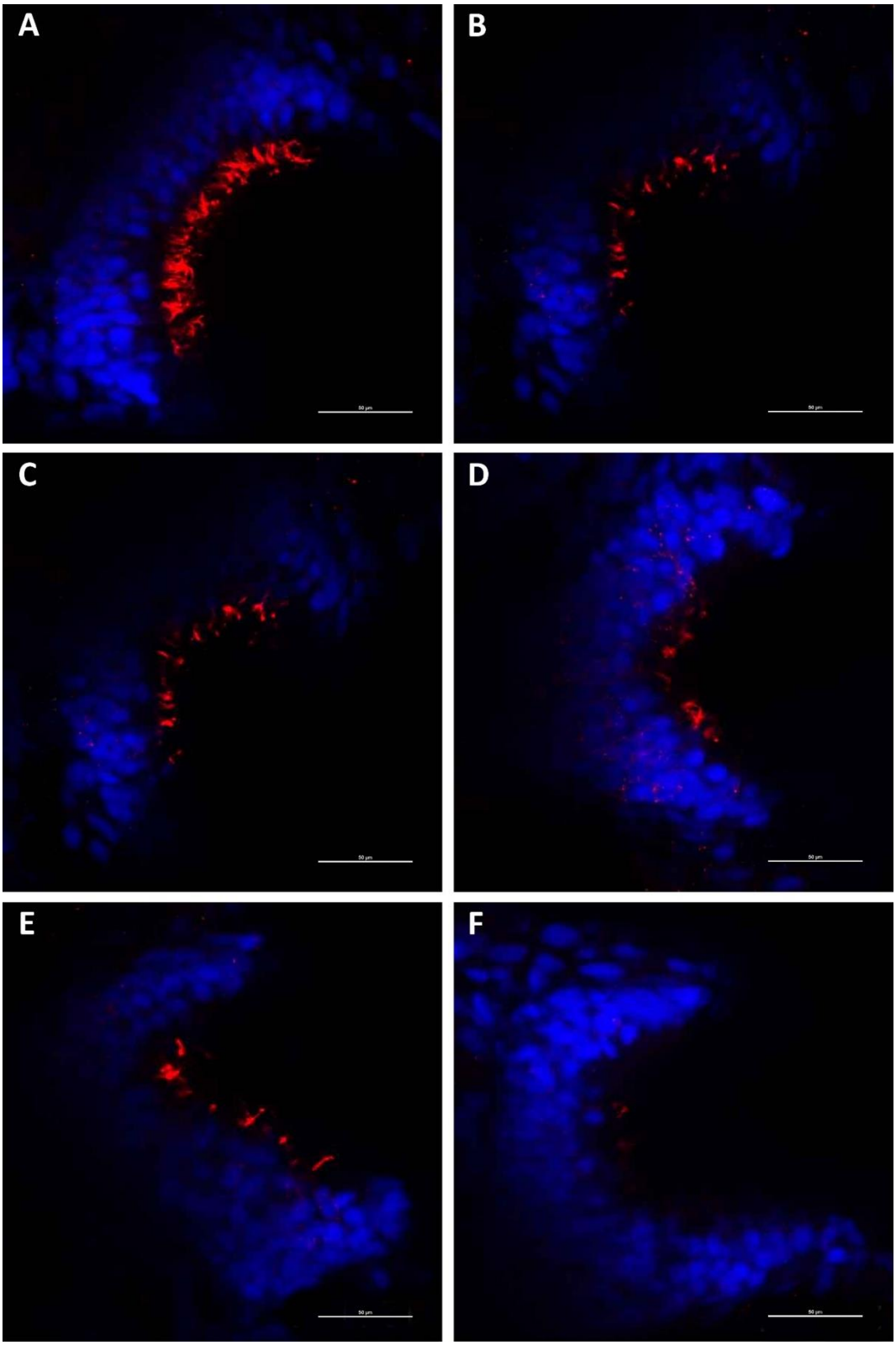

Figure 42: Olfactory epithelium of a zebrafish embryo (96 hpf) exposed to five different concentrations of copper sulfate for 30 minutes, stained with dk α rb A568 detecting GαSolf (indicating cillia, in red) and DAPI as a counter-staining (indicating nuclear layer, in blue) A: negative control, B: 2.49 mg/L, C: 4.98 mg/L, D: 9,97 mg/L, E: 19,95 mg/L and F: 39.9 mg/L copper sulfate; Bars 50 µM.

Screen for unknown olfactory toxicants

For 2,4-dinitrotoluene, 2,4-dichlorophenol and dichlorvos in all tested concentrations, the structural variability within the different concentrations and substances was too high to exclude the possibility of false-positive result. Additionally, none of the tested substances provoked either a loss of cilia or a change in the fluorescence signal of the marker protein $G_{\alpha Solf}$ in the tested concentrations (EC_{10}, Fig. 43).

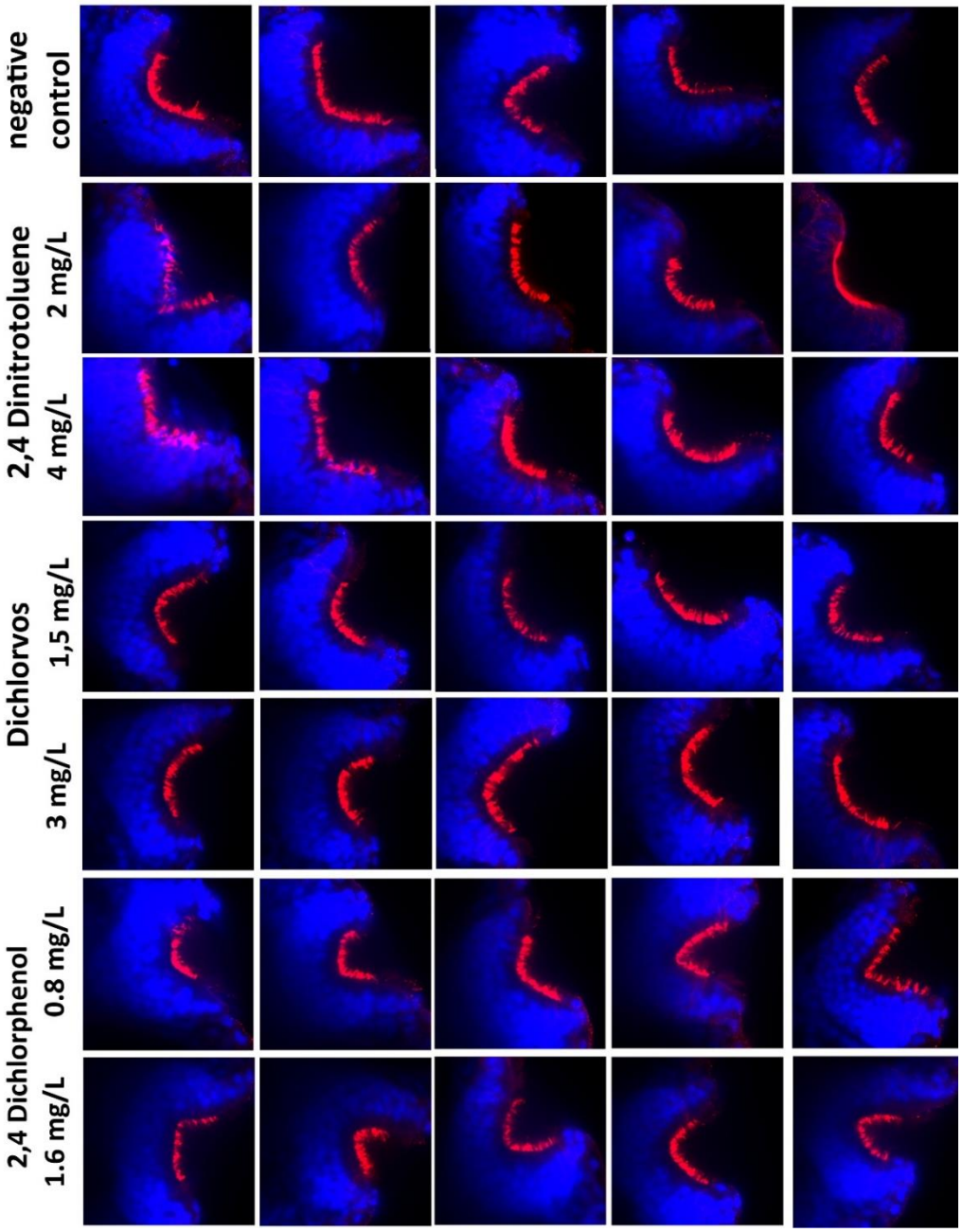

Figure 43: Olfactory epithelium of 35 zebrafish embryos (96 hpf) exposed to two different concentrations of 2,4-dinitrotoluoene (2 and 4 mg/L), dichlorvos (1.5 and 3 mg/L) and 2,4-dichlorophenol (0.8 and 1,6 mg/L) for 30 minutes, stained with dk α rb A568 detecting GαSolf (indicating cillia, in red) and DAPI as a counter-staining (indicating nuclear layer, in blue) 20x magnification.

