Effekte von in der Umwelt auftretenden Schadstoffen (Pestiziden, Pharmazeutika, Schwermetallen) auf den Zebrabärbling (*Danio rerio*) und weitere Süßwasserfische

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So long, and thanks for all the fish

(Douglas Adams)

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Zusammenfassung

1. Promotionsthema

Effekte von in der Umwelt auftretenden Schadstoffen (Pestiziden, Pharmazeutika, Schwermetallen) auf den Zebrabärbling (*Danio rerio*) und weitere Süßwasserfische.

2. Einleitung

2.1 Grundlagen

Stoffe, die anthropogen in die Umwelt eingebracht werden und dort Schäden hervorrufen können, sind als potentielles Problem schon lange bekannt, waren bis in die siebziger Jahre des vergangenen Jahrhunderts jedoch eher von akademischem Interesse und weniger Teil der öffentlichen Aufmerksamkeit (Strubelt 1996). Mit dem Auftreten und Bekanntwerden größerer Umweltverschmutzungen und vermehrter Information durch die Medien nahm auch das Interesse der Öffentlichkeit an umweltrelevanten Themen und das Bewusstsein für öko(toxiko)logische Fragestellungen zu (Alloway & Ayres 1996). Der Schutz der Umwelt vor schädlichen Substanzen fand Eingang in die Gesetzgebung, so führt z.B. das Strafgesetzbuch (StGB) der Bundesrepublik Deutschland in § 324a I (Bodenverunreinigung) auf:

(1) Wer unter Verletzung verwaltungsrechtlicher Pflichten Stoffe in den Boden einbringt, eindringen lässt oder freisetzt und diesen dadurch

1. in einer Weise, die geeignet ist, die Gesundheit eines anderen, Tiere, Pflanzen oder andere Sachen von bedeutendem Wert oder ein Gewässer zu schädigen, oder
2. in bedeutendem Umfang verunreinigt oder sonst nachteilig verändert, wird mit Freiheitsstrafe bis zu fünf Jahren oder mit Geldstrafe bestraft. (StGB 2007).

Die aktuellste Entwicklung im Rahmen der Gesetzgebung bezüglich möglicher Schadstoffe stellt die EU-Chemikalienverordnung REACH [oftmals korrekter bezeichnet: REACh] dar. Die Chemikalienverordnung REACH („Registration, Evaluation, Authorisation of Chemicals“) ist am 1. Juni 2007 in Kraft getreten und fordert, neben weiteren Auflagen, bei potentiell gefährlichen und besorgniserregenden Stoffen einen Stoffsicherheitsbericht mit Expositionsszenarien für Mensch und Umwelt sowie eine Beschreibung der toxikologischen und ökotoxikologischen Eigenschaften des Stoffes (Lahl und Hawxwell 2006).

Die umfangreichen Auflagen bei der Zulassung neuer Stoffe und nachträglichen Bewertung bereits zugelassener Stoffe im Rahmen von REACH

unterstreicht die Bedeutung der potentiellen Umweltgefährdung durch Chemikalien. Doch auch diese Verordnung bezieht sich, ihrer Natur gemäß, lediglich auf die Beurteilung einzelner Stoffe. Wechselwirkungen verschiedener Chemikalien oder auch Wechselwirkungen von Chemikalien mit abiotischen Faktoren werden in den vorgeschriebenen Standardtests in der Regel nicht berücksichtigt. Dies ist insofern von Bedeutung, als dass nur in den seltensten Fällen davon auszugehen ist, dass ausschließlich ein Schadstoff isoliert in einem Ökosystem auftritt und dieses Auftreten auch noch unter konstanten äußeren Bedingungen geschieht, wie es im Laborexperiment der Fall ist. Vielmehr ist im Freiland mit Wechselwirkungen von einer Vielzahl von (Schad-) Stoffen, die gemeinsam auf einen Organismus treffen, zu rechnen.

Bekannt ist, dass Stoffe, wenn sie gemeinsam auftreten, in ihrer Mischung andere Schadwirkungen haben können als Einzelsubstanzen. Abhängig sind die Wechselwirkungen von den Wirkmechanismen („*modes of action*“) bzw. vom Wirkort des Schadstoffes. Mischungen von Stoffen unterschiedlicher Wirkmechanismen können zunächst einmal als voneinander unabhängig wirkend betrachtet werden; Stoffe mit ähnlichen oder gleichen Wirkmechanismen führen zu einer additiven Schadwirkung, wenn sie nicht interagieren. Wechselwirkungen zweier oder mehrerer Schadstoffe miteinander können aber auch zu antagonistischen (im Vergleich zur unabhängigen Wirkweise geringeren) oder synergistischen (im Vergleich zur Additivität verstärkten) Schadwirkungen führen (Plackett & Hewlett 1952, Escher & Hermens 2002). Des Weiteren können Schadwirkungen von Stoffen auch von abiotischen Faktoren abhängen. So sind z.B. Halbwertszeit und Bioverfügbarkeit von Pestiziden abhängig von Temperatur, Boden- und Luftfeuchtigkeit, Sonnenstrahlung usw. (Aislabie & Lloyd-Jones 1995, Sukul & Spiteller 2001, Relyea & Hoverman 2006), und auch Stoffwechselprozesse, wie z.B. jene, die der Detoxifizierung von Schadstoffen in ektothermen Tieren dienen, hängen maßgeblich von der Außentemperatur ab (Campbell 1997).

Ob potenzielle Schadstoffe negative Auswirkungen auf Organismen haben, lässt sich mit Hilfe von sogenannten Monitororganismen abschätzen. Um den Gesundheitszustand eines solchen Monitororganismus' beurteilen zu können, nutzt die ökotoxikologische Forschung Biomarker. Biomarker sind nach van Gestel & van Brummelen (1996) biologische Antworten oder Reaktionen eines Organismus' auf Umweltveränderungen. Zu diesen Biomarkern gehören, neben weiteren, auch biochemische und histologische Parameter sowie Änderungen in der Entwicklung von Organismen. Ein Beispiel für einen biochemischen Biomarker ist der in dieser Arbeit untersuchte Hitzeschockproteingehalt von unterschiedlich belasteten Tieren.

Die Hitzeschockproteine, auch Stressproteine genannt, wurden in den siebziger Jahren des 20. Jahrhunderts entdeckt. Schon Ritossa (1962) hat Veränderungen in der Genexpression in Form von veränderten Puff-Mustern der Riesenchromosomen in der Speicheldrüse bei *Drosophila*-Larven beobachtet, wenn diese bei erhöhten Temperaturen gehalten wurden. Tissières et al. (1974) konnten nachweisen, dass parallel zu dem Auftreten der Puffs eine bestimmte Gruppe von Proteinen gebildet wurde. Aufgrund des Auftretens bei erhöhten Temperaturen wurden diese Proteine als Hitzeschockproteine (*heat shock proteins*, Hsp) bezeichnet. Die Hitzeschockproteine werden jedoch außer unter Temperaturstress auch bei Einwirkung von verschiedensten anderen Stressoren, die ebenfalls in einer Beeinträchtigung der Integrität intrazellulärer Proteine (=Proteotoxizität) resultieren, vermehrt produziert (einen Überblick geben z.B. Feder und Hofmann (1999)), so dass in diesem Zusammenhang allgemein auch von Stressproteinen gesprochen wird (Lewis et al., 1999). Über den Vergleich des Gehaltes von Stressproteinen im untersuchten Tier oder dessen Organen ist ein Rückschluss auf die „Gesamtmenge“ an proteotoxischem Stress, dem das Tier ausgesetzt war, möglich. Der direkte Vergleich des Hitzeschockproteingehaltes in einem unter Kontrollbedingungen gehaltenen Organismus’ mit dem in einem Organismus, welcher zusätzlich einem definierten, potentiell proteotoxischen Stressor ausgesetzt wurde, erlaubt es, das Stresspotential abzuschätzen, welches von diesem ausgeht (siehe z.B. Eckwert et al., 1997; Nadeau et al., 2001, Hallare et al. 2004, Scheil et al. 2008). Untersuchungen an Hitzeschockproteinen werden in Kapitel 2, 4 und 6 dieser Arbeit vorgestellt.

Vom Niveau biologischer Organisation höher anzusiedeln sind Veränderungen in Zellen oder Organen. Auch hier lassen sich Biomarker zur Schaderkennung nutzen. So zeigen die in der vorliegenden Arbeit untersuchten histopathologischen Veränderungen von Kiemen belasteter Fische Abweichungen vom Kontrollzustand und geben damit direkte Hinweise auf Schadwirkungen, welche, bei umfassender Untersuchung weiterer Organe, auch direkten Wirkmechanismen in bestimmten Organen zugeordnet werden können (Triebskorn et al. 2003 und Triebskorn et al. 2004, Kapitel 1). Noch eine Stufe höher im Organisationsniveau liegen Biomarker, denen makroskopische Veränderungen im Organismus zugrunde liegen. So können z.B. Veränderungen in der Embryonalentwicklung von Tieren als Reaktion auf Schadstoffbelastung erfasst werden. Beispielhaft sei hier der „Embryotest mit *Danio rerio*“ genannt, welcher von Nagel (2002) vorgestellt wurde und in modifizierter, erweiterter Form in Kapitel 2-5 angewandt wurde. Der Embryotest soll den akuten Fischtest ersetzen, welcher als

Endpunkt die Mortalität von Fischembryonen nutzt und zur Chemikalienbewertung eingesetzt wird. Da akut letale Konzentrationen von Schadstoffen nur selten in der Umwelt anzutreffen sind, soll der i.d.R. wesentlich sensitivere Parameter „Störung in der Embryonalentwicklung“ mögliche Wirkungen von Schadstoffen in der Umwelt besser voraussagen können (Nagel 2002). Alle diese Biomarker können im Freiland (eingeschränkt) und im Labor untersucht werden.

Viele der vom Menschen beabsichtigt (z.B. Pestizide in der Landwirtschaft oder der häuslichen Anwendung, unsachgemäße Entsorgung von Pharmazeutika) oder unbeabsichtigt (Unfälle) ausgebrachten Chemikalien gelangen entweder direkt oder indirekt über Abwässer, Auswaschungen oder Verdriftungen in Grundwässer und Oberflächengewässer (siehe z.B. Flury 1996, Ohe et al. 2005, Bloomfield et al. 2006) und sind dort potentiell toxisch für Flora und Fauna. Betrachtet man Oberflächengewässer und deren Fracht an Pestiziden bzw. Pharmazeutika, so findet man für Pharmazeutika Maximalkonzentrationen von z.B. 2 µg /L Diclofenac [Lehmann 2000] oder 2.2 µg /L Metoprolol (Ternes 2001) bzw. Pestizidkonzentrationen von 1.5 µg/L 3,4-Dichloranilin (EU, 2006; Planas et al. 2006) oder 1.5 µg/L Diazinon (Bailey et al. 2000). Mögliche Wirkungen solcher Pharmazeutika in diesen niedrigen, umweltrelevanten Konzentrationsbereichen werden in Kapitel 1 dargestellt.

Die vorliegenden Untersuchungen wurden an verschiedenen Fischarten durchgeführt, welche unterschiedliche Vorteile als Testfische für die Bewertung von Umweltbelastungen besitzen. Die einheimischen Fischarten *Oncorhynchus mykiss* (Regenbogenforelle), *Cyprinus carpio* (Karpfen), *Leuciscus cephalus* (Döbel) und *Chondrostoma nasus* (Nase) bieten sich für die Untersuchung von europäischen Gewässerbelastungen an, da sie natürlich in einheimischen Gewässern vorkommen bzw. zu Fischereizwecken eingesetzt werden. Ihre Laborhaltung ist jedoch, aufgrund ihrer Größe und den daraus resultierenden Haltungsbedingungen, problematisch und aufwändig. Der Zebrabärbling (*Danio rerio*) hingegen, einheimisch im östlichen Vorderindien (Riehl & Baensch, 2001), stellt aufgrund seiner geringen Größe, leichten sowie kostengünstigen Haltung und hohen Reproduktivität ein ideales Versuchstier für Laborversuche zur Ökotoxizität von in Gewässern auftretenden Belastungen dar. Ein weiterer Vorteil des Zebrabärblings sind dessen transparente Eier, die es ermöglichen, vom Zeitpunkt der Eiablage an die Entwicklung der Embryonen im Ei zu verfolgen und z.B. Veränderungen in der Entwicklung unter Schadstoffbelastung zu untersuchen (z.B. Nagel 2002, Hallare et al. 2004; Hallare et al. 2006, siehe auch Kapitel 3-6).

Um den realen Umständen im Laborversuch näher zu kommen, ist es naheliegend, neben reinen, standardisierten, Chemikalientests Experimente durchzuführen, die den natürlichen Gegebenheiten zumindest etwas näher kommen. So sind Experimente gefordert, die auch Chemikalienmischungen beinhalten oder aber eine oder mehrere Chemikalien mit unterschiedlichen abiotischen Faktoren (wie z.B. erhöhte oder erniedrigte Temperatur) kombinieren. In wissenschaftlichen Publikationen zur Ökotoxikologie von Stoffen tauchen Untersuchungen zur Mischungstoxizität von Chemikalien erst seit Mitte der neunziger Jahre vermehrt auf (z.B. Rayburn et al. 1995, Feron et al. 1995, Birnbaum & DeVito 1995), wobei die Bedeutung der näher an der Realität liegenden Mischungsszenarien im Vergleich zu den weniger realitätsnahen Einzelstofftests hervorgehoben wird (Feron et al. 1995).

Die vorliegende Arbeit beschäftigt sich zum einen mit Auswirkungen von ausgewählten Einzelstoffen (Pestiziden, Pharmazeutika, Schwermetallen) und Mischungen dieser Stoffe auf die Süßwasserfische *Danio rerio* (Zebrabärbling, Kapitel 2, 3 und 4) und *Oncorhynchus mykiss* (Regenbogenforelle, Kapitel 1), zum anderen mit Reaktionen verschiedener Süßwasserfische auf komplexe Belastungssituationen im Freiland (Kapitel 5 und 6). Zudem wird auf die Wechselwirkung von Chemikalien mit dem abiotischen Faktor Temperatur eingegangen (Kapitel 3). Die in Kapitel 1 bis 6 detailliert beschriebenen Versuche sind in größere Forschungsvorhaben eingebettet, deren Ziel es ist, bzw. war, zum einen Schädigungen in bestehenden Ökosystemen aufzuzeigen (Kapitel 6, Fluss Mureş, sowie Kapitel 5, Fluss Kizinga) zum anderen mögliche Schadwirkungen durch Chemikalien, die potentiell in die Umwelt gelangen können, zu untersuchen (Kapitel 1, Pharmazeutika; Kapitel 2-4, Schwermetalle und Pestizide). In allen Studien wurden mehrere Parameter untersucht, es wurden sowohl histologische, als auch biochemische Untersuchungen sowie Studien zur Embryotoxizität durchgeführt. Eine Aufstellung der Anteile dieser Promotionsarbeit an den jeweiligen Projekten kann dem Abschnitt „Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten“ ab Seite 24 entnommen werden.

2.2 Fragestellungen

Im Rahmen der vorliegenden Arbeit sollen Reaktionen von Süßwasserfischen auf (a) einzelne Schadstoffe, (b) Schadstoffe in Kombination mit unterschiedlichen Umgebungstemperaturen, (c) Mischungen von Schadstoffen und (d) komplexe Schadstoffbelastungen im Freiland untersucht werden. Grundlage für die

Beurteilung von Schadwirkungen sind dabei Untersuchungen auf histopathologischer, biochemischer und entwicklungsbiologischer Ebene. Eingebettet in größere Forschungsvorhaben werden Teilaspekte der jeweiligen Belastungssituationen untersucht und mit anderen Arbeiten in Verbindung gebracht.