4.5 Discussion

To identify possible molecular markers for the detection of functional and structural changes in the olfactory system of the zebrafish embryo, anti-ANO2, -Gap 43, -$G_{\alpha q}$ and -$G_{\alpha Solf}$ antibodies were tested.

Given that, Gap 43 protein of zebrafish shares 81 % homologies in amino acid sequence with Gap 43 of mouse (revealed with the Basic Local Alignment Search Tool, protein–protein Blast, NCBI), the original host of the Gap 43 antibody used, a high probability to specifically detect zebrafish Gap 43 with this antibody could be assumed. This assumption is supported by works of Bettini et al. (2006), Ebbesson and Braithwaite (2012), Ebbesson et al. (2003), Miyasaka et al. (2009) and Udvadia et al. (2001). However, in the present, study a signal of Gap 43 in the olfactory epithelium, as shown by Bettini et al. (2006), could not be detected. In the work of Bettini (2006), Gap 43 signals were detected in a few cells in the olfactory epithelium. This would lead to a rather small, and barely visible signal with fluorescent methods. Since the present thesis aimed at visualizing the structure of the olfactory epithelium, Gap 43 was excluded from the list of possible markers for this work.

The specificity of the rabbit hosted $G_{\alpha Solf}$ antibody in zebrafish had been proven in several studies by Ferrando et al. (2011), (2014), Braubach et al. (2012) and Hansen et al. (2003). These works documented specific ciliary olfactory receptor neurons in olfactory epithelia of adult channel catfish (Hansen, et al 2003), adult zebrafish (Ferrando et al. 2014) and 96 hours old zebrafish embryos (Braubach et al. 2012). In the present study, the $G_{\alpha Solf}$ signal was detected primarily in cilia, leading to a sufficient structure resolution of ciliary receptor neurons for further exposure experiments.

The ANO2 antibody has been shown to detect ANO2 in cilia of ciliated sensory neurons in mouse; the signal was restricted to the sensory area of the olfactory epithelium, but not detected in the respiratory area (Dauner 2012). In contrast, the ANO2 antibody used in the present work led to a signal in the ciliated non-sensory area of the olfactory rosette of adult zebrafish and in crypt cell within the sensory area of the olfactory epithelium. Thus, data on expression patterns and function of ANO2 in fish is rather scarce, a functional interpretation of these results appears difficult. For a structural observation of the sensory epithelium in fish, the ANO2 antibody proved not suitable for further experiments.

Anti-$G_{\alpha q}$ has been suggested to detect the $G_{\alpha q}$ protein specifically expressed in microvillous olfactory receptor neurons in catfish (Hansen et al. 2003). Hansen detected $G_{\alpha q}$ in a small band in the olfactory epithelium. In contrast, further works of Braubach et al. (2012) and Ferrando et al. (2011) failed to confirm these observations. The results in the present study in zebrafish embryos support the results of Braubach and Ferrando in adult zebrafish and led to the assumption of a possible species-specific difference in the detection sequence of the expressed antigen. Since $G_{\alpha q}$ was suggested to mediate signaling in endothelial cells of fish (Clouthier et al. 2010) and mouse (Kedzierski and Yanagisawa 2001), a more detailed characterization of $G_{\alpha q}$ in

zebrafish has to be undertaken in further studies. Thus, for the use as a marker to stain specifically microvillous olfactory receptor neurons, $G_{\alpha q}$ proved not suitable for further experiments.

After screening for possible specific markers, anti-$G_{\alpha Solf}$ was used to stain and detect specifically ciliary olfactory receptor neurons of 96 hours old zebrafish embryos. Staining revealed a specific signal in cilia of the olfactory epithelium of zebrafish embryos, comparable to the presentability of cilia in the adult olfactory epithelium. These result support data by Ferrando et al. (2014). After transfer of the adult anti-$G_{\alpha Solf}$ staining to zebrafish embryos, embryos were exposed to three different substances, Triton X-100, zinc sulfate and copper sulfate, to characterize upcoming effects in the ciliary olfactory sensory neurons. Triton X-100 led to a disrupted gross morphology of the olfactory pit, but sparing ciliary olfactory neurons. Paskin et al. (2011) revealed effects on olfactory neurons and olfactory bulb after chronic treatment of adult zebrafish to 0.7 % Triton X-100, directly applicated to the cavity of olfactory pit. In contrast to the here shown result, he showed a nearly complete loss of cilia in the olfactory epithelium. If compared to results of Parskin, Triton X-100 was used in concentrations of 0.025 – 0.4 % in a 30 minutes exposure scenario. It can be assumed that the difference in concentration led to a decreased effect on cilia in the olfactory epithelium.

Zinc sulfate had a negligible effect at concentrations up to 15 g/L in a 30 min exposure scenario. In contrast, Cancalon (1982) showed a loss of olfactory receptor cells of catfish after exposure to zinc sulfate above 30 mM (~4.8 g/L). Degeneration started within a few hours and reached 20 % of remaining neurons at day 4 after exposure. Compared to own results, the concentrations were similar, but differences in exposure time had an influence on the effect on ciliary olfactory neurons. Stronger effects on ciliary olfactory neurons after an increase in observation time above 30 minutes are therefore possible.

For copper sulfate, effects on ciliary olfactory neurons from 2.5 mg/L support result shown by Bettini et al. (2006) who illustrated effects by copper sulfate at doses ~15 µg/L. Additional effects for copper on the structure and functionality of the olfactory system have been shown by Baldwin et al. (2003), Baldwin et al. (2011), Sandahl et al. (2006), Moran et al. (1992); Julliard et al. (1996) and Hansen and Zeiske (1998), however, with variable effect thresholds. Lowest thresholds were about 1000 times lower than in here presented experiments, indicating a higher sensitivity of EOG (electroolfactogram) recordings and ultrastructural studies. EOG recordings show effects on a functional level by detecting field potentials by channel activations. Ultrastructural studies possess the possibility to detect subcellular changes. Nevertheless, anti-$G\alpha_{Solf}$ should be able to show effects at higher doses while testing mono substances.

For 2,4-dinitrolouene, dichlorvos and 2,4-dichorophenol a lack of effect could be shown. To the best of knowledge, 2,4-dinitrolouene, dichlorvos and 2,4-dichorophenol have not been tested yet with respect to olfactory effects. Other organophosphates such as chlorpyrifos (Maryoung et al. 2015, Tilton et al. 2011) showed an effect on olfactory neuronal function, determined *via* EOG measurement, at 0.5 µg/L with an additionally altered marker expression. This concentration is to the results of the present study (0.75 – 3 mg/L). Since assuming EOG as more sensitive when compared to gross morphological effects, an effect for dichlorvos in olfactory function at a molecular level cannot be excluded. Thus, the here used method to detect morphological effects in the olfactory epithelium of zebrafish embryos by cell specific antibody staining has shown to be useful, albeit less sensitive if compared to molecular methods.

4.6 Conclusion

Since it has been shown that the olfactory system expresses detectable molecular markers, the OE has shown to be suitable to visualize olfactory as well as non-olfactory structures. However, since pure substance tests did not lead to effects in the OE, additional tests have to be conducted to prove suitability of the test system.

Chapter V:

5. Characterization and visualization of structural alterations in the retinal layer arrangement of zebrafish embryos

5.1 Abstract

In order to clarify the suitability of sensory organs of zebrafish embryos (*Danio rerio,* 96 hpf) to detect neurotoxic effects induced by emerging anthropogenic pollutants, histopathological alterations in the retinal layer arrangement were used as a possible endpoint. Cryo sectioning with paraformaldehyde autofluorescence and DAPI staining, paraffin based slicing with rhodamin B and DAPI staining and paraffin-based slicing with classical hematoxilin/eosin staining (HE) were validated with respect to adequate structure visualization. To detect alterations in layer arrangement, embryos were exposed to EC_{10} concentrations of priority pollutants listed as relevant for the European water policy (amidotricoic acid, caffeine, 2,4-dichlorophenol, 2,4-dinitrotoluene, dichlorvos, 4-nonylphenole, perfluorooctanoic acid and perfluorooctansulfonic acid) following OECD TG 236. Cryo sectioning led to a loss of structure preservation and showed not suitable for the use in a test battery. Paraffin-based rhodamin B and DAPI staining led to a loss in structure resolution and cell discrimination and showed also not suitable for further testing. Paraffin-based sectioning with classical HE staining has shown the best structure resolution and preservation. However, none of the tested substances induced alterations in the layer arrangement of the retina at an EC_{10} concentration. Therefore, the test system is basically able to show effects on the retinal layer arrangement of zebrafish embryos, but is rather insensitive.

5.2 Introduction

The sense of vision plays a central role in the life of fish. Since the vertebrate retina is organized into defined layers, including three nuclear and two plexiform layers (Dowling 1987), it can be assumed to be suitable to detect layer abnormalities. At 72 hpf, all cell types are already present, correctly arranged, and the retina becomes completely functional. Sensory motor response starts soon after, as evidenced by visually evoked startle response at 68–79 hpf and tracking eye movements at 73–80 hpf (Easter and Nicola 1996). Since the retinal layers develop at 50 hpf (Schmitt and Dowling 1994), histopathological effects in retinal layer arrangement can be recorded from this point on. In recent years, the detection of effects in the visual system of fish has been frequently undertaken by use of a histopathological approach (Huang et al. 2013, Kim et al. 2013, Mecklenburg and Schraermeyer 2007, Mela et al. 2012, Triebskorn et al. 1994, Wang et al. 2012). For example, simple histopathology has shown to detect changes in eye pigmentation, induced by estrogens in a transgenetic medaka (*Oryzias latipes*) model (Lee et al. 2012), as well as changes in the optic tectum of the snakehead fish (C*hanna punctatus)* after being exposed to chlorpyrifos (Mishra and Devi 2014). Ultrastructural observations have shown to detect effects of PCB`s (Wang et al. 2012) and methylmercury (Mela et al. 2012) on neurons of the retinal photoreceptor layer. Since substance dependent alterations in the retina, observed

by histological methods, have a great scientific background, the present study used a histo-pathological approach to detect neurotoxic effects in the retinal layer arrangement indicated by structural alterations. Therefore, three different staining and preparation approaches have been verified in terms of usability, tissue preservation and structural resolution:

(1) Cryo-slicing technique with PFA autofluorescence and DAPI counter-staining.

(2) Paraffin based slicing with rhodamin B and DAPI counter-staining.

(3) Paraffin based slicing with classical hematoxilin / eosin (H/E) staining.

5.3 Material and Methods

Chemicals

For staining, rhodamin B (CAS: 81-88-9), DAPI (2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, CAS: 28718-90-3), hematoxilin and eosin were purchased from Sigma Aldrich (Dreisenhof, Germany). Rhodamin B was used from a 90 mg/L stock solution. Hematoxilin and eosin was prepared and used according to Romeis - mikroskopische Technik (Romeis 2010). DAPI was used from a 1 mg/ml aliquot stock and diluted to 1 ng/ml for staining. For fixation, paraformaldehyde (PFA) as well as staining by autofluorescence and ingredients for Davidson's fixative (Table 10) were purchased from Sigma-Aldrich in highest possible grade. For exposure experiments, amidotrizoic acid, caffeine, dichlorvos, 2,4-dichlorophenol, 2,4-dinitrotoluene, 4-nonylphenol, perfluorooctanoic acid, perfluorooctanesulfonic acid were used. For test concentrations in the fish embryo tests and neuromast assays, see Table 9. All toxicant stock solutions were prepared in artificial water according to OECD TG 236 (OECD 2013).

Fish

All adult zebrafish used for breeding were wild-type descendants of the "West-aquarium" strain and obtained from the Aquatic Ecology and Toxicology breeding facilities at the University of Heidelberg (licensed under no. 35-9185.64/BH). Details of fish maintenance, egg production and embryo rearing have repeatedly been described in detail (Kimmel et al. 1988, 1995, Nagel 2002, Spence et al. 2006, Wixon (2000) and have been updated for the purpose of the zebrafish embryo toxicity test by Lammer et al. (2009) and Sessa et al. (2008).

Zebrafish embryo toxicity tests (FET)

In order to avoid indirect effects by systemic acute toxicity potentially mimicking neurotoxic effects, the highest test concentrations in the neurotoxicity tests were set at the EC_{10} for any kind of effect in zebrafish embryos. For this end, fish embryo tests were performed according to the protocol specified by OECD TG 236 (OECD 2013). In brief, embryos were exposed at latest from 1 hpf (hour(s) post-fertilization) in glass vessels, which had been pre-incubated (saturated) for at least 24 h, to a series of dilutions of the respective toxicants. After control of the fertilization success, embryos were transferred to 24-well plates (TPP Renner, Dannstadt, Germany), which had been pre-incubated with 2 ml of the test solutions per well for 24 hours prior to the test start, and kept in an incubator at 26.0 ± 1.0 °C with a 10:14 h light regime. In order to prevent evaporation or cross-contamination between the wells, the plates were sealed with

self-adhesive foil (Nunc, Wiesbaden, Germany). Embryo tests were classified as valid, if the mortality in the negative control was ≤ 10 %, and the positive control (3,4-dichloroaniline) showed mortalities between 20 and 80 % (Lammer et al. 2009b). All fish embryo tests were run in in three independent replicates.

Table 9: Substances and solutions

Substance in [mg/L]	CAS	Stock	Exposure conc.
Amidotrizoic acid	117-96-4	8.9	2.25 - 8.9
Caffeine	58-08-2	1000	6.05 - 24.2
2,4-Dichlorophenol	120-83-2	1000	0.4 - 1.6
2,4-Dinitrotoluene	121-14-2	100	1 - 4
Dichlorvos	62-73-7	800	0.75 - 3
4-Nonylphenol	84852-15-3	6	0.047 - 0.189
Perfluorooctanoic acid	335-67-1	1000	112.5 - 550
Perfluorooctansulfonic acid	1763-23-1	~40 % #	1.66 - 6.6

#factory-provided

Zebrafish embryos were exposed to the test substances until 96 hours post-fertilization (hpf) in a semi-static fashion, i.e. the medium was changed every 24 hours subsequent to the daily inspection of the embryos. Every 24 hours, the embryos were controlled for lethal effects according to OECD TG 236 as well as for sublethal changes using the endpoint specified by Bachmann (2002), Schulte and Nagel (1994) as well as Nagel (2002). Both lethal and sublethal effects were used for the determination of EC values (cf. Chapter II, Results). Screening for staining methods and histopathological effects were run in 3 independent replicates with 20 embryos each. After exposure to according substances for 96 hpf, embryos were transferred to 1.5 ml Eppendorf tubes and rinsed three times in artificial water for 10 minutes each as well as anesthetized and euthanized by rapid cooling on ice (Wilson et al. 2009). After heart beat arrest, embryos were fixed in 500 µl 4 % paraformaldehyde (PFA) or Davidson's fixative (Table 10), respectively, over night at 7° C.

Table 10: Composition of Davidson`s fixative

Volume	**Substance**
220 ml	37 % formaldehyde
115ml	99 % acetic acid
330ml	95 % ethanol
335ml	H_2O bidest.

Cryo sectioningtechnique with PFA fixed embryos

After fixation with PFA, the embryos were washed three times with 1 x PBS and incubated, firstly with a 10 % sucrose solution in 1 x PBS for 3 h at room temperature and secondly with a 30 % sucrose solution at 4°C overnight, to prevent formation of ice crystals. The embryos (n=20) were moved into molding cups and embedded with Tissue Freezing Medium (Leica, Biosystems, Nussloch, Germany). Thereafter, the embryos were positioned for coronal sectioning using a forceps and afterwards they were frozen at -20°C with the Leica cryostat CM 3050S (Leica, Biosystems) and stored in a freezer at -20°C. Coronal cryo sections of 25 µm thickness were made using the Leica cryostat CM 3050S. The cryo sections were collected on gelatinized microscope slides, and stored in a freezer at -20°C.

For slicing, cryo sections were encircled with Super PAP Pen (Daido Sangyo, Osaka, Japan) and dried at room temperature in a moistening chamber for 10 min. Afterwards, the sections were additionally fixed on the slide with 4 % PFA for 5 min and subsequently washed three times with 1 x PBS for 10 min each. The sections were blocked with 2 % BSA in PBST for 1 h at room temperature. Thereafter, sections were incubated in 70 µL 1 ng/ml DAPI for 1 min, then washed three times for 10 minutes each and lastly mounted with GelMount Aqueous Mounting Medium (Sigma Aldrich) onto the slides. The slides were kept light-excluded until microscopy.

Paraffin based slicing and H/E staining

After fixation with Davidson's fixative, embryos were rinsed three times in 1 x PBS and embedded in agarose molds according to Sabaliauskas et al. (2006) to embed 20 embryos at ones. Diverging from Sabaliauskas, the amount of agarose was reduced while casting the molds from 1218 µl to 788 µl 1 % agarose, ensuring uniform tissue infiltration in the steps thereafter. After casting, the molds were dried for 45 minutes bevor embedding. Embryos were transferred from Eppendorf tubes to the molds with glass pipettes and orientated with Dumont forceps no. 5. After orientation, embryos were covered with agarose, dried for 45 minutes, transferred in histo cassettes (Leica Microsystems, Wetzlar, Germany) and stored in 70 % ethanol overnight.

On the next day, agarose molds were processed with a tissue infiltrating device, Leica TP 1020 (Leica Microsystems, Wetzlar, Germany), in a 48 h program using Histoplast. After tissue infiltration, agarose molds were embedded in paraffin with the Leica EG 1140 H (Wetzlar, Germany) embedding station and stored at room temperature until further processing (slicing and staining).

Slicing was processed on a slicing microtome HN 40 (Reichert-Jung, Heidelberg, Deutschland) with 4 µm thickness. After slicing, each slice was transferred to a water bath heated to 40 °C to stretch the slices bevor transferring to glycerol albumin (Serva Electrophoresis GmbH, Heidelberg) coated object slides. Object slides were dried in an incubator at 38 °C overnight.

Staining was processed with a widely used hematoxylin / eosine (H/E) staining (Romeis - mikroskopische technik; Romeis (2010)), whereas hematoxylin stains alkaline structures like nuclei in blue and eosin, acidic structures like cytoplasm and connective tissue in red. Thus, the

dyes are water soluble, the slides were deparaffinated with X-Tra Solv and rehydrated with a rising ethanol series. Afterwards, slides were stained with hemalaun and contrasted in tab water, stained in eosine, washed, dehydrated, resolved in x-tra-solv and fixed under cover slips with X-TRA-Kitt (Medite Histotechnic, Burgdorf, Deutschland) (Table 11) to prevent oxidation of the tissue.