3. Material und Methoden

3.1 Experimenteller Aufbau

Für die in Kapitel 1 beschriebenen Experimente wurden 1,5-1,8 Jahre alte Regenbogenforellen (*Oncorhynchus mykiss*) sowie, für die Untersuchungen mit Carbamazepin, 1,5 Jahre alte Karpfen (*Cyprinus carpio)* aus einer Zucht des Bayerischen Landesamtes für Umweltschutz gegenüber den angegebenen Pharmazeutikakonzentrationen in Quellwasser exponiert. Die Experimente wurden durch das Bayerische Landesamt für Umweltschutz durchgeführt. Die Exposition dauerte 28 Tage, sie wurde in Durchflusssystemen mit 100 L- (Diclofenac-Experimente) bzw. 160 L-Aquarien (übrige Experimente) mit einer Durchflussrate von 9 L / Stunde durchgeführt. Die Versuche fanden unter einem Lichtregime von 12 Stunden Helligkeit und 12 Stunden Dunkelheit statt, die Tiere wurden jeden zweiten Tag gefüttert. Kontrollen mit reinem Quellwasser und, falls im Experiment erforderlich, zusätzliche Kontrollen mit Quellwasser und Lösungsmittel wurden parallel zu den Expositionen durchgeführt.

Die in Kapitel 2-5 beschriebenen Embryotests erfolgten in Labors der Universität Tübingen. Die eingesetzten Eier stammen aus eigener Nachzucht eines Zebrabärblingsstammes (Wildtypstamm WIK, ZFIN ID: ZDB-GENO-010531-2). Die Versuchsdauer war so angelegt, dass die Eier vom Zeitpunkt der Befruchtung bis kurz nach dem Schlupf gegenüber den Schadstoffen, Schadstoffmischungen bzw. Freilandproben bei konstanter Temperatur und einem Licht- / Dunkelwechsel von 12:12 Stunden exponiert waren. Die Exposition fand in Glaspetrischalen (mit Ausnahme der Versuche in denen Nickelchlorid eingesetzt wurde, diese erfolgten in Plastikpetrischalen) statt. Während dieser Zeit wurden zu festgelegten Zeitpunkten verschiedene Parameter zur Embryonalentwicklung aufgenommen. Parallel dazu wurden für die Experimente, die in Kapitel 2-4 beschrieben sind, Eier bzw. Embryonen für eine Woche gegenüber den jeweiligen Stoffen und Mischungen sowie Kontrollwasser exponiert und anschließend auf ihren Gehalt an Stressproteinen hin untersucht. Stressproteinuntersuchungen fanden auch bei den

in Kapitel 6 beschriebenen Freilanduntersuchungen statt. Hier wurden in dem rumänischen Fluss Mureş (einem Zufluss der Theis (Tisza), welche wiederum in die Donau mündet) an vier Stellen dort einheimischen Fischen (Döbeln (*Leuciscus cephalus*) und Nasen (*Chondrostoma nasus*)) Leber und Kiemenproben entnommen. Diese wurden vor Ort in flüssigem Stickstoff gefroren und anschließend in Tübingen auf ihren Stressproteingehalt hin untersucht.
Die in Kapitel 5 mittels Embryotest untersuchten Freilandproben stammen aus Tansania. Im direkten Ausfluss einer Textilfabrik, welcher in den Fluss Kizinga mündet, und dort im Verhältnis von etwa 1:5 verdünnt wird, wurden Proben gesammelt. Die Tests wurden einerseits mit reinem Abwasser der Textilfabrik, andererseits mit polaren Fraktionen, welche chromatographisch gewonnen, gefriergetrocknet und in Tübingen wieder zur ursprünglichen Konzentration mit Kunstwasser für die Embryotests gelöst wurden, durchgeführt. Für eine erfolgreiche Versuchsdurchführung mit dem Abwasser bzw. seinen Auszügen musste eine Verdünnungsreihe der Proben hergestellt und verschiedene Konzentrationen der Originalproben im Embryotest getestet werden.

3.2 Histologische Untersuchungen

Nach der Exposition der Tiere wurden diese anästhesiert und mit einer Perfusionslösung aus Glutardialdehyd und Formaldehyd fixiert. Nach der Perfusion wurden Proben von Kiemen, Niere und Leber entnommen, diese wurden in kleine Stücke von 1-2 mm Länge geschnitten und in die auch für die Perfusion genutzte Fixierlösung gegeben. Für die anschließenden elektronenmikroskopischen Untersuchungen wurden die Proben in einem zweiten Fixans mit Glutardialdehyd in Cacodylatpuffer und weiter in Osmium-Ferrocyanid fixiert. Nach Waschen in Cacodylat- und Maleatpuffer erfolgte eine *en-bloc* Kontrastierung der Proben in Uranylacetat. Nach Entwässerung über eine aufsteigende Alkoholreihe wurden die Proben in Epon-Kunstharz eingebettet. Ultradünnschnitte der Proben mit einer Dicke von 50-100 nm wurden mit Bleizitrat gefärbt und an einem Transmissions-Elektronenmikroskop Philips Tecnai 10 ausgewertet. Die Auswertung erfolgte einerseits descriptiv, andererseits semiquantitativ über eine Kategorisierung der Schädigungsgrade.

3.3 Embryotests

Für die Embryotests wurden Eier von Zebrabärblingen gewonnen und nach möglichst kurzer Zeit nach Befruchtung exponiert. Die Eiablage wurde durch Anschalten des Lichtes der Aquarien am Morgen induziert, als Laichsubstrat

dienten Laichboxen, über denen die Weibchen ihre Eier ins Wasser geben. Ein Siebeinsatz in den Laichkästen verhinderte, dass adulte Fische die frisch gelegten Eier fraßen. Die Exposition der Eier begann eine Stunde nach Einschalten des Lichts, nur befruchtete Eier wurden untersucht. Die gewonnenen Eier wurden zufällig auf Petrischalen mit Kontrollwasser bzw. den jeweiligen Expositionskonzentrationen verteilt. Die Versuche wurden in Klimaschränken durchgeführt, um eine konstante Temperatur zu gewährleisten, die Beleuchtung wurde auf einen Hell-Dunkel-Rhythmus von 12h:12h eingestellt. Zu festgelegten Zeitpunkten (alle 12 Stunden, am ersten Tag des Versuchs erfolgte eine zusätzliche Kontrolle 8 Stunden nach der Befruchtung der Eier) wurden eine Reihe von Entwicklungs-Endpunkten betrachtet, um die Embryonalentwicklung der Tiere unter Belastung mit derjenigen unter Kontrollbedingungen zu vergleichen. Beobachtet wurde das Überleben der Embryonen sowie Schädigungengen und Fortschritte in der Entwicklung. Zu den Entwicklungs-Endpunkten zählen die erfolgreiche Gastrulation, Entwicklung von Augen, Somiten und Otolithen sowie die Ablösung des Schwanzes vom Dottersack, Herzschlag und regelmäßige Herschlagfrequenz. Zu den protokollierten Schädigungen zählen Veränderungen in der Herzschlagfrequenz im Vergleich zu Kontrolltieren, das Auftreten von Ödemen an Herz und Dottersack sowie Fehlentwicklungen von Wirbelsäule und Schwanz. Zudem wurde die Stärke der Pigmentierung der Zebrabärblingslarven protokolliert. Weitere Auffälligkeiten (z.B. Verhaltensauffälligkeiten) wurden abhängig von ihrem Auftreten zusätzlich vermerkt.

3.4 Stressproteinanalysen

Für die Stressproteinanalysen in Kapitel 2 und 4 wurden jeweils 10 Replika von je 8 gepoolten Zebrabärblingslarven, die von der Befruchtung der Eizelle bis sieben Tage nach der Befruchtung exponiert waren, für die weitere Untersuchung in Stickstoff schockgefroren. Für die Stressproteinanalysen in Kapitel 6 wurden Kiemen- und Leberproben nach Elektrobefischung vor Ort für jedes Tier individuell entnommen und ebenfalls in Stickstoff schockgefroren. Alle Proben wurden anschließend mit einer jeweils adäquaten Menge Extraktionspuffer homogenisiert und zentrifugiert. Der Gesamtproteingehalt des Überstandes wurde nach Bradford (1976) ermittelt. Zur Proteinauftrennung wurde eine modifizierte SDS-PAGE nach Laemmli (1970) durchgeführt, darauf folgte ein Western-Blot mit Peroxidasefarbreaktion. (Erster Antikörper: mouse anti-human hsp70 IgG, zweiter Antikörper: goat anti-mouse IgG, Peroxidase-Konjugat) Die Auswertung der Färbung der Proteinbanden erfolgte densitometrisch.

4. Ergebnisse und Diskussion

4.1 Kapitel 1: *Triebskorn R, Casper H, Scheil V, Schwaiger J (2007): Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout* (Oncorhynchus mykiss) *and common carp* (Cyprinus carpio). *Analytical and Bioanalytical Chemistry 387:1405-1416.*

Die Studie zeigte, dass mit allen untersuchten Pharmazeutika (mit Ausnahme der Clofibrinsäure) schon in sehr niedrigen, umweltrelevanten, Konzentrationen Effekte in Organen von Fischen hervorgerufen werden können. Die Untersuchungen der Kiemen der mit Metoprolol und Clofibrinsäure belasteten Regenbogenforellen (diese Teile der Studie sind der Eigenanteil an den Untersuchungen) zeigten, dass nach Belastung mit niedrigen Konzentrationen der jeweiligen Stoffe bereits Schädigungen der Kieme auftreten. So zeigten die Kiemen eine Ablösung des Epithels, Hyperplasien und Hypertrophien von Schleimzellen (bei 20 µg/L Metoprolol und höheren Konzentrationen) und Chloridzellen (bei 50 µg/L Metoprolol und höheren Konzentrationen) sowie Erweiterungen des Endoplasmatischen Retikulums in Chloridzellen unter Metoprololbelastung. Die gleichen Symptome waren unter Belastung mit Clofibrinsäure zu verzeichnen, diese traten jedoch in stärkerem Maße auf, eine signifikante Verschlechterung der Kiemen im Vergleich zur Kontrolle zeigte sich ab einer Clofibrinsäurekonzentration von 5 µg/L. Vergleichbar zu den Reaktionen der Kiemen auf Metoprololbelastung waren diejenigen nach Exposition gegenüber Carbamazepin. Wesentlich stärker waren die Schädigungen der Kieme nach Belastung mit Diclofenac: hier traten neben den oben genannten Reaktionen auch Nekrosen von Pfeilerzellen auf.

Auch Leber und Niere wurden durch Diclofenac am stärksten geschädigt, gefolgt von schwächeren Schädigungen durch Carbamazepin und Metoprolol. Am schwächsten waren die Reaktionen in den mit Clofibrinsäure (keine Effekte in der Niere) exponierten Tieren. Die Schädigungen in den Lebern der Tiere umfassten erhöhte Makrophagenzahlen, verminderte Glykogengehalte, Auftreten von Membranmaterial im Cytoplasma, Vesikulierungen des Endoplasmatischen Retikulums, zelluläre Desintegration im Disse'schen Raum sowie Zusammenbrüche der Zellkompartimentierung. Die Nieren der Tiere zeigten verdickte Basalmembranen in den Nierenkörperchen, Vesikulierungen und Verdickungen des Endoplasmatischen Retikulums und vergrößerte Mitochondrien im proximalen und distalen Tubulus, erhöhte Makrophagenzahlen und vermehrt auftretende sekundäre Lysosomen an den Zellbasen. Ausschließlich unter Diclofenacbelastung

traten Nekrosen in den Glomeruli sowie eine hyalintropfige Degeneration in den Zellen des proximalen Tubulus 1 auf.

Insgesamt zeigt sich ein organspezifisches und schadstoffspezifisches Reaktionsbild bei den Untersuchungen. Betrachtet man die LOECs („*lowest observed effect concentrations*“, die niedrigste untersuchte Schadstoffkonzentration, die einen signifikanten Effekt hervorruft), so zeigt sich, dass, mit Ausnahme der Clofibrinsäure, alle untersuchten Pharmazeutika in Konzentrationen, die auch in der Umwelt gefunden wurden (Rohweder & Friesel 2005, Sacher 2002, Lehmann 2000, Ternes 2001), Effekte bei einheimischen Fischen hervorrufen. Zudem wird deutlich, dass die gefundenen LOECs wesentlich niedriger liegen (Faktor 10-100), als dies in Standardtests mit *Daphnia magna* (Ferrari et al. 2003, Cleuvers 2005) oder *Danio rerio* (Hallare et al. 2004) der Fall ist. Basierend auf diesen großen Unterschieden in der Empfindlichkeit gegenüber Pharmazeutika zeigt sich, dass neben den akuten Standardtests auch chronische Tests mit einheimischen Spezies notwendig sind, um eine Risikoabschätzung im Bezug auf ungewünschte Nebenwirkungen von Pharmazeutika in der Umwelt durchzuführen. Dies wird u.a. auch von Fent et al. (2006) betont, welche zudem auch auf die Bedeutung von Mischungstoxizitätstests eingehen.

Kapitel 2: *Scheil V, Zürn A, Triebskorn R, Köhler H-R (eingereicht): Embryo development, stress protein (Hsp70) responses and histopathology in zebrafish* (Danio rerio) *following exposure to nickel chloride, chlorpyrifos and binary mixtures of them. Environmental Toxicology.*

Die Untersuchungen zu den Effekten von Nickelchlorid ($NiCl_2$) und Chlorpyrifos auf Zebrabärblinge erbrachten, abhängig vom betrachteten Parameter, unterschiedliche Ergebnisse. So führte eine $NiCl_2$-Belastung während der Embryonalentwicklung zu einer mit der $NiCl_2$-Konzentration korrelierenden Abnahme des Schlupferfolges. Dieser Effekt konnte auch bei anderen Fischarten unter Nickelbelastung nachgewiesen werden (Nebeker et al. 1985, Dave und Xiu 1991). Chlorpyrifos alleine hatte keinen Effekt auf die Embryonalentwicklung der Zebrabärblinge, Mischungen von $NiCl_2$ und Chlorpyrifos führten zu den gleichen Auswirkungen wie $NiCl_2$ allein, dies spricht für eine unabhängige Wirkung der beiden Stoffe, da sich keine Hinweise auf eine gegenseitige Abhängigkeit ergaben. Ein gleiches Bild zeigen die Stressproteinanalysen: hier führte Nickel mit zunehmender Konzentration erst zu einem ansteigenden, dann, bei weiter steigenden $NiCl_2$-Konzentrationen zu einem im Vergleich zur Kontrolle abnehmenden Hsp70-Gehalt. Im Versuch mit Chlorpyrifos wurde ein Anstieg des

Stressproteinlevels durch Belastung verzeichnet. In Mischungen konnte ein additiver Effekt der beiden Substanzen im Hinblick auf die Stressproteinreaktion beobachtet werden. Dies deckt sich mit den Ergebnissen aus dem Embryotest und weist erneut auf eine unabhängige Wirkung der Substanzen hin. In histologischen Untersuchungen (durchgeführt von R. Triebskorn, nicht Bestandteil der Dissertation) zeigte sich nur ein geringer Effekt der beiden Einzelsubstanzen. In Mischungen der beiden Substanzen wurde ein eher unabhängiger oder gering additiver Effekt der beiden Substanzen nachgewiesen. Eine statistische Bewertung der Mischungstoxizität mit dem Modell von Jonker et al. (2005), welches auf der Grundlage von Konzentrations-Wirkungsbeziehungen theoretische Mischungstoxizitäten errechnet und diese mit tatsächlichen Werten vergleicht, war nicht erfolgreich, da die Resultate der Einzelstoffuntersuchungen zu komplex für das genannte Modell waren.

Die Tests zeigten einen über alle betrachteten Parameter insgesamt additiven Effekt der beiden Substanzen bei generell moderaten Effekten sowohl der Einzelsubstanzen wie auch der Mischungen. Eine Ausnahme bildet dabei der verminderte Schlupferfolg unter Nickelchloridbelastung. wie auch der zusammenbrechende Hsp70-Level. Diese beiden Effekte traten schon weit unterhalb von Konzentrationen auf, die in der Natur anzutreffen sind. Im Freiland findet man bis zu 183 mg Ni/L in der Nähe von Nickel verarbeitender Industrie (Kasprzak 1987), in nicht von der Nickelindustrie beeinflussten Gewässern liegen die Nickelkonzentrationen deutlich niedriger, so berichtet Murkherjee (1998) von Konzentrationen von 0.14 to 4.0 µg in finnischen Flüssen. Chlorpyrifos scheint für Fische nur wenig toxisch zu sein, das Pestizid ist auch, zumindest theoretisch, auf seine Funktion als Insektizid für wirbellose Schädlinge zugeschnitten (U.S. EPA 2002). Nichtsdestotrotz führen beide Stoffe, alleine und in Mischungen, zu subletalen Schädigungen in der Embryonalentwicklung von Fischen und können deshalb, auf lange Sicht, zu Veränderungen im Lebenszyklus oder auf Populationsebene führen.