Analysis

For tissue analysis, slides of paraffin- and cryo-sectioning technique were observed under a Nikon ECLIPSE 90i Microscope (Nikon Instruments, Europe B.V., Amsterdam, Netherlands). Representative pictures were made with an attached high resolution color camera, Nikon DS-Ri 1 (Nikon Instruments, Europe B.V., Amsterdam, Netherlands) and arranged with Adobe Photoshop CS5 (Adobe Systems, San Jose, USA)

Table 11: Steps for hematoxylin - eosine-staining

step	reagent	duration
Deparaffination	X-TRA-Solv	3 x 10 min.
Rehydration	100 % isopropyl alcohol	2 x 5 min.
	96 % ethanol	3 min.
	90 % ethanol	3 min.
	80 % ethanol	3 min.
	70 % ethanol	3 min.
	Aqua dest.	Short
Staining	hematoxylin after Mayer	15 min.
	contrasting in tap water	10 min.
	Aqua dest.	Short
	0,1 % erythrosine	5 min.
	Aqua dest.	Short
Dehydration	70 % ethanol	Short
	80 % ethanol	Short
	90 % ethanol	Short
	96 % ethanol	Short
	100 % isopropyl alcohol	2 x 5 min.
	X-TRA Solv	3 x 10 min.

5.4 Results

Cryo-technique with PFA autofluorescence and DAPI staining.

First staining results using PFA autofluorescence and DAPI as a nuclear counterstaining on 24 µm retinal cryo-sections revealed autofluorescence mainly in receptor cell (RCL) and plexiform layers (OPL and IPL; Fig. 44 A, white arrows 1 - 3). However, each stained negative control embryo showed unexpected structural abnormalities in plexiform layers (Fig. 44 D, white dots). Staining with DAPI led to expected staining of nuclear cell layers (Fig. 44 D, ONL,

INL and GCL), however, similar abnormalities as shown for the plexiform layers appeared in nuclear layers as well. The inner nuclear layer (INL) was composed of two distinguishable areas, one area with high nuclear density (Fig. 44 D, asterisk) and one with low nuclear density (Fig. 44 D, hash).

Exposure experiments with amidotrizoic acid, dichlorvos, 2,4-dinitrotoluene and perfluorooctanoic acid revealed disrupted structures in all tested substances, independent of chosen concentrations (Fig. 45). Thus, disruptions in retinal morphology among negative controls was too great to ensure structure related effects by influence of substances, the development of the PFA autofluorescence based staining of cryo slides was aborted and changed to a paraffin based slicing method supplemented with a rhodamin b and DAPI staining.

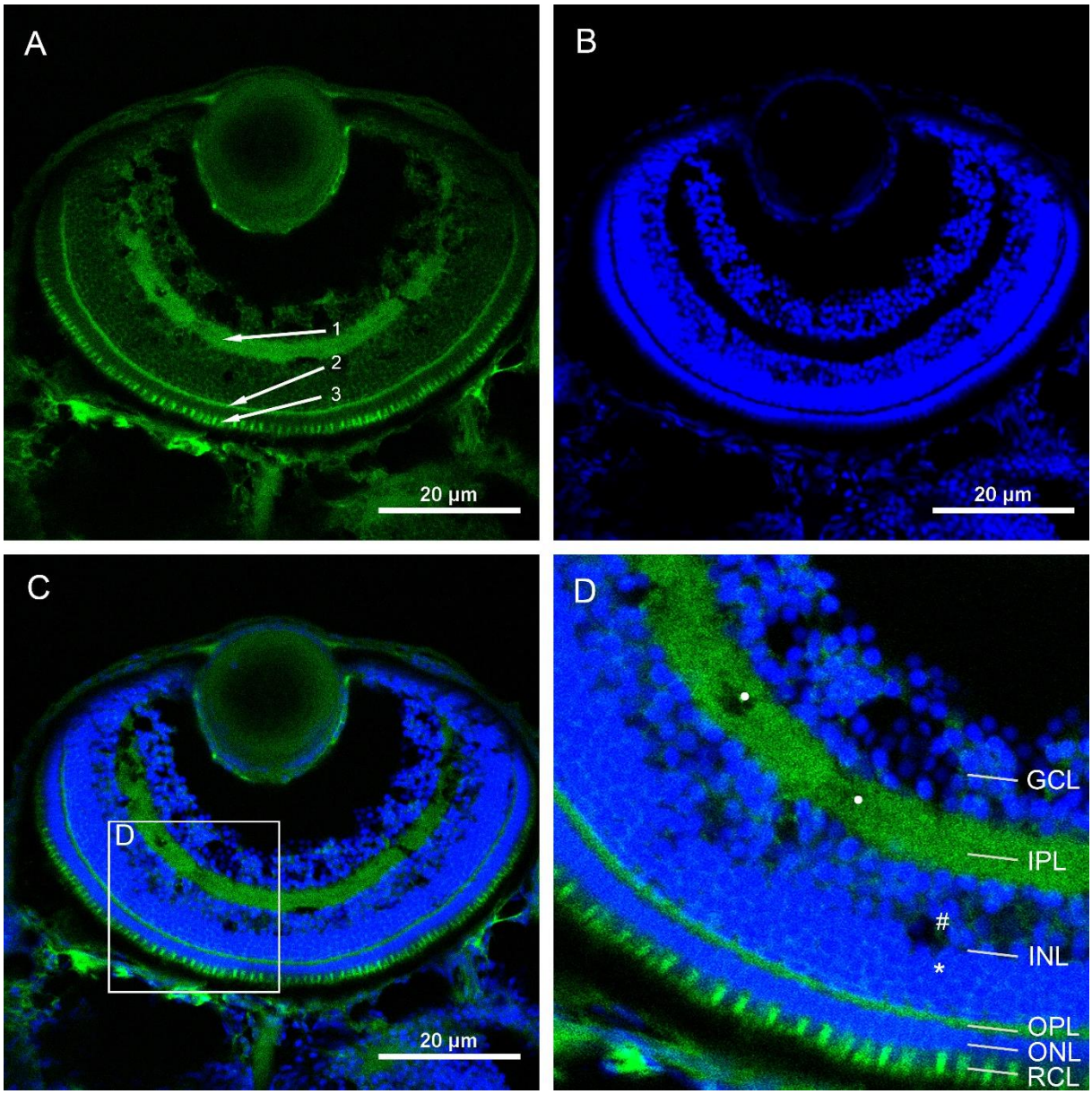

Figure 44: Eye of an untreated zebrafish embryo (96 hpf) shown in PFA-autofluorescence (A, green), DAPI staining (B, blue) and merge of PFA and DAPI (C). (D) shows a higher magnification of C (white frame). Arrows (A) indicate areas with high PFA autofluorescence (1: INL, 2: ONL, 3: RCL). (D) structural abnormalities are indicated by dots in the inner plexiform layer and asterisk/hash for the inner nuclear layer. RCG: receptor cell layer, ONL: outer nuclear layer, OPL: outer plexiform layer, INL:

inner nuclear layer, IPL: inner plexiform layer, GCL: ganglion cell layer.

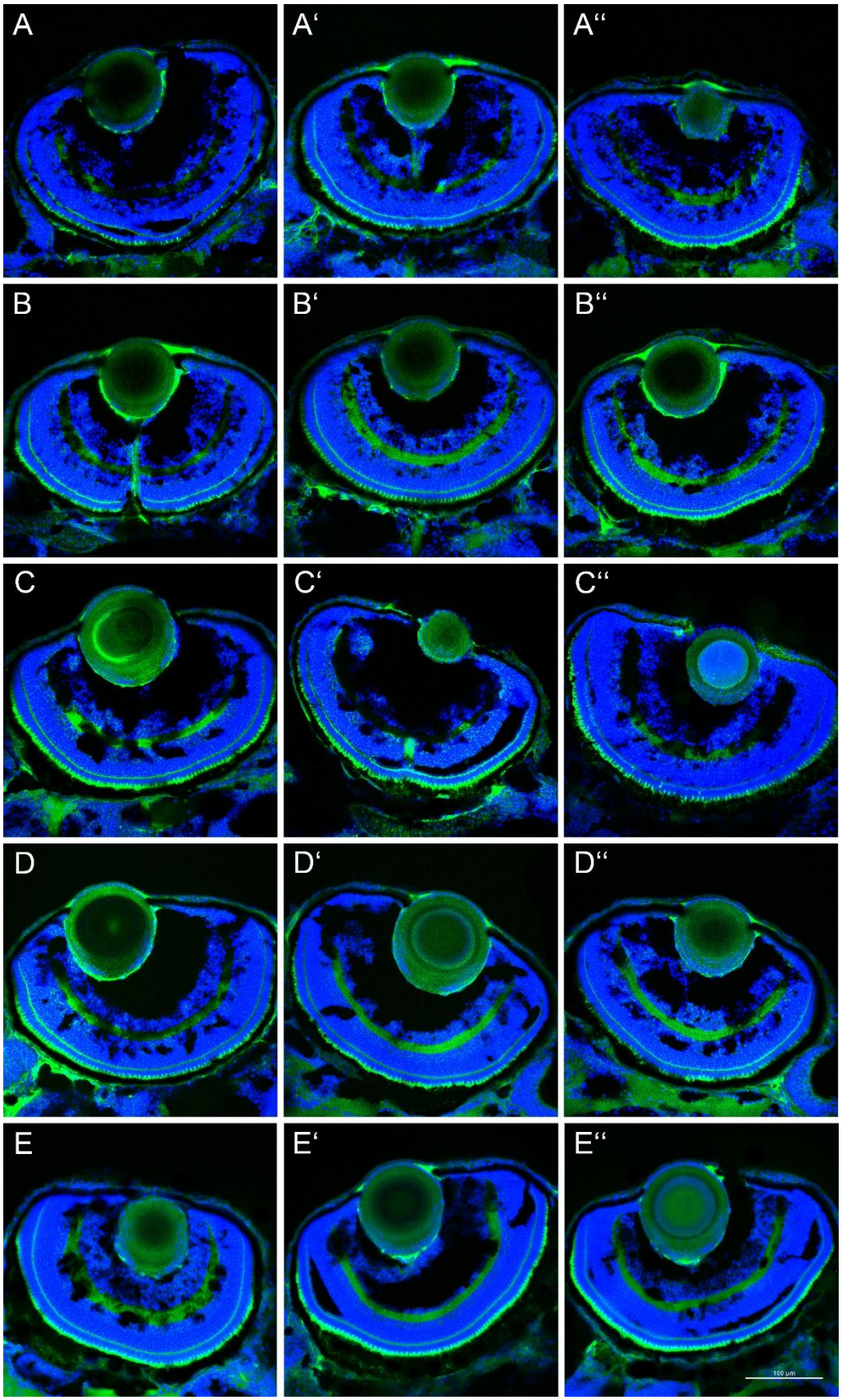

Figure 45: Eyes of zebrafish embryos (A) unexposed, B – E exposed to (B) amidotrizoic acid, (C) dichlorvos, (D) 2,4-dinitrotoluene and (E) perfluorooctanoic acid for 96 hours shown in PFA-autofluorescence (green) and DAPI (blue). Concentrations are indicated as non-apostrophe (EC_{10}), apostrophe (EC_5) and double apostrophe ($EC_{2.5}$); bar 100µM.

Staining with rhodamin B and DAPI of paraffin sections and comparison to H/E staining.

Staining with rhodamin B showed an unspecific, high and brightly stained background (Fig. 46). Supplemented with DAPI, the co-staining revealed a sufficient structure resolution. The change in slicing technique to paraffin based slicing led to an increased structure preservation even in a smaller slide thickness of 5 µm.

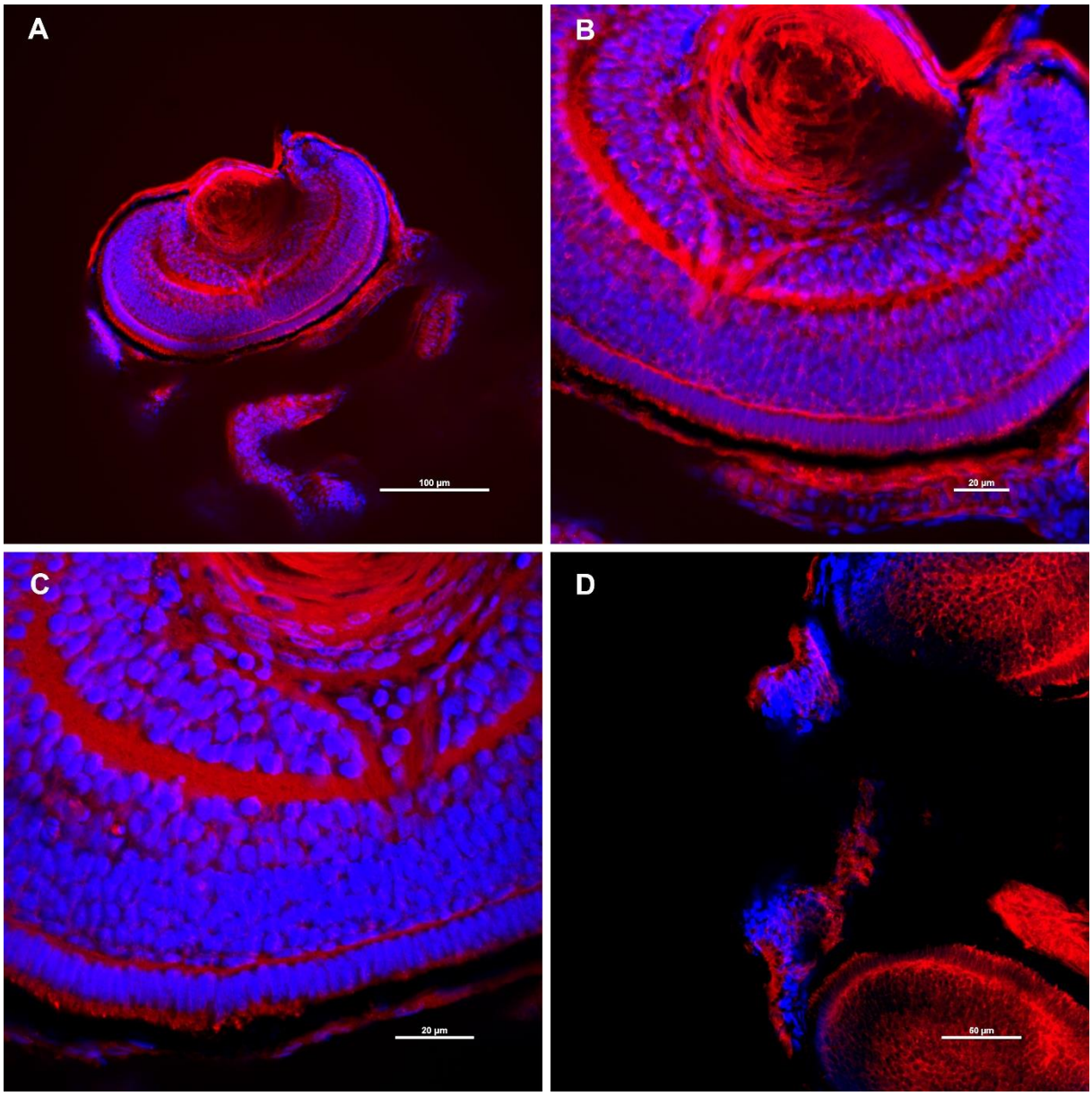

Figure 46: Eyes (A-C) and the olfactory epithelium (D) of untreated zebrafish embryos (96 hpf) shown in Rhodamin b (red) and DAPI staining (blue) with paraffin sectioning Bars A/100 µM, B/20 µm, C/20 µm, D/50 µm.

Comparison of cryo- and paraffin-sectioning techniques with paraformaldehyde, rhodamin B and HE staining

Thus, structure resolution of a rhodamin B and DAPI staining had no significant advantages compared to classical, well studied and widely used HE staining method (Fig. 47 C), methodical approach was changed to a paraffin based HE staining and compared to previously used methods. Compared to previously used and tested techniques, paraffin based sectioning (Fig. 47 B, C, and B', C') provides a higher structure preservation if compared to cryo- sectioning (Fig. 47 A and A'). HE staining and rhodamin B / DAPI staining revealed a similar and higher structure resolution if compared to the PFA-autofluorescence / DAPI.