Kapitel 3: *Scheil V, Köhler H-R (eingereicht): Influence of nickel chloride, chlorpyrifos and imidaclopride in combination with different temperatures on the embryogenesis of the zebrafish,* Danio rerio. *Archives of Environmental Contamination and Toxicology.*

Nachdem Vortests ergaben, dass eine im Vergleich zur Standardtemperatur (26°C, nach Nagel (2002) und OECD (1992)) erniedrigte Wassertemperatur bereits allein zu Effekten auf die Embryonalentwicklung führte (Auftreten von Ödemen und

erhöhten Mortalitäten), wurden die Versuche zur Schadstoffauswirkung unter verschiedenen Temperaturen mit im Vergleich zur Standardtemperatur erhöhten Temperaturen durchgeführt. Nickelchlorid führte bei allen Temperaturen zu vermindertem Schlupferfolg bzw. zu Schlupfverzögerungen. Mit steigender Temperatur verstärkte sich dieser Effekt. Berücksichtigt man einerseits, dass der Effekt des Schlupfverzuges sowohl bei anderen Fischarten als auch unter Belastung mit anderen Schwermetallen beobachtet wurde (Nebeker et al. 1985, Dave & Xiu 1991, Hallare et al. 2005), und andererseits, dass in Zukunft mit global steigenden Temperaturen zu rechnen ist, so ist der mit steigender Temperatur zunehmende Effekt alarmierend.

Die Insektizide Chlorpyrifos und Imidacloprid hatten bei allen untersuchten Temperaturen keinen Effekt auf die Embryonalentwicklung der Zebrabärblinge. Lediglich in den höchsten untersuchten Chlorpyrifos-Konzentrationen waren unkontrollierte Zuckungen der Larven zu beobachten. Sobald der Test für andere Untersuchungen (bei 26°C) verlängert wurde, führten 600 und 100 µg/L Chlorpyrifos zum Tode der Larven. Studien von Levin et al. (2003 und 2004) zeigten, dass Chlorpyrifos in Konzentrationen von 100 ng/L und höher während der frühen Embryonalentwicklung zu Veränderungen im Schwimmverhalten von älteren Larven sowie zu Beeinträchtigungen in der räumlichen Wahrnehmung adulter Zebrabärblinge führen können. Imidacloprid scheint negative Wirkungen, die bei adulten Zebrabärblingen nachgewiesen wurden (96h LC50 10 mg/L (unpublizierte Daten, zitiert in Jemec et al. (2007)), bei Embryonen und Larven nicht zu entfalten.

Zusammenfassend ist festzuhalten, dass Nickelchlorid unter verschiedenen Temperaturen unterschiedlich starke, mit der Temperatur korrelierende Effekte, hervorruft, während Imidacloprid und Chlorpyrifos keine Effekte (mit Ausnahme von unkontrollierten Zuckungen bei sehr hohen Chlorpyrifoskonzentrationen) hervorrufen.

Kapitel 4 *Scheil V, Kienle C, Osterauer R, Gerhardt A, Köhler H-R (eingereicht): Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish* (Danio rerio) *embryos and larvae. Aquatic Toxicology.*

Ein Pestizid (Diazinon) und ein Abbauprodukt diverser Pestizide (3,4-Dichloranilin, 3,4-DCA) wurden in dieser Studie auf ihre Auswirkungen auf die Embryonalentwicklung (4 Tage Embryotest bzw. 11 Tage subchronischer Test), die Hsp70 Stressproteinreaktion und das Verhalten von Zebrabärblingsembryonen und Larven untersucht (Gegenstand der Dissertation sind die Stressproteinanalysen

(Hsp70) und die Embryotests bezüglich 3,4-DCA und der Mischungsexperimente. Die Verhaltenstests sowie die Untersuchungen zu Diazinon alleine wurden von C. Kienle bzw. R. Osterauer durchgeführt). Studien zu diesen beiden Substanzen bezogen sich bisher nur auf ihre Einzelwirkung, nicht jedoch auf das Verhalten von Mischungen der beiden Stoffe. Untersucht wurden die oben genannten Parameter unter Nutzung eines definierten Zebrabärblingsstammes (Wildtypstamm WIK, ZFIN ID: ZDB-GENO-010531-2, wie auch in Kapitel 2,3 und 5.). Die LOECs für 3,4-DCA lagen, über alle Parameter betrachtet, zwischen 0,25 mg/L (Hsp70 und Ödembildung im subchronischen Test) und >2 mg/L (Parameter des Embryotests außer Ödembildung). Für Diazinon wurden LOECs von 0,05 mg/L (Hsp70) bis 2 mg/L (Verhalten, Parameter des Embryotests) Diazinon ermittelt.

In Mischungen zeigten die beiden Substanzen additives Verhalten, dies war aufgrund der unterschiedlichen Wirkweise (3,4-DCA ist ein nichtspezifischer Stoffwechselhemmer während Diazinon ein spezifischer Acetylcholinlesterase-Hemmer ist) zu erwarten. Eine gegenseitige Beeinflussung der beiden Substanzen mit resultierendem antagonistischen oder synergistischen Effekt bezüglich ihrer Toxizität wurde nicht beobachtet. Die gefundenen Effekte entsprachen denen der Einzelstoffe. Eine statistische Bewertung der Mischungstoxizität mit dem Modell von Jonker et al. (2005) war auch in dieser Untersuchung nicht erfolgreich, da die Resultate der Einzelstoffuntersuchungen zu komplex für das genannte Modell waren.

Die gefunden LOECs liegen für beide Substanzen um den Faktor 10-100 über den in der Natur vorhandenen Maximalkonzentrationen von 1,5 µg/L (Planas et al. 2006; Bailey et al. 2000). Auch bei den Mischungen traten Effekte erst in nicht-umweltrelevanten Bereichen auf. Auch wenn diese Ergebnisse wenig für eine Gefährdung von aquatischen Ökosystemen durch diese beiden Substanzen sprechen, ist zu berücksichtigen, dass Zebrabärblinge im Vergleich zu anderen einheimischen Fischarten bekanntermaßen relativ unsensitiv auf Chemikalienbelastungen reagieren. Für adulte Regenbogenforellen wurden z.B. 96h LC_{50} Werte ermittelt, die für 3,4-DCAund Diazinon 4,5 bis 6 mal niedriger liegen als entsprechende Werte für adulte Zebrabärblinge (Keizer et al. 1979, Meier et al. 1979, Hodson 1985, Becker 1990).

Zusammenfassend zeigte sich, dass sowohl die Einzelsubstanzen, wie auch die Mischungen beider Stoffe in hohen Konzentrationsbereichen zu Schädigungen bei sich entwickelnden Zebrabärblingen führen. Der vielseitige Ansatz mit einer großen Bandbreite an Parametern zeigte, dass verschiedene Parameter, je nach

eingesetzter Substanz, unterschiedlich sensitiv reagieren und es somit empfehlenswert ist, möglichst breit angelegte Testbatterien einzusetzen.

Kapitel 5 *Kruitwagen G, Scheil V, Pratap HB, Wendelaar Bonga, SE (eingereicht): Developmental toxicity in zebrafish embryos* (Danio rerio) *exposed to textile effluents. Environmental Monitoring and Assessment.*

Untersuchungen von Kruitwagen et al. (2006) in Mangrovegebieten in der Nähe von Dar-es-Salaam (Tansania) haben gezeigt, dass Schlammspringer (*Periophthalmus argentilineatus*), die in durch Abwasser einer Textilfärberei verschmutzten Gebieten lebten, drastische Entwicklungsstörungen, die vor allem im Bereich der Augen auftraten, zeigten. Um Ursachen für diese Störungen, zu finden, wurden über eine Gegenstromchromatographie Auszüge aus Abwasserproben hergestellt, und es sollte überprüft werden, ob auch bei Zebrabärblingen Störungen in der Embryonalentwicklung auftreten, wenn sie diesen Proben gegenüber exponiert werden. (entspricht dem Eigenanteil an der Arbeit). Die Zebrabärblinge stellten eine Alternative für die nur schwer aufzuziehenden und zu haltenden Schlammspringer dar. Sowohl die reinen Abwässer wie auch die untersuchten polaren Fraktionen des Abwassers (welche auf die Ursprungskonzentration verdünnt wurden) führten, selbst in starker Verdünnung zu drastischen Effekten während der Embryonalentwicklung der Fische. So war der Schlupf sowie die Mortalität unter Vollabwasserbelastung ab einer Verdünnung von 1:50 oder geringer negativ beeinflusst, der Herzschlag sogar ab einer Verdünnung von 1:1000 und geringer.

Die polaren Extrakte hatten geringere Auswirkungen, signifikante Änderungen zeigten sich bei den Herzschlagraten ab einer Verdünnung von 1:30. Geringere Verdünnungen wurden nach den Erfahrungen mit der hohen Toxizität des Gesamtabwassers nicht getestet. Im Gegensatz zum Gesamtabwasser führten die polaren Extrakte zu keiner erhöhten Mortalität in den Verdünnungen 1:50 und 1:30. Dies weist darauf hin, dass die hohe Toxizität entweder von den apolaren Bestandteilen des Abwassers stammt oder aber die Mischung der Schadstoffe in ihrer Gesamtheit ein höheres toxisches Potential hat.

Ähnliche Untersuchungen mit Textilfabrikabwässern liegen für Enzymaktivitäten von Tilapien (Gadagbui und Goksøyr 1996) und Schlammspringern (Chhaya et al. 1997) vor. Auch hier konnten negative Auswirkungen der Abwässer gezeigt werden. Die von Kruitwagen et al. (2006) beobachteten Fehlentwicklungen der Augen von Schlammspringern konnten im

Embryotest mit Zebrabärblingen nicht hervorgerufen werden. Mögliche Erklärungen hierfür sind die unterschiedliche Entwicklungsdauer und damit Expositionsdauer der Zebrabärblinge im Vergleich zu den Schlammspringern während der Embryonalentwicklung oder auch mögliche weitere Verschmutzungen des Lebensraumes der Schlammspringer, welche im Rahmen der vorliegenden Studie nicht erfasst wurden. Auch eine spezifische Beeinflussung der genetischen Kontrolle der Augenbildung bei Schlammspringern ist nicht auszuschließen.

Kapitel 6 *Köhler H-R, Sandu C, Scheil V, Nagy-Petrica EM, Segner H, Telcean I, Stan G, Triebskorn R (2007): Monitoring pollution in river Mureş, Romania, Part III: Biochemical effect markers in fish and integrative reflection. Environmental Monitoring and Assessment. 127, 47-54.*

Im Rahmen eines Monitorprogrammes wurden in dieser Studie an Freilandproben von Döbeln und Nasen aus dem Fluss Mureş in Rumänien Stressproteinanalysen (Hsp70) durchgeführt und die Cytochrom P450 (CyP IA1)-Aktivität gemessen. Proben wurden an vier Stellen entlang des Flusses genommen, zwei Probestellen lagen vor der rumänischen Stadt Arad, zwei nach dieser Stadt und damit auch nach Einleitungen der städtischen Kläranlage. Beprobt wurden Lebern (Hsp70 und CyP IA1-Untersuchungen) und Kiemen (Hsp70-Untersuchungen) der Fische, begleitend wurden histologische Untersuchungen durchgeführt (Triebskorn et al. *im Druck*). Gegenstand der vorliegenden Promotionsarbeit sind die Stressproteinanalysen (Hsp70) in den Freilandproben.

Gefunden wurden erhöhte Stressproteinlevel an Probestelle 1 (weit vor Arad) und 3 (direkt nach Arad) in den Kiemen der Döbel. Bei den Nasen wurden erhöhte Stressproteinlevel an Probestelle 3 gefunden, während der Stressproteinlevel an Probestelle 1 sehr niedrig lag. Die Stressproteinlevel der Lebern der beiden Fischarten waren an allen Probestellen gleich. Die erhöhten Stressproteinwerte an zwei Probestellen und der stark erniedrigte Stressproteingehalt an einer Probestelle (er weist auf eine Störung in der Stressproteinsynthese hin) zeigen proteotoxischen Stress der Tiere. Probestelle 1 liegt im Einzugsgebiet transsilvanischer Mienen und Metall verarbeitender Industrie, welche Cadmium und Kupfer in die Umwelt freisetzen. Probestelle 3 liegt im Bereich der Abwasserbelastungen durch Arads städtische Kläranlage, an beiden Probestellen ist demnach mit chemischen Belastungen zu rechnen.

Die Cytochrom P450 Aktivität, gemessen als 7-Ethoxyresorufin-ODeethylase-Aktivität (EROD-Aktivität) war in den Lebern der Nasen höher als in denen der

Döbel, unabhängig von der Probestelle. Erhöhte EROD-Aktivitäten wurden an Probestelle 3 bei Döbeln und an Probestelle 4 bei Nasen gefunden. Beide Probestellen liegen stromabwärts von der Stadt Arad. Dass die EROD-Aktivität, welche Hinweise auf organische Schadstoffbelastungen gibt (Stegemann und Hahn 1994, van Veld et al. 1997, Whyte et al. 2000, Navas et al. 2003), bei Nasen höher lag als bei den Döbeln, mag mit deren unterschiedlichen Fraßverhalten zusammenhängen. So fressen Döbel bevorzugt freischwimmendes Plankton, während Nasen Aufwuchs- und Detritusfresser sind und damit verstärkt organischen Schadstoffen, welche im Sediment akkumulieren, ausgesetzt sind. Die erhöhten EROD-Aktivitäten flußabwärts der Stadt Arad weisen auf organische Schadstoffe hin, die über die lokale Kläranlage in den Fluss gelangen.

Gemeinsam mit weiteren Untersuchungen (Sandu *im Druck*, Triebskorn et al *im Druck*) zeigt sich, dass unter Einbeziehung mehrerer Parameter die Charakterisierung des ökotoxikologischen Zustandes des Flusses Mureş möglich ist. Vor allem in Regionen, in denen ökotoxikologische Daten zur Wasserqualität fehlen, kann eine solche Untersuchung wichtiges Datenmaterial zur Güteklassifizierung eines Gewässers bereitstellen. Aufgrund der integrierenden Form der untersuchten Biomarker ist es möglich, einen Gesamteindruck der Wasserqualität eines Fliessgewässers zu erlangen ohne chemische Analysen zu nutzen, welche im Zweifelsfall nicht alle potentiell vorhandenen Schadstoffe erfassen können.So zeigte die vorliegende Untersuchung in Kombination mit den oben genannten Paralleluntersuchungen, dass die Probestellen unterschiedlich stark belastet sind. An Probestelle 4 wurde lediglich eine erhöhte EROD-Aktivität nachgewiesen, was für eine relativ schwache Belastung spricht, während an Probestelle 3 die Stressproteinlevel sowie die EROD-Aktivität Auffälligkeiten zeigten. Probestelle 1 und 2 scheinen am stärksten belastet zu sein, hier wurden histopathologische Schädigungen (Triebskorn, *im Druck*) sowie Reaktion auf molekularer Ebene (Hsp70 und EROD)-Aktivität) festgestellt.

Abschließende Betrachtungen

In Kapitel 1 bis 6 konnte gezeigt werden, dass Biomarker, speziell die Embryotests sowie die Stressproteinuntersuchungen, geeignet sind, zum einen Schadwirkungen von Einzelsubstanzen und definierten Mischungen, zum anderen Effekte von komplexen Schadstoffbelastungen im Freiland aufzuzeigen. Möglichst mehrere Parameter müssen untersucht werden, um ihren unterschiedlichen, aber nicht immer vorhersagbaren Sensitivitäten bei unterschiedlichen Schadstoffen gerecht zu werden. Die Untersuchungen zu Mischungstoxizitäten zeigen den Bedarf weiterer

Forschung zu dem Thema auf, insbesondere auch den Bedarf an mathematischen Modellen, die dazu dienen können, die Mischungstoxizitäten besser beschreiben. Da die vorhandenen Modelle meist mit sehr einfachen Annahmen arbeiten und komplexere Antworten auf Schadstoffbelastung nicht oder nur schwer simulieren können, ist in diesem Bereich großer Handlungsbedarf angezeigt. Nichtsdestotrotz ist auch die Bewertung sehr komplexer und nur bedingt bekannter Belastungen im Freiland mit den angewandten Methoden möglich.