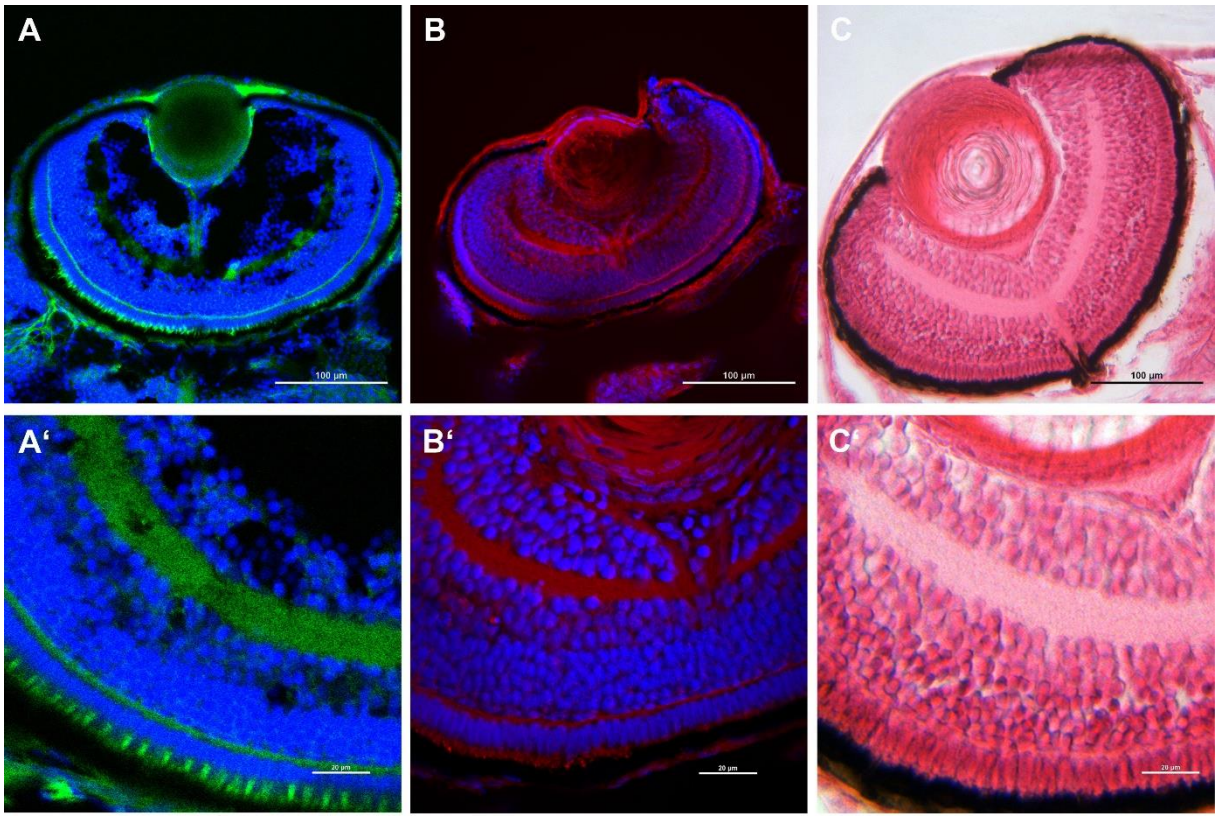

Figure 47: Eyes of untreated zebrafish embryos (96 hpf) shown in A: PFA-autofluorescence (green) and DAPI staining (blue), B: Rhodamin b (red) and DAPI staining (blue) and C: Hematoxilin / eosin staining. Bars A – C 100 µm, higher magnification of tissues are shown in A' – C' respectively; bar 20 µm.

Application of H / E technique to exposure tests with single substances

Exposure of 20 embryos, in three independent replicates, for 96 hpf to amidotrizoic acid, caffeine, 2,4-dichlorophenol, 2,4-dinitrotoluene, dichlorvos, 4-nonylphenol, perfluorooctanoic acid and perfluorooctansulfonic acid at concentrations of EC_{10} and below, did not show significant substance specific effects in the retina of zebrafish embryos.

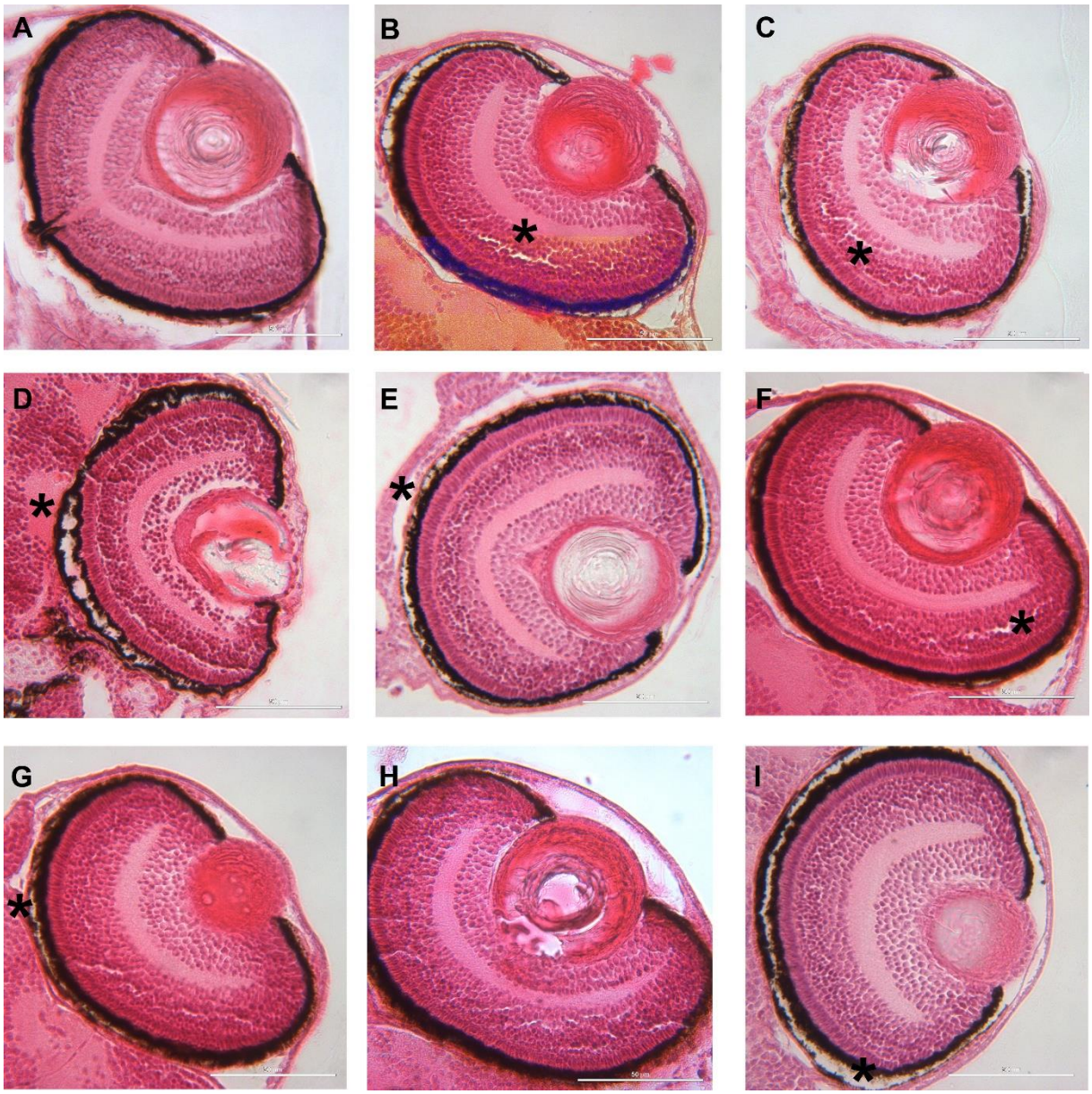

Figure 48: Eyes of zebrafish embryos exposed to (B) 2,4-dichlorophenol, (C) 2,4-dinitrotoluene, (D) dichlorvos, (E) caffeine, (F) amidotricoic acid, (G) 4-nonylphenole, (H) perfluorooctanoic acid and (I) perfluorooctansulfonic acid for 96 hours shown in H/E staining. (A) Represents negative control; bars 50 µm.

5.5 Discussion

In this approach, the retina was used as a possible marker to show structural alteration by exposure to different substances, which are assumed as neurotoxic (Huang et al. 2013, Kim et al. 2013, Mecklenburg and Schraermeyer 2007, Mela et al. 2012, Triebskorn et al. 1994, Wang et al. 2012). It was attempted to visualize morphological changes in the retina by three different staining and preparation methods: (1) Cryo sectioning technique with PFA autofluorescence and DAPI staining, (2) paraffin-based slicing with rhodamin b and DAPI staining and (3) paraffin based slicing with classical hematoxilin / eosin staining (HE). To validate the established methods, embryos were exposed to chosen pure substances and results were analyzed in terms of reproducibility.

Cryo-sectioning with PFA autofluorescence and DAPI staining.

Cryo-sections, which were fixed with paraformaldehyde, revealed an expected autofluorescence as shown in different tissues of human samples and cell cultures (Baschong et al. 2001, Ganguly et al. 2011, Neumann and Gabel 2002, Poulsen et al. 2013) mostly restricted to plexiform layers of the retina. However, structural deficits were observed in negative controls as well as in following exposure test. Regarding the present results, it can be assumed that PFA fixation combined with cryo-sectioning technique led to an insufficient structure preservation. This may have the following reason: inadequate technical management, at which shortened fixation time or incorrect temperature while slicing, led to a loss of structure stability and, therefore, to non-evaluable results. Nevertheless, beside structural loss, a detailed evaluation of cellular alterations would not be possible with this method, since staining did not result in detectability of different cell types by morphological discrimination.

Staining with rhodamin B and DAPI of paraffin sections and comparison to H/E staining.

After excluding cryo-sectioning, embryos were processed with a paraffin based slicing approach with additional rhodamin B staining, supplemented with a DAPI counterstaining. Although, the paraffin-based approach included similar PFA fixation steps, the results revealed a normal structure of the retina, supporting the idea of a cryo preparation-dependent reduction of tissue preservation or, at least, an improved structure preservation by paraffin slicing technique which is independent of PFA. In the present thesis, rhodamin B has been used for staining, since it has been frequently used to stain for keratin in dermal tissues (Pinkus et al. 1980, Wessely et al. 1981) and cumarin derivates had shown to stain retinal cells (Watanabe et al. 2010). However, further literature of specific staining is scarce. Nevertheless, rhodamine B staining led to a fine structure resolution of paraffin sections, but also to a loss of cell type discrimination as described for PFA autofluorescence.

Comparison of approaches

Based on the present results and in comparison of the here shown staining techniques, the classic hematoxilin / eosin staining on paraffin-based sections revealed the best results in structure preservation, resolution and cell differentiation. This led to the use of the classic hematoxilin / eosin staining on paraffin based section for following exposure experiments.

Effect determination at an EC_{10} concentration.

The tested substances, namely 2,4-dichlorophenol, 2,4-dinitrotoluene, dichlorvos, caffeine, amidotricoic acid, 4-nonylphenol, perfluorooctanoic acid and perfluorooctanesulfonic acid, revealed a lack of significant effects if compared to the negative control at concentrations at EC_{10} and below. Data on effects on eye morphology in fish for all tested substances is scare or missing. But studies with dichlorvos and nonylphenole indicate the existence of possible mechanisms effecting eye morphology in different species. Mishra and Devi (2014) observed an induction of vacuolization in tectum opticum of the snake head fish *Channa punctatus* by chlorpyrifos at concentrations of 1.79 and 0.538 µg/L, a family member of organophosphates like dichlorvos. These results could not be repeated in the present study. Even if the concentration range was similar, effects on the retina induced by dichlorvos might be covered by the observation of artefacts in all concentrations as well as the negative control. Additionally, the retina might react differently if compared to the tectum opticum, leading to a lack of visible effects in the retina. Since it has been shown that the retinal marginal cells increase the production of new retinal cells after mechanical or neurochemical damage (Reh 1987, Reh and Tully 1986), a masked effect would possibly occur by regeneration. Additionally, Yu et al. (2008) observed apoptosis in retina of mouse treated with 63 mg/kg chlorpyrifos (~3.8 mg/mouse, compared to 3 mg/L exposure concentration in the present study), but did not observe a gross morphological aberration. It can be assumed, that, while testing in similar concentrations, the results of the present study show a "false negative" result, by lower sensitivity if compared to apoptotic markers used by Yu et al. (2008).

For nonylphenol, there aren`t any works on altered retinal structure below a sublethal concentration. But, as already shown for dichlorvos, Chandrasekar et al. (2011a) revealed an increased apoptosis pattern in retina of zebrafish embryos (33 hpf) with additional strong notochord malformations for a concentration of 2 µM nonylphenol (~440 µg/L). Compared to the here shown result, this concentration ranges higher than the EC_{10}, which is about 2 times higher than the here tested concentrations. However, a lack of apoptotic effects on the retina at an EC_{10} concentration in the here shown result cannot be certainly proven since the presented method does not specifically detect apoptotic processes.

To the best of knowledge, there are no studies observing histopathological effects on the eye induced by PFOS, PFOA, amidotrizoic acid, caffeine, 2,4-dichlorophenol or 2,4-dinitrotoluene. Since there is a lack of data, the presented study is the first to show a lack of effect in retinal structures induced by either of these compounds at EC_{10}.

5.6 Conclusions

The test system is basically able to show morphological effects on the retina of zebrafish embryos, however not at a concentration below EC_{10}. Truly neurotoxic effects could therefore not be identified.

Conclusion

The present thesis aimed at establishing and evaluating methods to serve within a test battery for neurotoxicity in early developmental stages of zebrafish. Specifically, the neuromast and the acetylcholinesterase assay proved to be promising tools for monitoring of neurotoxicity. In practice, a combination of several methods to detect neurotoxicity with a method to account for general (acute) toxicity seems able to comprehensibly identify the risk of neurotoxic action. As a result, the present thesis combined the fish embryo toxicity test (FET) with the neuromast and acetylcholinesterase assays. Each of these methods detected single specific modes of action, a small selection or even multiple mechanisms of toxicity, thus leading to a wide range of detectable substances and targets.

A closer analysis of the specificity of targets and their integration into cellular and/or physiological processes allows a distinction between truly neurotoxic modes of mechanism or a generalized toxic effect, i.e. an impact of not specifically neurotoxic nature. The combination of methods selected supports the differentiation between neurotoxic and non-neurotoxic effects by either reduction of physiological side-effects or by clarifying underlying toxic mechanisms. Given that each method can be localized at different levels of biological organization (organismal: FET; molecular: AChE inhibition; cellular: (neuromast assay), the combination of methods allows an evaluation of the effect relevance. Thus, the advantages of the selected combination of methods can be summarized as follows: (1) detection of neuronal target mechanisms; (2) prevention of false negative results; (3) evaluation of the relevance of the effects detected.

5.7 Detection of neuronal target mechanisms

The extended fish embryo toxicity test detects a wide range of endpoint, for instance heart-beat, formation of somites, eye development, spontaneous movements, general malformations and others. This information allows a general insight into processes interfering with neurotoxic endpoints. General developmental retardation, e.g., may have an impact on every organ, while the substance triggering the process may act unspecifically. Without knowledge of such unspecific modes-of-action, modifications in the layout of retinal layers could easily be misinterpreted as an endpoint of neurotoxicity. Therefore, a test such as the fish embryo toxicity test primarily aims at gathering information about general toxicity, but also serves as a range-finder for subsequent testing into more specific endpoints *via* definition of LC and EC values. In the present study, EC_{10} values were picked as the highest test concentrations for subsequent neurotoxicity tests on the expectation that interference with physiological side-effects should be minimal.

The inhibition of acetylcholine esterase (AChE) activities has repeatedly been used both in the context of *in vivo* or *in vitro* approaches. However, as documented for cypermethrin, which induces AChE stimulation *via* regulatory processes, an exclusive focus on AChE reduction may result in a loss of information. This example illustrates that chemical substances may cause unexpected effects by manipulating remote mechanisms, which are independent of the actual target mechanism. E.g., chemicals suppressing AChE gene expression will lead to an inhibition of enzyme activities in an *in vivo* approach in embryos. In contrast, in conventional biochemical

assays, the inhibitory effect would not necessarily be detectable *in vitro*. As a conclusion, only a combination of both *in vivo* and *in vitro* approaches allows for the detection of additional underlying mechanisms of cypermethrin neurotoxicity.

In the neuromast assay, the present studied considered neuromasts located in both the anterior and the posterior lateral lines as individual neuromasts, since there were significant differences in sensitivity and susceptibility. In contrast, most previous studies used neuromasts from either the anterior or the posterior system (Linbo et al. 2009, Montalbano et al. 2014). Such approaches provided target-specific information, but inevitably led to a partial loss of information for overall effect assessment. As illustrated for nonylphenol, the detection limit for effects in the posterior lateral line was 4 times lower than for those in the anterior lateral line.

In the present layout, the neuromast assay also potentially provides the opportunity to detect indirect modes of action: In addition to known ototoxic substances such as neomycin and copper sulfate, neuromasts also proved sensitive to an endocrine disruptor (4-nonylphenol) and an AChE inhibitor (dichlorvos). However, unspecific effects related to acute toxicity on hair cells need to be considered. For instance, chemicals inducing an inflammatory response in the dermal epithelium, may also lead to decreased hair cell viability. Again, the isolated use of a single method might lead to the false conclusion of specific neurotoxicity, even if the cause is unspecific inflammation.

5.8 Prevention of false negative results

Given the different endpoints addressed, the acetylcholinesterase assay, the neuromast assay and the fish embryo toxicity test showed different specificities. However, the grade of specificity can be related to the organizational level (Fig. 49). Testing strategies addressing more apical endpoints at higher levels of organization (organismal level, e.g. embryo toxicity) are more likely able to detect a larger number of chemicals by incorporating a wider spectrum of possible targets. In contrast, most molecular markers (e.g. AChE) will − by definition − only detect one single mechanism and will, thus, capture only a narrow range of possible toxicants (Fig. 49). As a consequence, the use of a suite of highly specific molecular endpoints would inevitably result in an increased number of chemicals, the neurotoxic nature of which would be overlooked.

The acetylcholinesterase assay, which only detects conversion rates of a specific AChE substrate, covers one mode of action and shows the highest specificity. Thus, the acetylcholinesterase assay only identified dichlorvos, chlorpyrifos and cypermethrin as neurotoxic. For cypermethrin, there were differences between *in vivo* and *in vitro* results. Using either an *in vivo* or *in vitro* approach, the result would thus be either false-positive (*in vivo* only) or false-negative (*in vitro* only) due to mechanisms that regulate the amount, but not the activity of enzyme available.

In contrast, the analysis of neuromast viability *via* changes in fluorescence intensity is subject to modulation by more processes than AChE activity. The higher the number of regulatory processes involved, the more pronounced the reduction in specificity may be expected. In fact, neuromasts were affected by AChE inhibitors, heavy metals, antibiotics as well as endocrine disruptors; most likely, these chemicals interfere with different mechanisms, which, however, all end up in a decrease of neuromast viability. The wider range of processes covered thus leads to a reduction of the number of false negative results.