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten

Kapitel 1:

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Kompletter Eigenanteil an der Probengewinnung, -aufbereitung und -bewertung der Kiemenproben aus den Experimenten mit Clofibrinsäure und Metoprolol. Die Gewinnung, Bearbeitung und Auswertung des weiteren Probenmaterials wurde von R. Triebskorn, H. Casper und J. Schwaiger durchgeführt. Fachliche Betreuung durch Prof. Dr. R. Triebskorn (Universität Tübingen).

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Scheil V, Zürn A, Triebskorn R, Köhler H-R (eingereicht): Embryo development, stress protein (Hsp70) responses and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos and binary mixtures of them. Environmental Toxicology.

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Kompletter Eigenanteil an der Probenaufbereitung und -bewertung der Stressproteinproben (Hsp70) unter Mitwirkung der Praktikantin E. M. Nagy-Petrica. Probenahme und Bearbeitung der Proben, die nicht auf Hsp70 hin untersucht wurden, erfolgten durch die weiteren Autoren (Köhler, Sandu, Segner, Telcean, Stan, Triebskorn). Fachliche Betreuung durch Prof. Dr. H.-R. Köhler (Universität Tübingen).

Kapitel 1: Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*)

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Abstract

In order to assess potential effects of human pharmaceuticals in aquatic wildlife, laboratory experiments were conducted with carbamazepine, clofibric acid, metoprolol and diclofenac using fish as test organisms. For each substance, at least one environmentally relevant concentration was tested. In liver, kidney, and gills of trout and carp exposed to carbamazepine, clofibric acid, and metoprolol, ultrastructural effects were qualitatively described and semi-quantitatively assessed. The obtained assessment values were compared with previously published data for diclofenac-induced effects in rainbow trout tissues. Quantitative analyses of protein accumulated in kidneys of diclofenac-exposed trout corroborated previously published data which indicated diclofenac to induce a severe glomerulonephritis resulting in a hyaline droplet degeneration of proximal kidney tubules. The investigations provided information on the general health status of the pharmaceutical-exposed fish, and allowed a differential diagnosis of harmful effects caused by these human pharmaceuticals in non-target species. For the different cytological effects observed, LOECs for at least three of the test substances (diclofenac, carbamazepine, metoprolol) were in the range of environmentally relevant concentrations (1 µg/L).

Keywords: pharmaceuticals, liver, gills, kidney, trout, carp

Analytical and Bioanalytical Chemistry 387,1405-1416

Introduction

According to the directive 2001/83/EU modified by the directive 2004/27/EU, the application for authorization of human pharmaceuticals has to include an environmental risk assessment [1] which shall be conducted according to the guideline on the environmental risk assessment of medicinal products for human use [2]. It should be based on evaluations of predicted environmental concentrations (PEC) of the respective substances in the environment and expected predicted no-effect concentrations (PNEC) in exposed species. During the last decade, large amounts of analytical data for human pharmaceuticals in aquatic environments were collected [3, 4, 5, 6, 7, 8]. In surface waters, e.g., maximum concentrations of 1 – 2 µg /L diclofenac [9], 1.6 µg /L carbamazepine [6], 1.1 µg /L clofibric acid [4], and 2.2 µg /L metoprolol [10] were found. In contrast, effect data for the chronic toxicity of human pharmaceuticals in wildlife are still scarce. In our opinion, however, such data are also necessary for a realistic environmental risk assessment of pharmaceuticals, since these substances were designed to exert distinct molecular modes of actions in cells, they often are effective when applied in low concentrations – as e.g. hormonally acting products – and they are not expected to exert a high general toxicity. Furthermore, standard toxicity tests have already been shown to be less sensitive than selected non-standard tests which particularly take into account the specific modes of action of these substances [11]. Consequently, a risk assessment exclusively on the basis of routine effect tests, e.g. on *Daphnia* motility would likely underestimate the toxicity of pharmaceuticals for wildlife species.

Our approach to investigate cellular effects in pharmaceutical-exposed fish is based on the knowledge that histological and cytological investigations are suitable and sensitive tools to assess the health of exposed organisms and to determine pollutant-specific syndromes possibly related to distinct modes of action of the respective chemicals in their organs [12, 13, 14].

In the present paper, we present sublethal effects of diclofenac, carbamazepine, clofibric acid and metoprolol in liver, kidney, and gills of exposed fish, and compare the obtained LOECs to those published on the basis of conventional effect tests for environmental risk assessment analyses.

Experimental

Experimental design

At the experimental station of the Bavarian Environmental Agency in Wielenbach, Germany, four laboratory experiments were carried out using carbamazepine (purity: > 98%), clofibric acid (purity: > 97%), metoprolol (purity: >99%), and diclofenac (purity: >98%) as test substances. All test substances were purchased from Sigma Aldrich (Deisenhofen, Germany). For the tests with clofibric acid, metoprolol and diclofenac, 1.5-1.8-years-old rainbow trout (*Oncorhynchus mykiss*) (average body weight: 180.4 ± 20.9 g; average body length: 26.6 ± 1.03 cm) were exposed to nominal concentrations of 1, 5, 20, 50, or 100 µg/L clofibric acid, or 1, 5, 20, 50, or 100 µg/L metoprolol, or 1, 5, 20, 100, or 500 µg/L diclofenac, respectively, for 28 d under flow-through conditions (water flow rate: 9 L / h). Due to the lack of rainbow trout of adequate size and health quality, the experiments with carbamazepine were conducted with 1.5 years-old carp (*Cyprinus carpio*) (average body weight: 370 g; average body length: 27 cm). These were exposed to 1, 5, 20, 50, or 100 µg/L carbamazepine also for 28 d under flow-through conditions (water flow rate: 9 L/h). Taking together all experiments, deviations of measured real concentrations from the nominal concentrations were between 0.54 % and 10.6%. Fish for all experiments were obtained from the breeding stock of the Bavarian Environmental Agency and were reared under disease-controlled conditions. During the experiments, fish were fed a commercially available food (Trouvit, F4-Proaqua 18) every second day (1 % of body weight). The photoperiod was maintained in a 12:12 h light-dark regime including a half-light phase of 30 minutes every morning and evening. Sex of all fish, in which gonads were only slightly to moderately developed, were individually recorded. Experiments took place in either 100 L aquaria (diclofenac experiment) or 160 L aquaria (other experiments) each containing 24 fish. For the experiments with all pharmaceuticals, control fish were kept in natural well water which was regularly checked for chemical and physical parameters (water controls). Ammonia, nitrate and nitrite were far below critical limits. Other parameters such as oxygen saturation (70 %), hardness (378.6 mg/L $CaCO_3$), and conductivity (730 µS/cm) were also in the well tolerable range. For the experiments with diclofenac and carbamazepine, which required the use of dimethylsulfoxid (DMSO) as a solvent, additional control fish were exposed to 0.012 % DMSO (diclofenac experiment) or 0,002 % DMSO (carbamazepine experiment), respectively, as a solvent control. The concentration of DMSO in the solvent control corresponded to the DMSO concentration present in

the test water containing the highest drug concentration. Chemical concentrations in the test waters were determined once a week throughout the exposure period by GC/MS (clofibric acid, metoprolol), HPLC/DAD (diclofenac), or both (carbamazepine) at DSG Biotec, Aschau, Germany, and residue analyses in fish organs were performed at the end of the experiment. Part of these analytical data have been published by Schwaiger and colleagues [15].

Anaesthetization, perfusion and dissection of fish

After 28 days of exposure, 6-8 fish per group were anaesthetized in a solution of ethylenglycol monophenylether (Merck, Darmstadt) in water at a concentration of 1:1000. After anaesthetization, fish were perfused *in situ* via the ventricle with ice-cold perfusion fixative containing 1.5 % glutardialdehyde and 1.5 % formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M sodium phosphate buffer (pH 7.6). The fixative contained 2.5 % polyvinylpyrrolidone (PVP). After perfusion, the two outer lamellae of the left gills, a middle portion of the posterior kidney, and an anterior portion of the liver were excised. The tissues were cut into pieces of about 1-2 mm length and then transferred into a fresh portion of perfusion fixative. Generally, the perfusion method was conducted according to [16] which was optimized for studies in liver and kidney. However, in order to avoid rupture of the gill lamellae which also were investigated in the present study, we renounced flushing with physiological fish saline and perfusion took place only about 1 min with low pressure, resulting in livers not to be completely perfused.

Sample preparation for electron microscopic studies, protein staining and quantification

For sample preparation, a published protocol [17, 18] was used including a second fixation in 2.5% glutardialdehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.6) containing 4% PVP and 0.05% calcium chloride for several days, and a third fixation in 1% osmium ferrocyanide for 1 h at 4°C [19]. After washing in 0.1 M cacodylate and 0.05 M maleate buffer (pH 5.2), tissue samples were stained *en bloc* with 1% uranyl acetate (dissolved in 0.05 M maleate buffer) overnight at 4 °C. The specimens were then dehydrated in a graded series of ethanol and embedded in Epon resin. Ultrathin sections (50-100 nm) were stained with 2.7% alkaline lead citrate [20] for about 1 min and examined in a Zeiss CEM 9 (diclofenac and carbamazepine samples) or in a Philips Tecnai 10 (clofibric acid and metoprolol samples) electron microscope. Per animal and organ, two samples, and per sample, 5-7 sections were investigated. Additionally, for each diclofenac-exposed individual,

semi-thin sections (1 µm) were cut at twelve different regions of a each kidney sample. These were stained for protein with Ponceau S (according to Gori [21]). The minimum distance between two sections was 100 µm. The stained protein was quantified using computer-based quantitative morphometry (Openlab 2.2.5 connected to Zeiss Axioplan) at the light microscope level. The protein content was expressed as % of the tubulus area.

Assessment of cytopathology

In the three organs, the following functional units (organelles in the liver, distinct cell types in the gills and more complex functional portions of the kidney and the gills) were examined:

Liver: In the hepatocytes, the cellular compartmentation, the cytoplasm, the nuclei, the ER, the Golgi apparatus, mitochondria, peroxisomes, lysosomes, glycogen and lipid storage, macrophage infiltration, and cellular debris, the bile canaliculi and the spaces of Disse were examined and assessed as functional units. Per animal, a total area of about 20 hepatocytes was analysed.

Kidney: Renal corpuscles (RC) (based on the structure of podocytes, endothelial cells, and the basal lamina), and sections of anterior (PI) and posterior portions (PII) of proximal tubules, and distal tubules (DI) (based on the structure of cytoplasm, nuclei, endoplasmic reticulum, Golgi apparatus, mitochondria, cell apices with microvilli, cell bases with basal labyrinth, basal lamina, vesicles and vacuoles, pinocytotic activity and storage products) were investigated as functional units. Per animal, 8 kidney sections were analysed.

Gills: Entire primary filaments (PF) and secondary lamellae (SL) (based on the structure of cytoplasm, nuclei, ER, Golgi apparatus, mitochondria, macrophage infiltration, cellular debris and intercellular spaces in epithelial cells) as well as chloride and pillar cells (based on the structure of nuclei, microvilli, mitochondria, ER, and Golgi apparatus) of the secondary lamellae were examined and assessed as functional units. Per animal, three primary filaments and 20 secondary lamellae were examined.

The health status of these functional units was qualitatively described and, in a second step, semi-quantitatively assessed by means of a classification into the following three categories: (category 1): 'control' state, (category 2): deviations from the 'control' state indicating a reaction of the animal to the exposure and/or alterations of the metabolism with slight pathologies visible, (category 3): major changes from the 'control' state with strong reactions or clearly visible damage. In the liver, for example, the structure of the ER was evaluated as "category 1" when

long, in parallel arranged ER cisternae were present, as “category 2”, when only parts of the ER showed slight reactions like vesiculation or degranulation, and as “category 3” when, in all hepatocytes, the ER was strongly vesiculated or the cisternae were disintegrated. All criteria for the classification of ultrastructural effects in the respective functional units can be taken from the work of Gernhöfer et al. [18].

For each organ, the cytopathology of each functional unit was assessed according to one of the three categories. Then, for each exposure group, a mean assessment value [MAV] was calculated for each functional unit in order to allow a differential diagnosis of the specific symptoms in the respective organs of the exposure groups. In order to assess the overall integrity of the respective organ, in addition, a second MAV for the entire organ was calculated as a mean of all MAVs which have been calculated for the respective functional units [22,23].

Statistical analyses

Data were tested for normal distribution using the Shapiro-Wilk W test. Since data were not normally distributed, significance of differences between two respective test groups was tested by the non-parametric Kruskal Wallis test using SAS JMP 4.0.0. Possible correlations between sex and weight of the test animals and the glycogen content in their liver were examined using the same software. Levels of significance were set to $p \leq 0.001$ (*** highly significant), $0.001 < p \leq 0.01$ (** significant), and $0.01 < p \leq 0.05$ (* slightly significant).

Results and discussion

Liver

In control fish of both species, the hepatocytes were well compartmented and characterized by a centrally located nucleus surrounded by tubular and a few vesicular endoplasmic reticulum (ER), numerous mitochondria and by large glycogen storage sites (Fig. 1). The contact areas with blood vessels were characterized by short microvilli of the hepatocytes and flat extensions of endothelial cells. The spaces of Disse were narrow and rarely, macrophages (Kupffer cells) were found within them (Fig. 2).

When comparing the four pharmaceuticals tested, the most prominent reactions in the liver were found in diclofenac-exposed trout with significant differences from the solvent control at 1µg/L diclofenac and higher (Fig. 3). The diclofenac-related symptoms were described, documented and discussed in detail by Triebskorn and colleagues [23].

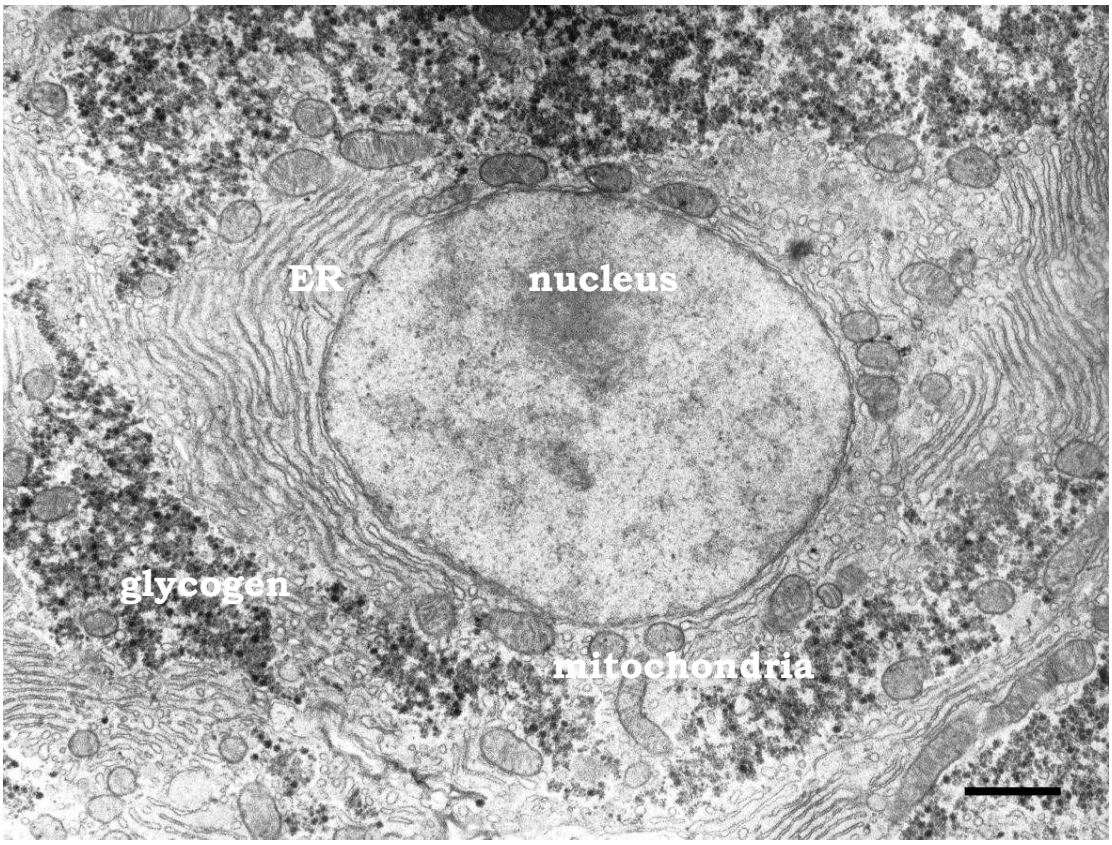

Fig. 1: Liver of rainbow trout (control animal) with large amounts of mitochondria, ER and glycogen. Scale bar: 1 µm.

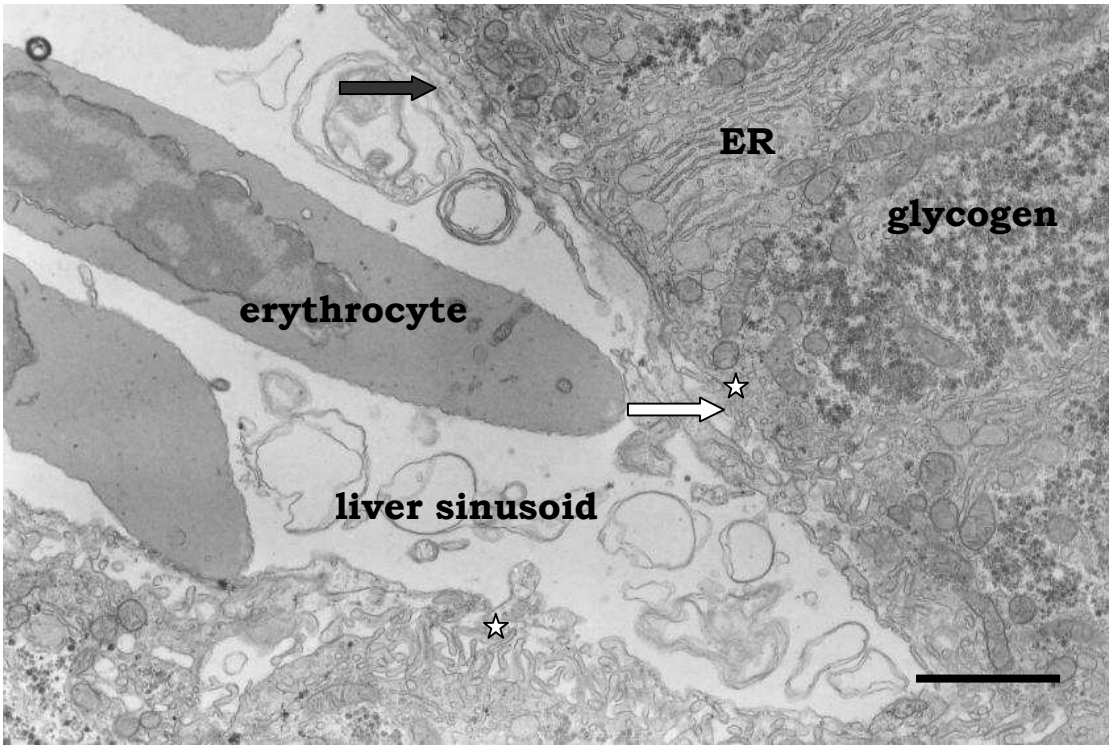

Fig. 2: Basal parts of hepatocytes adjacent to a blood vessel with short microvilli (asterisks) in a control rainbow trout. White arrow: space of Disse; black arrow: endothelial cell. Scale bar: 1 µm.

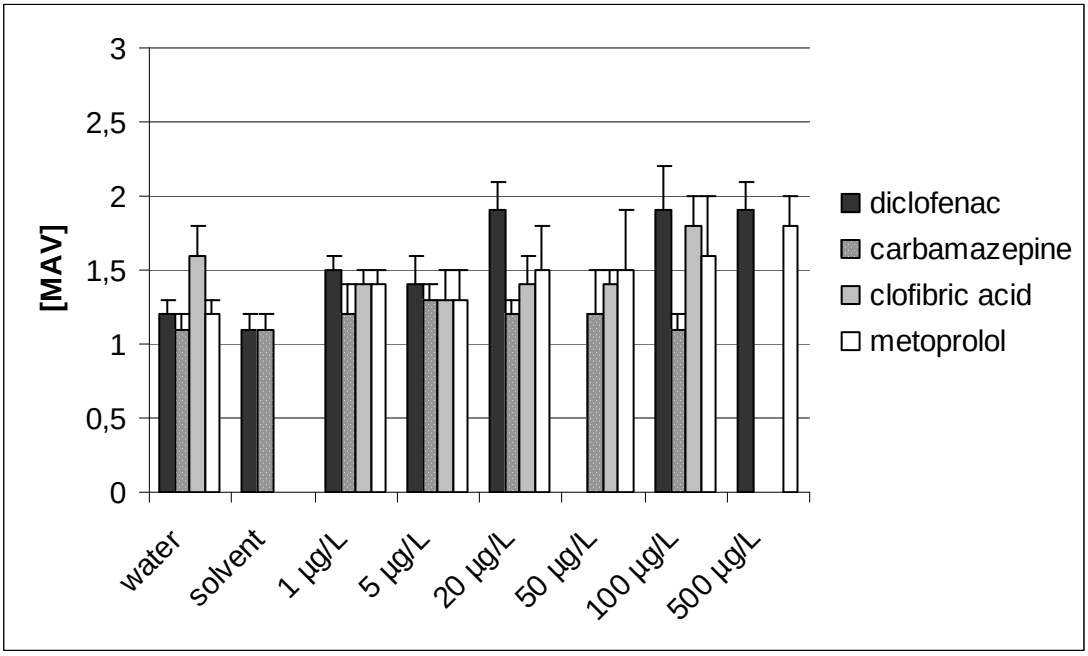

Fig. 3. Semi-quantitative assessment of cytopathology in the liver. "1" indicates the control state. The following significances of difference were found (w: water control, s: solvent control): diclofenac: w,s/5 (*); w,s/1,20,100,500 (**); metoprolol: w/1,50,100 (*); w/20 (**); w/500 (***). Data for diclofenac were extracted from Triebskorn et al. [23].

In carbamazepine-exposed carp, cellular reactions in response to the pharmaceutical were much less pronounced than in diclofenac-exposed trout. Only very few fish showed an increased number of macrophages in their livers and a slight increase in the amount of membrane material in the cytoplasm. No clear concentration-effect relationships became obvious for these effects in the liver of carbamazepine-exposed fish.

In clofibric-acid exposed trout, only in fish exposed to the highest concentration (100 µg /L) moderate effects were found in the liver, which, however, were not significantly different from controls. These included a slight dilation of blood vessels and the occurrence of membrane material in intercellular spaces. Since clofibric acid is known to be metabolized in the liver of mammals via glucuronidation [24], and since enzymes involved in this biotransformation process (UGTs) are localized in the ER, we expected structural responses of the ER, as e.g. proliferation, vesiculation, degranulation, which, however, could not be found. In addition, no significant proliferation of peroxisomes in the hepatocytes became obvious, probably since quantitative methods were not applied. This may have been be expected since clofibric acid has been described to induce the peroxisome proliferator-activated receptor (PPAR) leading to peroxisome proliferation [25, 26].

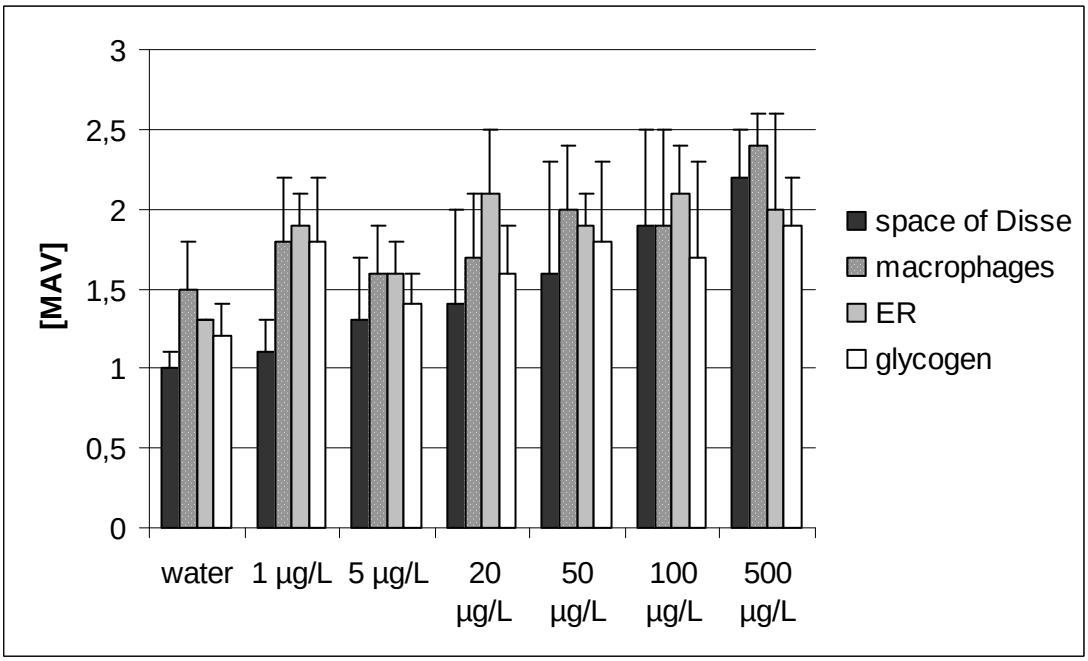

Fig. 4. Semi-quantitative assessment of reactions in distinct functional units of the liver in metoprolol-exposed trout. "1" indicates the control state. The following significances of difference were found (w: water control): space of Disse: w/100,500 (***); macrophages: w/50 (*); w/500 (***); ER: w/1,5,10 (**); w/20, 500 (***); glycogen: w/50 (*); w/1,20,100 (**); w/500 (***).

This lack of such an expected cellular response, however, in our case cannot be attributed to a possible deviation of nominal from real chemical concentrations in the exposure tanks, since real and nominal concentrations were proven to be very similar (e.g. real 487±2.52 µg/L vs. nominal 500 µg/L clofibric acid).

In metoprolol-exposed trout, clear concentration-related effects were observed in their livers with significant differences between controls and fish exposed to 1 µg /L metoprolol or higher concentrations (Fig. 4). Symptoms which were already found in fish exposed to 1 µg /L metoprolol included the reduction of glycogen stores combined with the occurrence of membrane material within the cells, plus a vesiculation, dilation and irregular orientation of the ER (Fig. 5, 6) As a result of these reactions, the compartmentation of the cells was less developed than in the controls. Generally, in intercellular spaces and the spaces of Disse, many macrophages occurred (Fig. 6) In addition, already in fish exposed to 1 µg /L metoprolol, the cell surfaces lining the spaces of Disse were characterized by long, irregularly oriented microvilli, whereas in controls only short or no microvilli were observed (comp. Figs. 2, 7, 8). This symptom became much more pronounced in fish exposed to higher concentrations. In addition, in the spaces of Disse and in the intercellular spaces between the hepatocytes, moderately electron-dense, flocculent

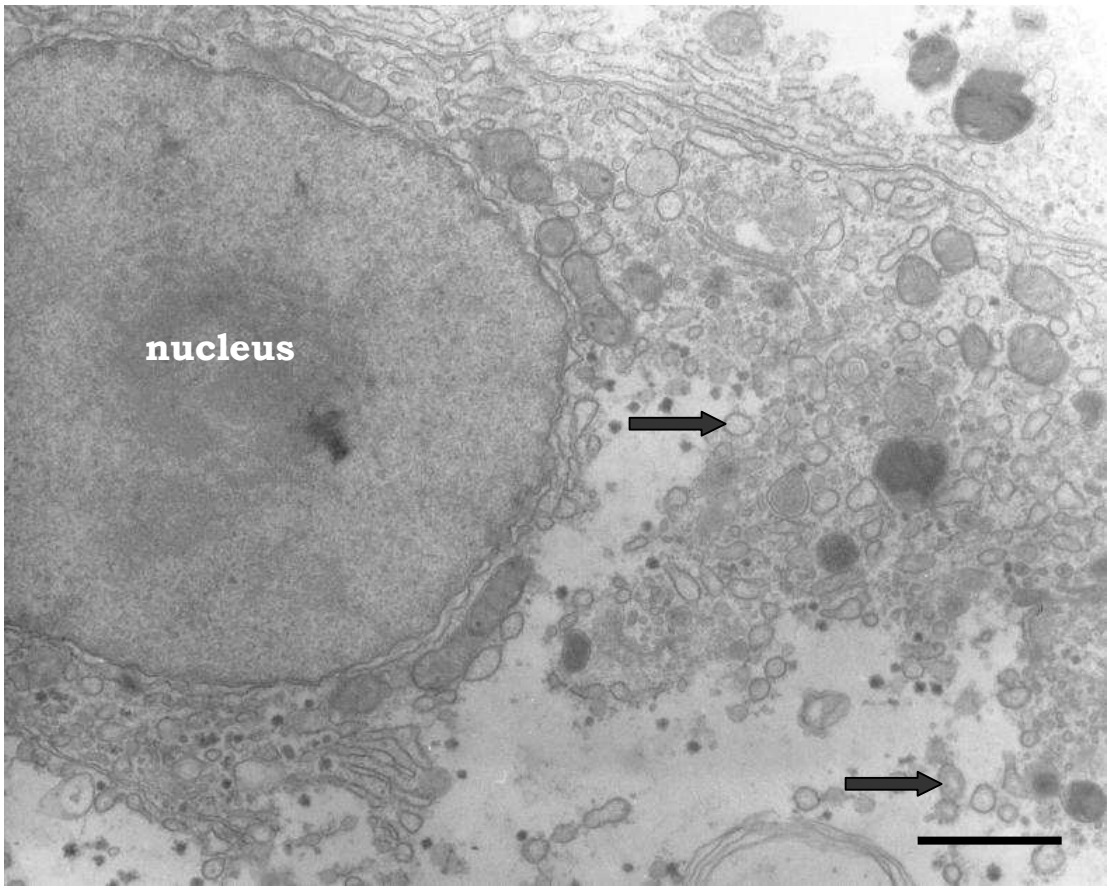

Fig. 5: Hepatocyte of a metoprolol-exposed rainbow trout (500 µg /L) with reduced glycogen content and vesiculated ER (black arrow). Scale bar: 1 µm.

material (Figs. 8, 9) and macrophages were found. The cytoplasm of the hepatocytes close to the spaces of Disse became completely vesiculated (Fig. 9).

Since trout has been shown to contain β_2–receptors in the heart and liver [27] which are structurally very similar to other vertebrate homologues [28] it is likely that β_2-receptor-antagonists, like metoprolol exert their specific action also in fish. In humans, metoprolol causes a reduction in the liver blood flow due to a decrease in the heart rate and the cardiac output [29]. Possibly, the observed cellular alterations in the vicinity of the spaces of Disse in metoprolol-exposed trout could be interpreted as structural reactions compensating for a reduced supply with oxygen and nutrients. In a recent publication, Larsson and colleagues [30] showed that the β -blocker propranolol did not influence the heart rate in rainbow trout – however, after a short-term exposure of 48 h only. Whether or not a longer exposure – like in the present study - would lead to a similar result remains to be investigated. With respect to metabolic degradation, it is known for mammals that metoprolol undergoes oxidative metabolism in the liver primarily by the microsomal

CYP2D6 isoenzyme [31]. In the metoprolol-exposed trout, the structural reactions of the ER (severe vesiculation and dilation) in cellular areas adjacent to the hepatic vessels might indicate an activation of enzymes equivalent to mammalian cytochromes of the CYP family, and thus, also an induction of biotransformation processes in the fish liver.