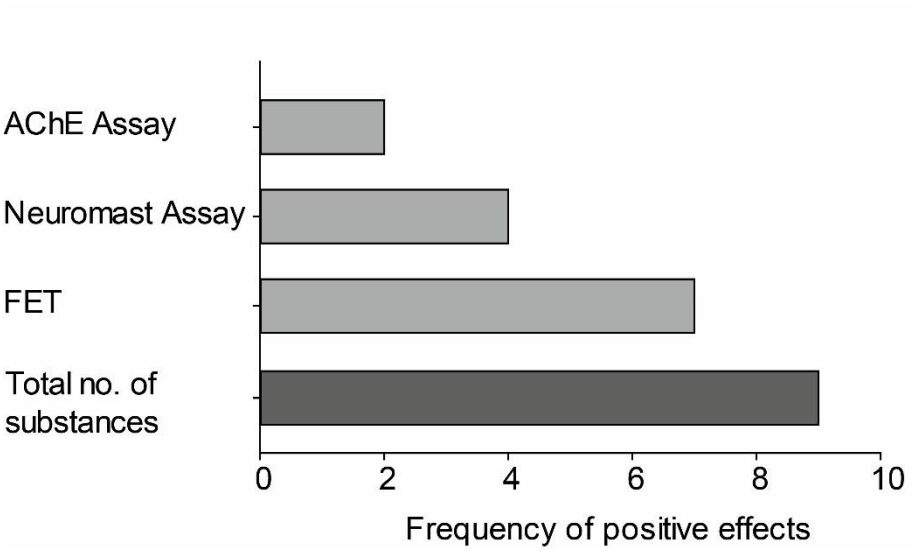

Figure 49: Frequency of positive effect seen with the test chemicals selected evidenced by the number of pure substances giving positive results in either the FET, the neuromast or the AChE-assays.

The fish embryo toxicity test represents the highest level of organization used in this study and comprises multiple mechanisms leading to changes in the physiological state of the embryos. Multiple molecular mechanisms addressed by an increasing variety of chemicals will have a complex impact on apical physiological endpoints such as developmental retardation. On the other hand, this is related to relatively low specificity. Again, an increasing number of underlying mechanisms leads to a decrease in the frequency of false negative results.

In summary, the higher the organizational level, the more mechanisms are potentially involved leading to a reduced specificity. The exclusive use of highly specific methods will most likely increase the risk of producing false negative results. For a comprehensive assessment of the neurotoxic nature of a chemical, the combination of different mechanisms at different levels of organization is, therefore, indispensable.

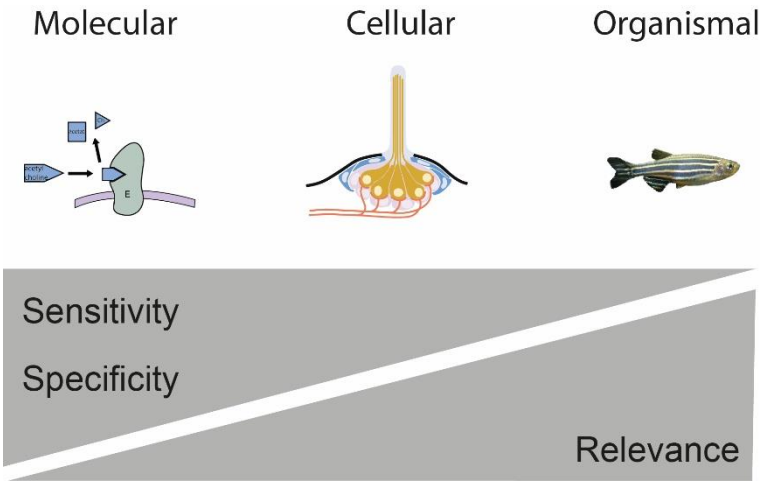

Figure 50: Comparison of sensitivity, specificity of test systems and relevance of results with organizational level the used assays. Sensitivity and specificity decrease whereas relevance of determined results rises with increasing complexity.

5.9 Relevance of the effects observed

In the present study, only dichlorvos produced effects in the AChE assay, the neuromast assay and the fish embryo test. As a consequence, these methods can be rated with respect to their sensitivity: Based on EC_{10} values, the relative sensitivities of the test systems are as follows: AChE > neuromast assay > fish embryo test (Fig. 51). With respect to the level of biological organization, the tests compare as follows: molecular > cellular > organismal level.

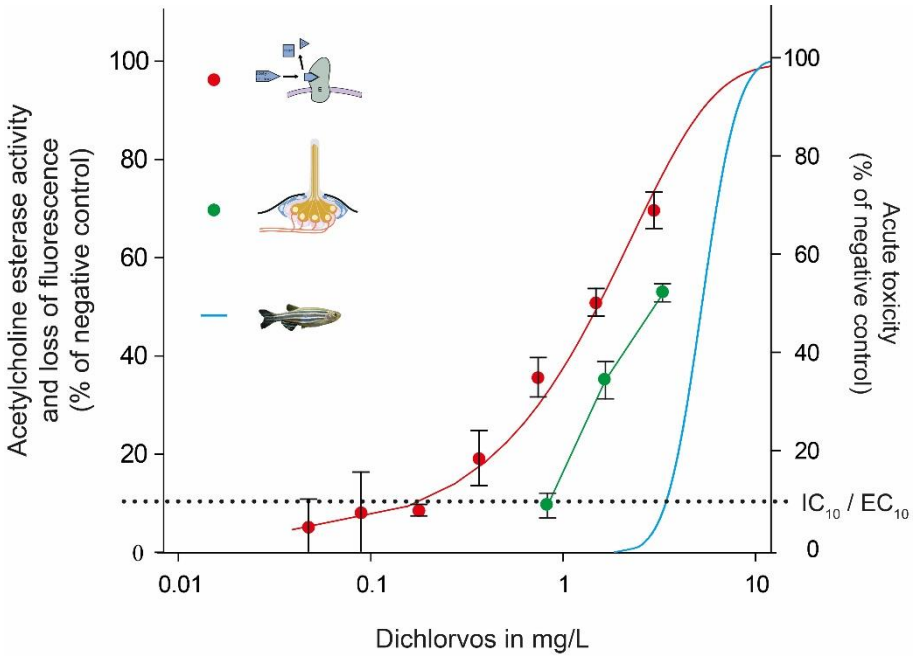

Figure 51: Comparison of IC_{10} (AChE) and EC_{10} values (neuromast assay, FET) for dichlorvos: The higher the level of biological organization, the less sensitive the tests tends to be. IC_{10} AChE: 0.18 mg/L, EC_{10} neuromast assay: 0.75 mg/L; EC_{10} FET: 2.86 mg/L.

As a molecular marker, AChE activity showed lower EC_{10} values than neuromasts or entire embryo viability. This may have several reasons: moderate AChE inhibition does not necessarily have to lead to a decrease in cell viability or disruption of neuronal tissues. Both neurons and non-neuronal cells have a diversity of regulatory mechanisms maintaining cellular homeostasis. Thus, the cellular systems are buffered by various adaptation processes and will endure periods of increased stress, leading to a delay in the appearance of effects from one level of organization to the next one. In the case of dichlorvos, the IC and EC_{10} concentrations of AChE, neuromast assay and FET showed a gradual delay in concentration following the level of biological organizational:

IC_{10} AChE		EC_{10} Neuromast		EC_{10} FET
0.1822 mg/L	>	0.75 mg/L	>	2.86 mg/L

Given the complexity of molecular regulation, especially at the molecular level, chemically induced effects do not necessarily result in effects at higher levels. In many cases, effects at higher levels of organization will results in a temporal delay of effects at higher levels: For example, chemicals affecting the DNA may lead to strongly delayed lethality *via* the development of cancer over years. The more complex a biological system is, the more adaptation mechanisms can reduce the extent of effects and the less important one mechanism can become.

The relative importance of adverse effects on the survival of the organism plays a major role for risk assessment. The relevance of an effect depends on the questions: (1) How many systems are affected? and (2) How important is system each for the survival of the organism? As documented *via* the gradual decrease in sensitivity (Fig. 51): the lower the organizational level, the lower the relevance for survival. However, this has to be confirmed for other mechanism sand modes of action. With an increase in the number of systems affected, the risk of lethality increases. In other words: The more systems are affected, the higher the relevance for the organism (Fig. 50).

Figures

Tables

Publications

Chapter I is based on the publication:

Kais, B., Stengel, D., Batel, A. and Braunbeck, T. (2015a) *Acetylcholinesterase in zebrafish embryos as a tool to identify neurotoxic effects in sediments*. Environmental Science and Pollution Research 1-11.

Chapters II-V are based on unpublished data and the following publications:

Braunbeck, T., Kais, B., Lammer, E., Otte, J., Schneider, K., **Stengel, D**. and Strecker, R. (2014) ***The fish embryo test (FET): origin, applications, and future.*** Environmental Science and Pollution Research 1-15.

Grummt, T., Kuckelkorn, J., Bahlmann, A., Baumstark-Khan, C., Brack, W., Braunbeck, T., Feles, S., Gartiser, S., Glatt, H., Heinze, R., Hellweg, C., Hollert, H., Junek, R., Knauer, M., Kneib-Kissinger, B., Kramer, M., Krauss, M., Kuster, E., Maletz, S., Meinl, W., Noman, A., Prantl, E.-M., Rabbow, E., Redelstein, R., Rettberg, P., Schadenboeck, W., Schmidt, C., Schulze, T., Seiler, T.-B., Spitta, L., **Stengel, D**., Waldmann, P. and Eckhardt, A. (2013) ***Tox-Box: securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany***. Environmental Sciences Europe 25: 27.

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