In contrast to severe cytotoxic effects of diclofenac, carbamazepine and clofibrate which have been reported for cultured fish cells [32], the *in vivo* reactions in the livers of pharmaceutical-exposed fish observed in the present study did not represent severe lesions but were more likely related to metabolic responses of this organ. Nevertheless, the effects resulting from an exposure to diclofenac or metoprolol were much more pronounced than those related to clofibric acid or carbamazepine. Generally, they point out (1) the energy demand of exposed fish for coping with the respective chemical (resulting in glycogen reduction), (2) an adaptation of the cellular functions to an activated drug metabolism (alterations of the ER) and (3) cellular adaptations in the vicinity of the blood vessels probably related to an activated metabolism and/or an altered blood pressure (alterations of cellular portions close to the spaces of Disse).

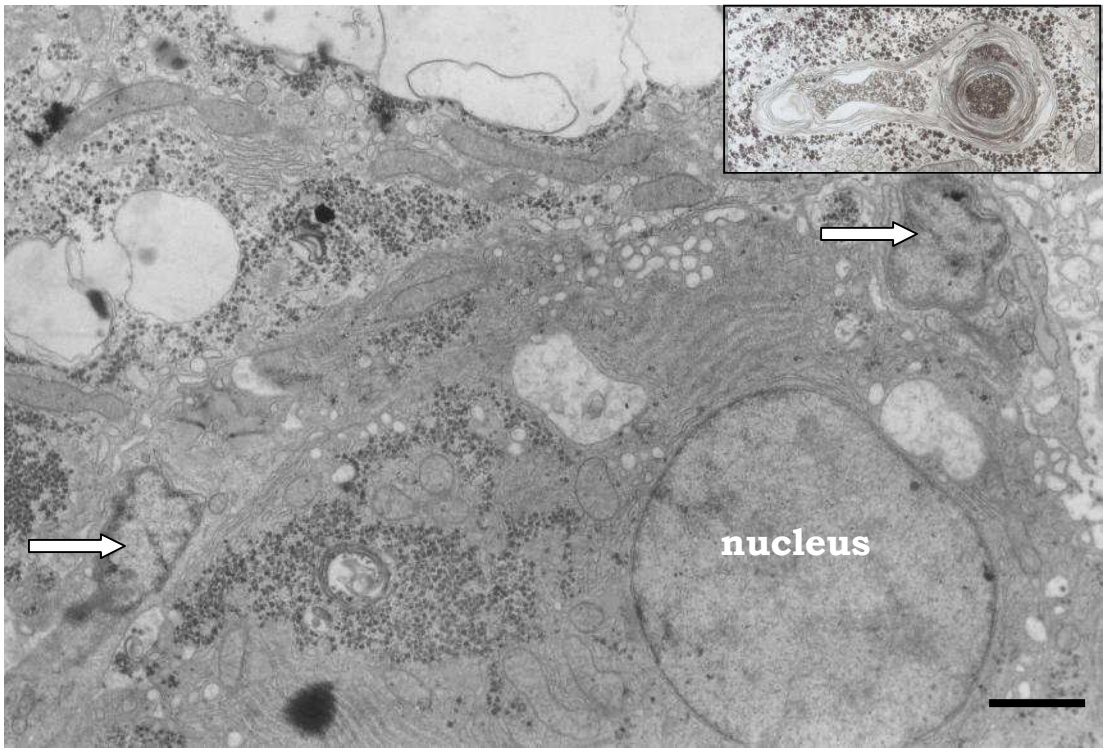

Fig. 6: Hepatocyte of a metoprolol-exposed rainbow trout (100 µg /L) with membrane material occurring in the areas where glycogen has been reduced (black arrows and inlet).Two macrophages are shown (white arrows). Scale bar: 1 µm.

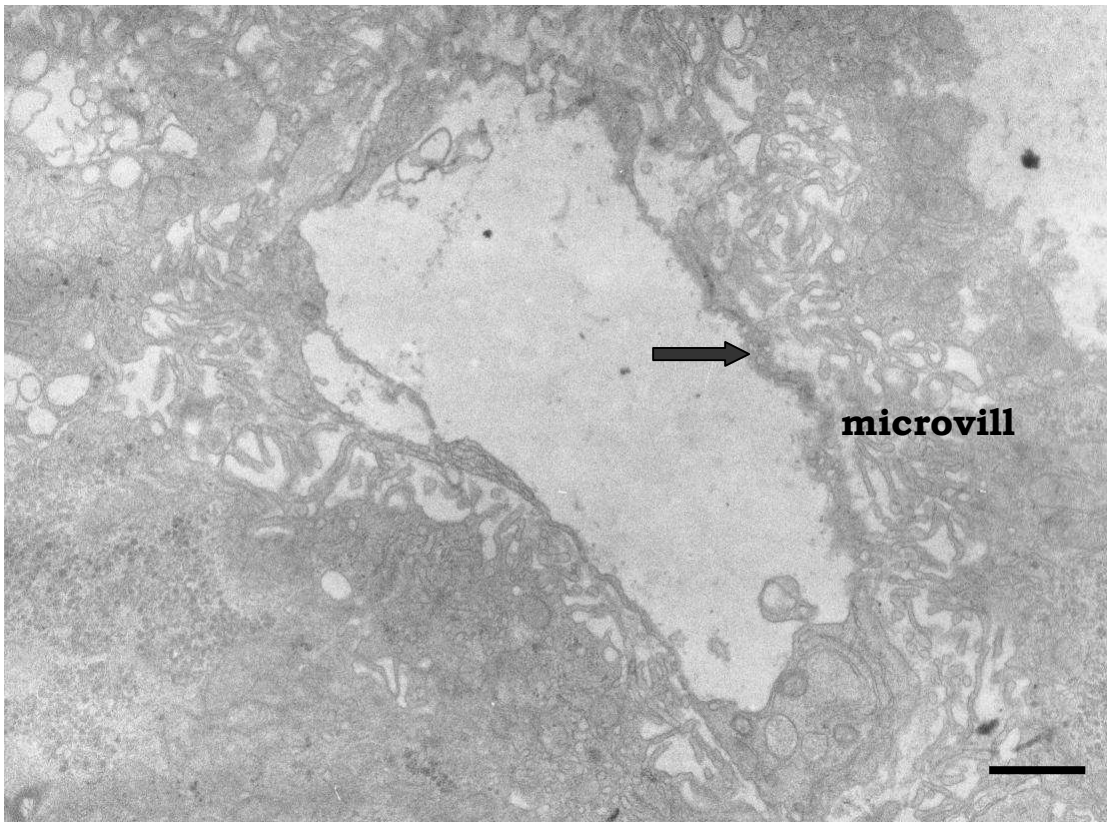

Fig. 7: Overview over a sinusoid in a metoprolol-exposed rainbow trout (500 µg /L) with an endothelial cell (black arrow) and irregularly oriented microvilli of the hepatocytes. Scale bar: 1 µm.

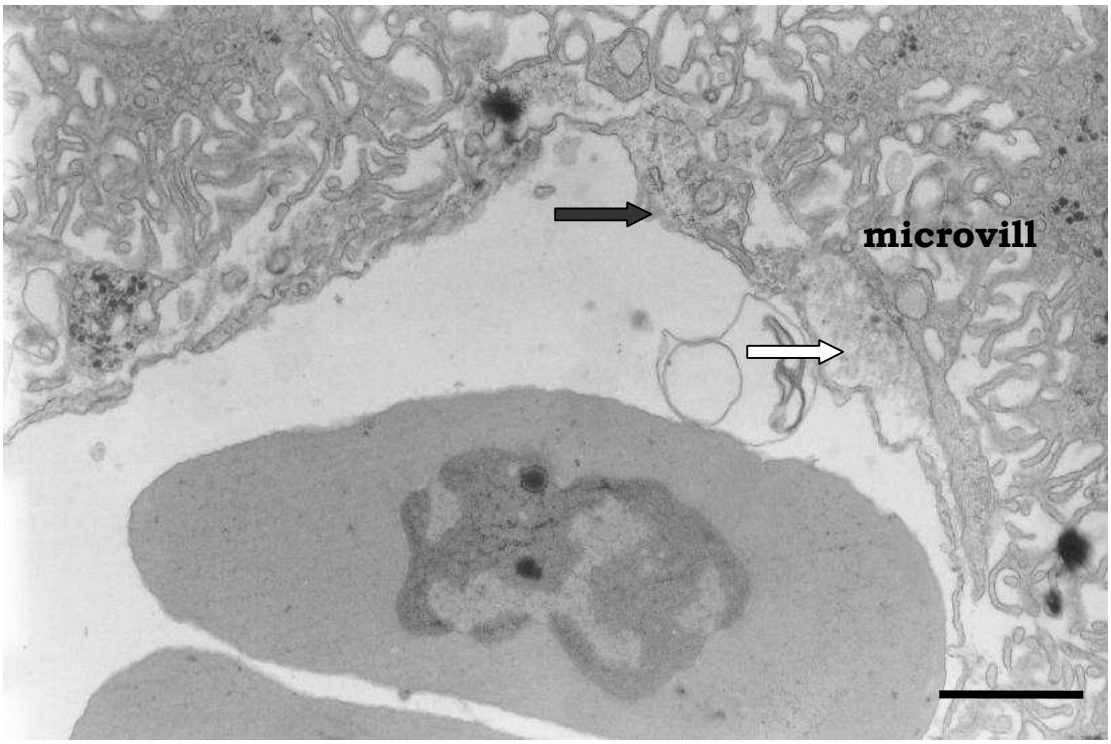

Fig. 8: Liver sinusoid in a metoprolol-exposed rainbow trout (500 µg /L) with irregular microvilli of the adjacent hepatocyte. In the space of Disse flocculent hyaline material can be observed (white arrow); black arrow: endothelial cell. Scale bar: 1 µm.

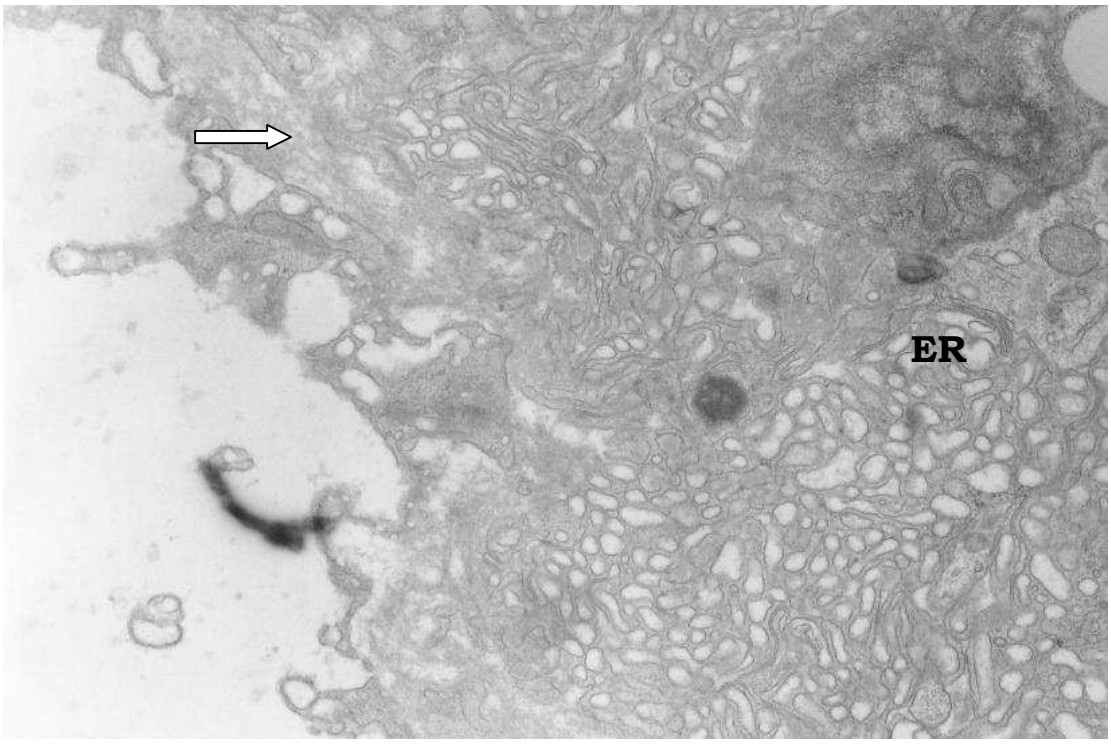

Fig. 9: Basal part of a hepatocyte in a metoprolol-exposed rainbow trout (500 µg /L) with dilated and vesiculated ER in the cytoplasm and flocculent hyaline material in the space of Disse (white arrow). Scale bar: 1 µm.

Trunk kidney

In control fish, four major portions of the kidney were investigated: the renal corpuscle (RC), the relevant site for ultra-filtration and formation of the primary urine, the proximal tubule 1 (PI) mainly responsible for the re-absorption of organic molecules, the proximal tubule 2 (PII) mainly involved in the re-absorption of inorganic molecules and bivalent ions, and the distal portions of the tubules (DI), in which univalent ions are re-absorbed. Except for the occurrence of few hyaline droplets in the PI in some fish only, no cytopathological changes were observed in these four functional units of the kidney in control fish.

A comparison of the semi-quantitative evaluation data recorded for these four kidney portions of fish exposed to the four test pharmaceuticals makes evident that, in the trunk kidney, the most prominent reactions were found in diclofenac-, and carbamazepine-exposed fish (Fig. 10).

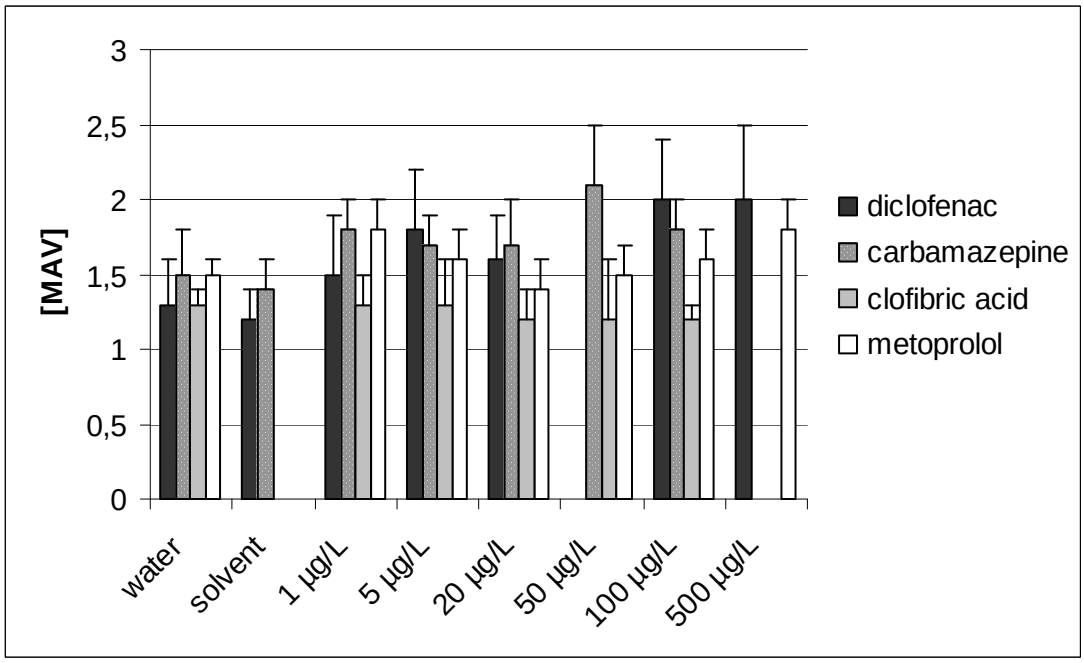

Fig. 10. Semi-quantitative assessment of cytopathology in the entire trunk kidney in pharmaceutical-exposed fish. "1" indicates the control state. The following significances of difference were found (w: water control, s: solvent control): diclofenac: w/20 (*): s/1 (*); w,s/5,100,500 (**); w,s/1,20,100,500 (**); carbamazepine: s/5 (*); w/50 (*); s/1,20,50,100 (**); metoprolol: w/1,500 (*). Data for diclofenac were extracted from Triebskorn et al. [23].

Whereas in diclofenac-treated trout the anterior portions of the nephrons, i.e. the RC and the PI were severely impaired, the strongest reactions in carbamazepine-exposed carp were found in the PII and the DI.

In the kidney of diclofenac-exposed trout, symptoms of a severe glomerulonephritis and a resulting hyaline droplet degeneration in the PI were previously described by Triebskorn et al. [23]. In the present study, quantitative data for this hyaline droplet degeneration are provided. It became obvious that fish exposed to 1 µ/L or higher concentrations of diclofenac showed significantly higher amounts of hyaline droplets in their PI cells which were positively Ponceau-stained for protein than control fish (Fig. 11, 12).

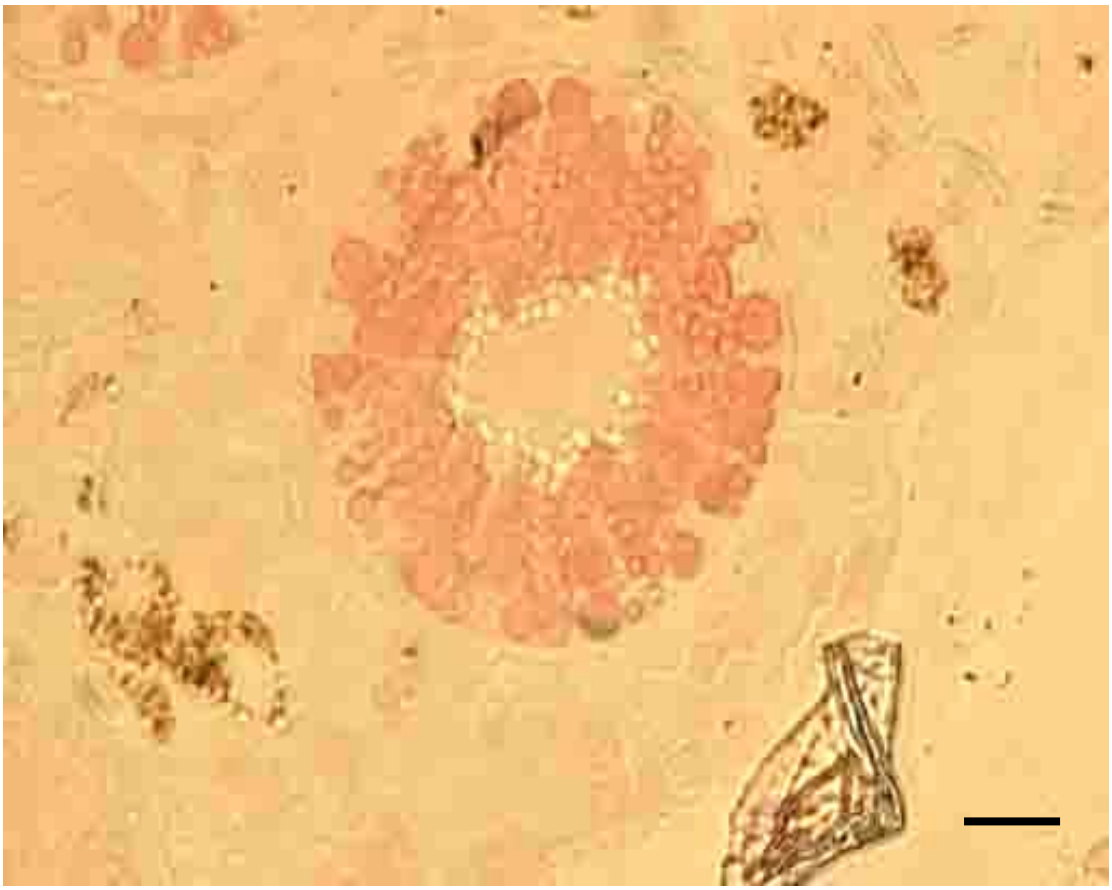

Fig. 11: Protein staining in a PI of rainbow trout exposed to 100 µg/L diclofenac. Scale bar: 30 µm.

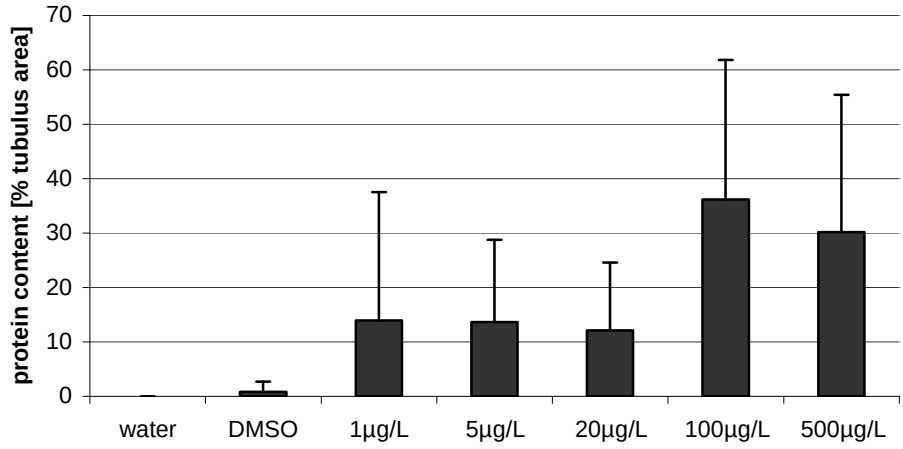

Fig. 12: Quantification of protein in the proximal tubule 1 of the trunk kidney in rainbow trout. The following significances of difference were found (w: water control, s: solvent control): w,s/1,5,20,100,500 (***).

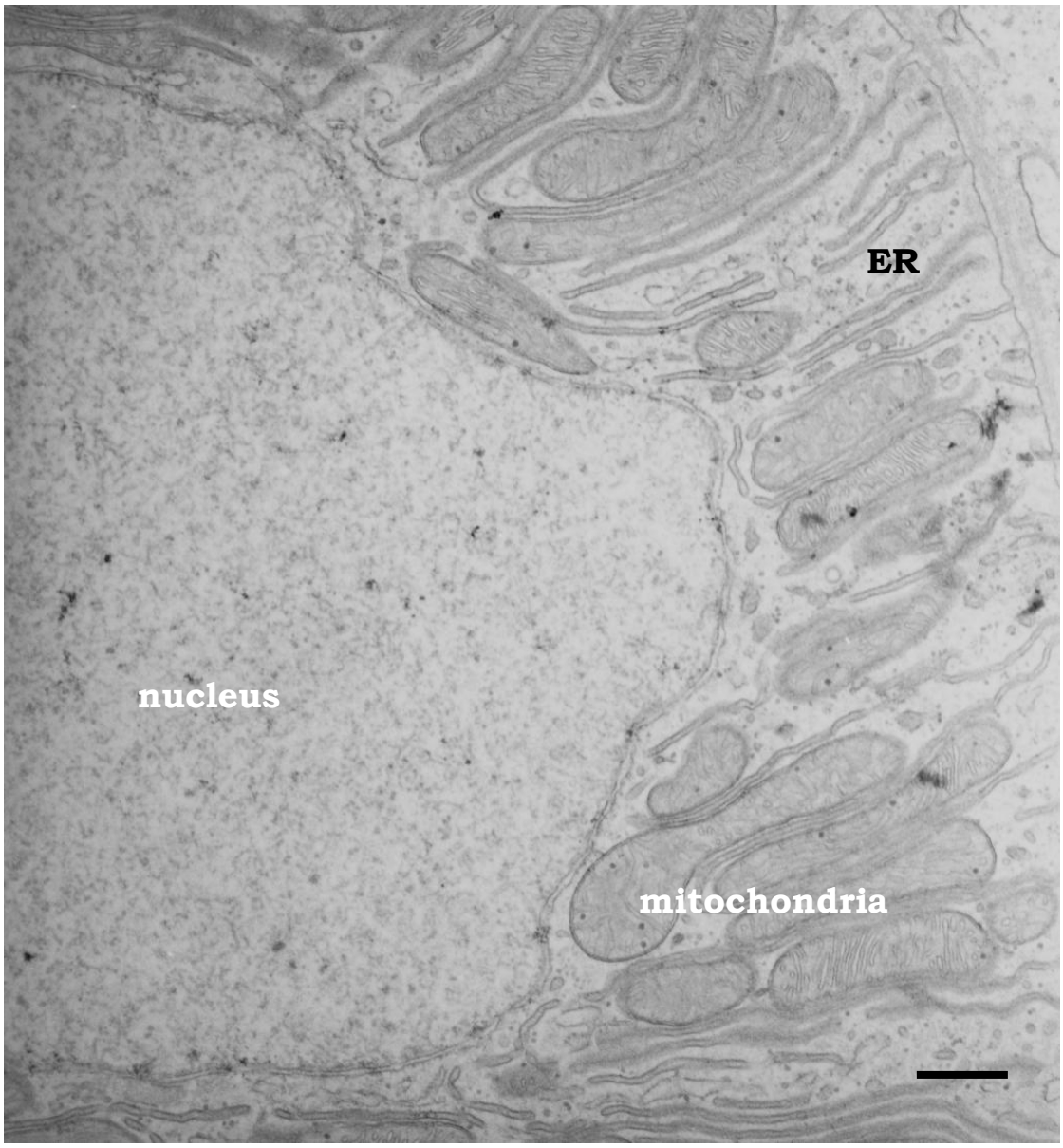

Fig. 13: Cell in the distal part of a nephron in control carp with long cisternae of ER and large mitochondria. Scale bar: 1 µm.

In mammals, diclofenac inhibits the synthesis of prostaglandins via inhibition of cycloxygenases 1 and 2 [33, 34], which catalyse the formation of prostaglandins from arachidonic acid [35]. Prostaglandins, in turn, are of importance as regulators of renal blood flow and are responsible for renal homeostasis [35]. Since Hoeger et al.[36] could show a diclofenac-induced inhibition of the stimulation of prostaglandin synthesis in head kidney macrophages of brown trout, and since Oaks et al. [37] correlated a renal failure in vultures with residues of diclofenac in their kidneys, we assume the mechanism of diclofenac action not to vary considerably across a variety of taxa. Flower [35], however, also has mentioned

toxic potentials of anti-inflammatory drugs for the kidney independent from the inhibition of prostaglandins.

Whereas, in carbamazepine-exposed carp, the above-mentioned diclofenac-induced reactions in the RC and the PI were not striking, symptoms in the PII and the DI were similar to those in diclofenac-exposed trout. These reactions included a prominent vesiculation of the ER, an increased amount of cellular debris in the intercellular spaces and secondary lysosomes in the basal cytoplasm of these cells (comp. Figs. 13 and 14). In addition, in some cells of the DI, mitochondria appeared enlarged. In carbamazepine-exposed fish, however, more macrophages were found in PII and DI than in diclofenac-exposed fish.

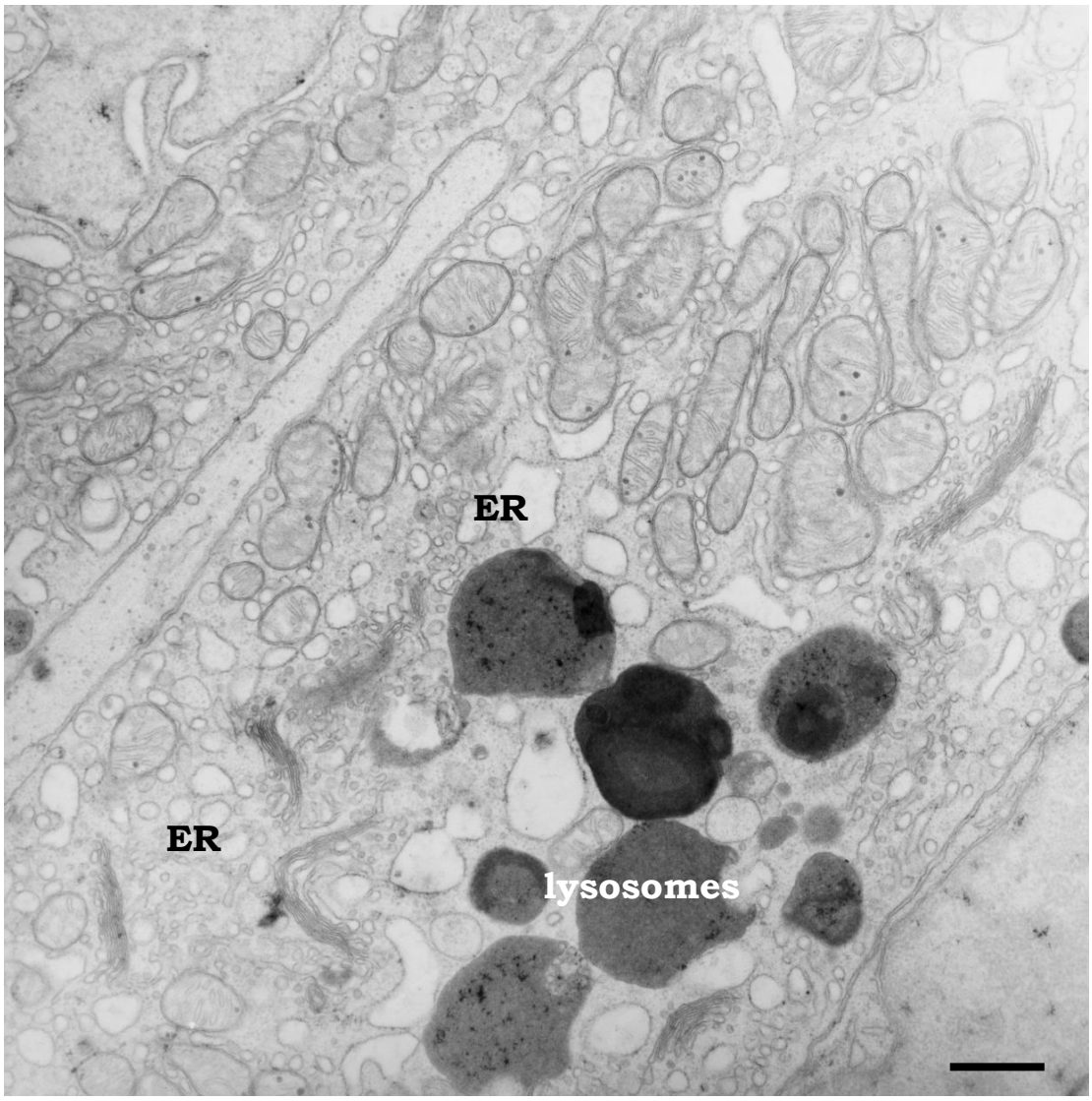

Fig. 14: Cell in the distal part of a nephron in a carp exposed to 50 µg/L carbamazepine. Large electron-dense lysosomes occurred and the ER was heavily dilated and vesiculated Scale bar: 1 µm.

Possibly, these reactions in the PII and DI were independent from the reactions in the RC and PI and were rather related to an influence of the chemicals on the salt and water balance in the kidney. Especially for carbamazepine which is a voltage-sensitive sodium channel blocker, an influence on Na^+ homeostasis and resulting cellular effects in the kidney portions related to this function could be expected.
Exposure of trout to clofibric acid did not result in any pronounced cellular effects in the four investigated compartments of the kidney.
After exposure to metoprolol, slight reactions were found in fish exposed to the lowest (1 µg /L) and highest concentration of this chemical (500 µg /L). The observed symptoms included a slight thickening of the basal membrane in the RC, slightly elongated and more branched endocytotic channels in the PI, and an increased amount of macrophages in all investigated kidney portions. However, no clear concentration-effect relationship could be found. Possibly, the observed reaction could be a result of an altered blood pressure caused by metoprolol.
In contrast to all reactions observed in the livers of pharmaceutical-exposed fish, diclofenac-induced kidney cytopathology reflected severe lesions which undoubtedly affect the function of this organ. Effects observed in carbamazepine- and metoprolol-exposed fish were moderate and can be interpreted as cellular adaptations to modifications of distinct kidney functions as, e.g., ion metabolism.

Gills

In control fish, the gills were well-structured and the cells of the primary and secondary lamellae (epithelial/pavement cells, pillar cells, mucous cells, chloride cells) were in a good condition (Fig. 15, 16). Due to the perfusion process, in some few cases, the capillaries in the secondary lamellae were artificially dilated.

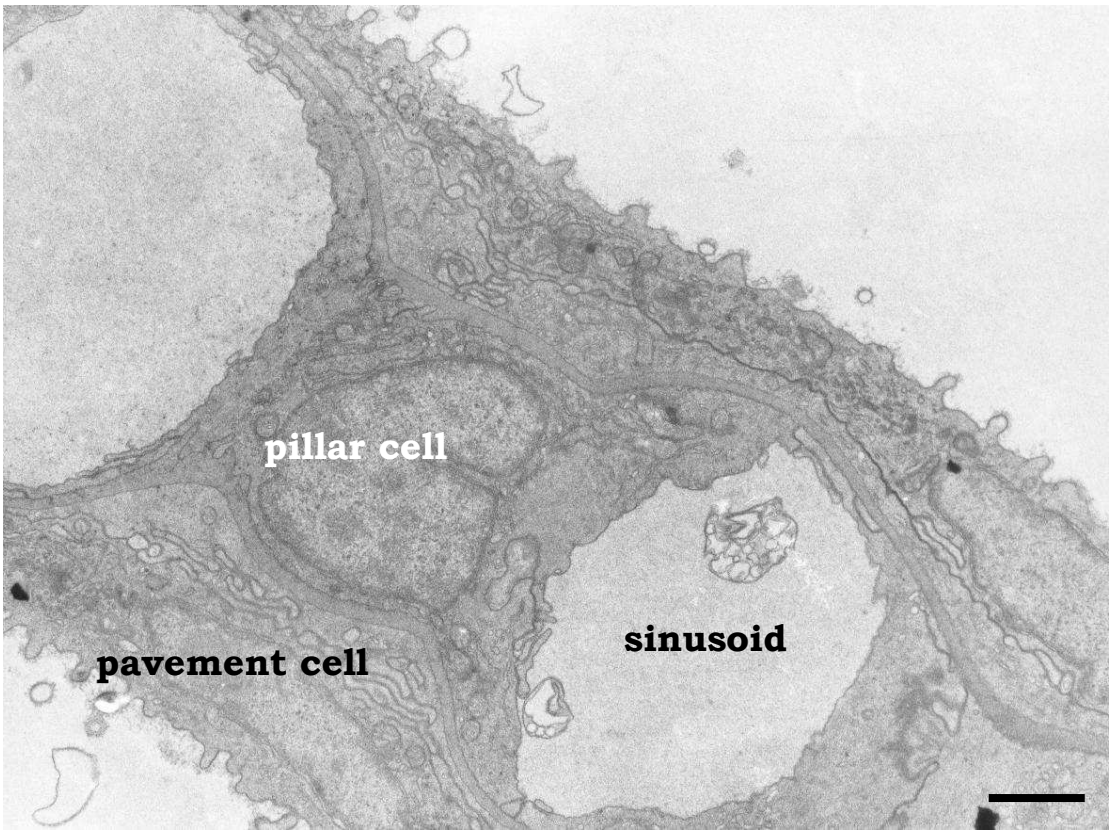

Fig. 15: Secondary lamella in the gill of a control rainbow trout. Scale bar: 1 µm.

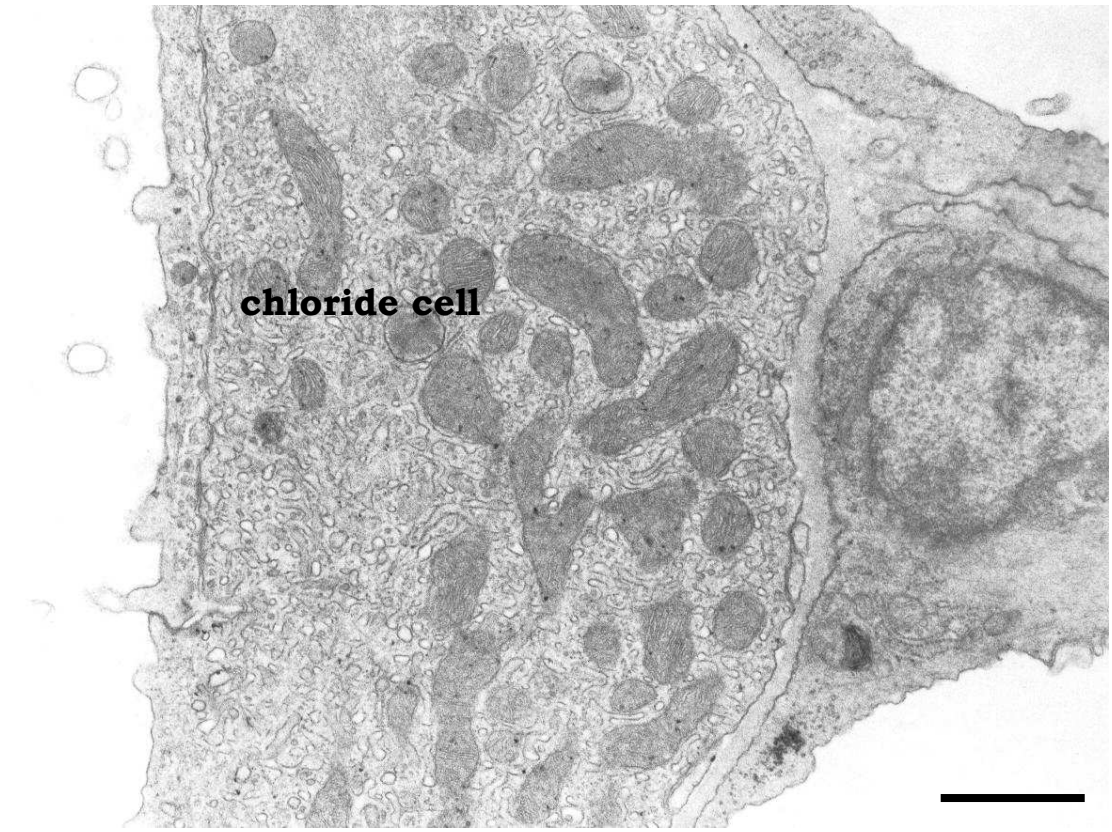

Fig. 16: Chloride cell in the gill of a control rainbow trout with electron-lucent lumen of the ER. Scale bar: 1 µm.

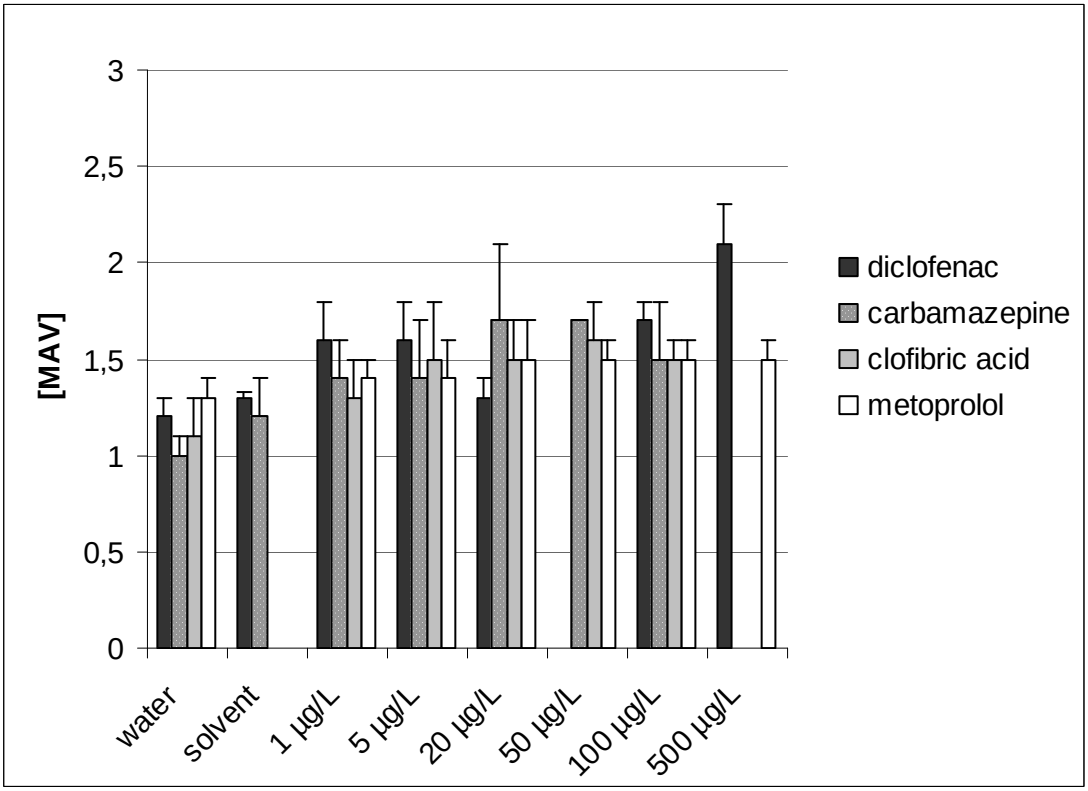

Fig. 17: Semi-quantitative assessment of cytopathology in the entire gills in pharmaceutical- exposed fish. "1" indicates the control state. The following significances of difference were found (w: water control, s: solvent control): diclofenac: w/20 (*); w,s/1,5,100,500 (**); carbamazepine: w/100 (*); s/20,50 (*); w/1,5,20,50 (**); clofibric acid: w/5,20,50,100 (**);metoprolol: w/20,50,100,500 (*).Data for diclofenac were extracted from Triebskorn et al. [23].

Like in liver and kidney, the most prominent reactions in the gills were found in diclofenac-exposed fish with significant differences between solvent control and exposure groups starting at 1 µg /L diclofenac (Fig. 17). The respective symptoms were also described earlier by Triebskorn and colleagues [23]

In carbamazepine-exposed fish, the reactions in the gills were less pronounced than in diclofenac-exposed fish. Most prominent were the epithelial lifting and the hypertrophy and hyperplasia of mucus cells. Regarding the semi-quantitative MAV data for the total gill reactions and the mucus cell proliferation, effects were significantly different from the solvent controls at 20 µg /L carbamazepine or higher concentrations. The epithelial lifting and the occurrence of oedema was already significantly pronounced at 5 µg /L carbamazepine (Fig. 18).

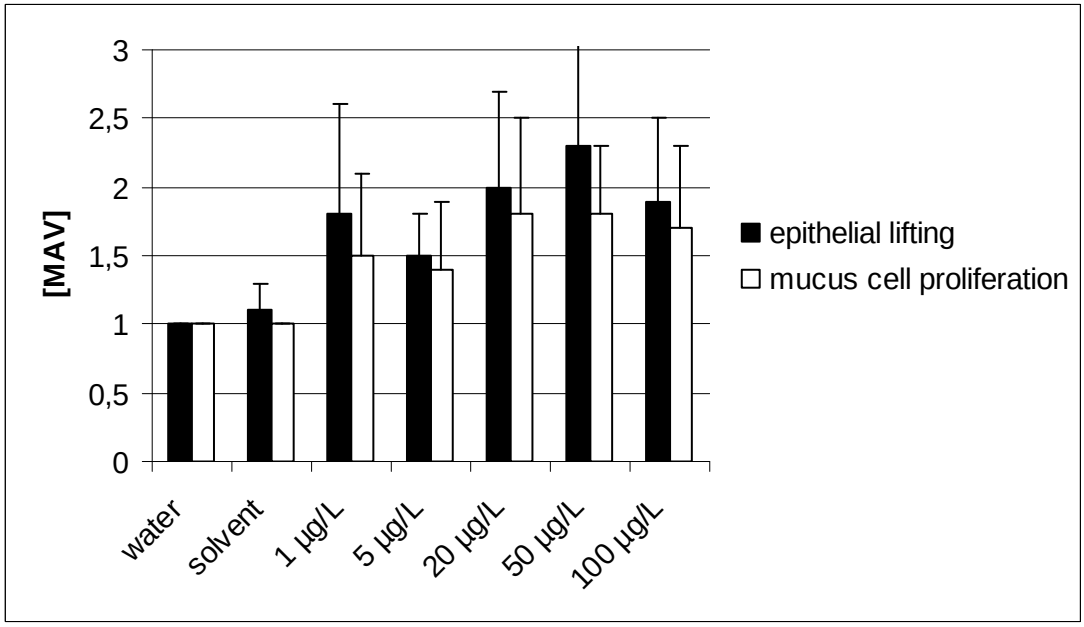

Fig. 18: Semi-quantitative assessment of reactions in distinct functional units of the gills in carbamazepine-exposed carp. "1" indicates the control state. The following significances of difference were found (w: water control, s: solvent control): epithelial lifting: w/1,5 (*); s/5,20,50,100 (*); w/20,50,100 (**); mucus cell proliferation: w/100 (*); s/20,100 (*); w/20,50, (**); s/50 (**).

In fish treated with clofibric acid, the MAV for the total organ was significantly different from the control value at 5 µg /L clofibric acid or higher concentrations (Fig. 19). Hereby, the most prominent reactions were the hypertrophy and hyperplasia of mucus cells and a moderate epithelial lifting with electron-dense granules and membrane whorls appearing in the epithelial cells of the primary filament and the secondary lamellae. The hypertrophy and hyperplasia of chloride cells, however, was found to be significantly pronounced already at 1 µg /L clofibric acid. In the chloride cells, a proliferation of the ER became evident and the lumen of the ER was often electron dense.

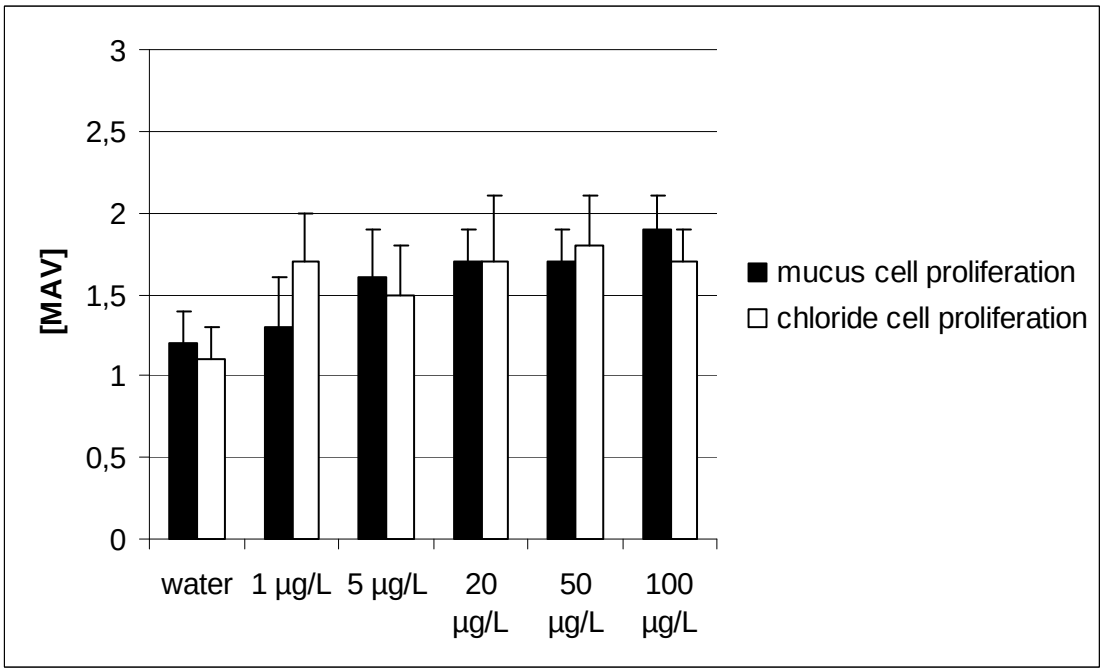

Fig. 19: Semi-quantitative assessment of reactions in distinct functional units of the gills in clofibric acid-exposed rainbow trout. "1" indicates the control state. The following significances of difference were found (w: water control): mucus cell proliferation: w/5 (*); w/20,50 (**); w/100 (***); chloride cell proliferation: w/5 (*); w/20 (**); w/1,50,100 (***).

In metoprolol-exposed fish, with respect to the quality, reactions were similar to those observed in clofibric acid–exposed fish. However, the MAVs for the total organ and the mucus cell proliferation were significantly different from the control values first at 20 µg /L or higher concentrations of metoprolol, and for the chloride cell hypertrophy and hyperplasia at 50 µg /L or higher concentrations of this pharmaceutical. The observed reactions included the epithelial lifting with a related formation of oedema (Figs. 20, 21), hypertrophy and hyperplasia of mucus and chloride cells and macrophage infiltrations in the secondary lamellae (Figs. 21). In the enlarged chloride cells, the ER was dilated and partly showed an electron-dense lumen (Fig. 22).

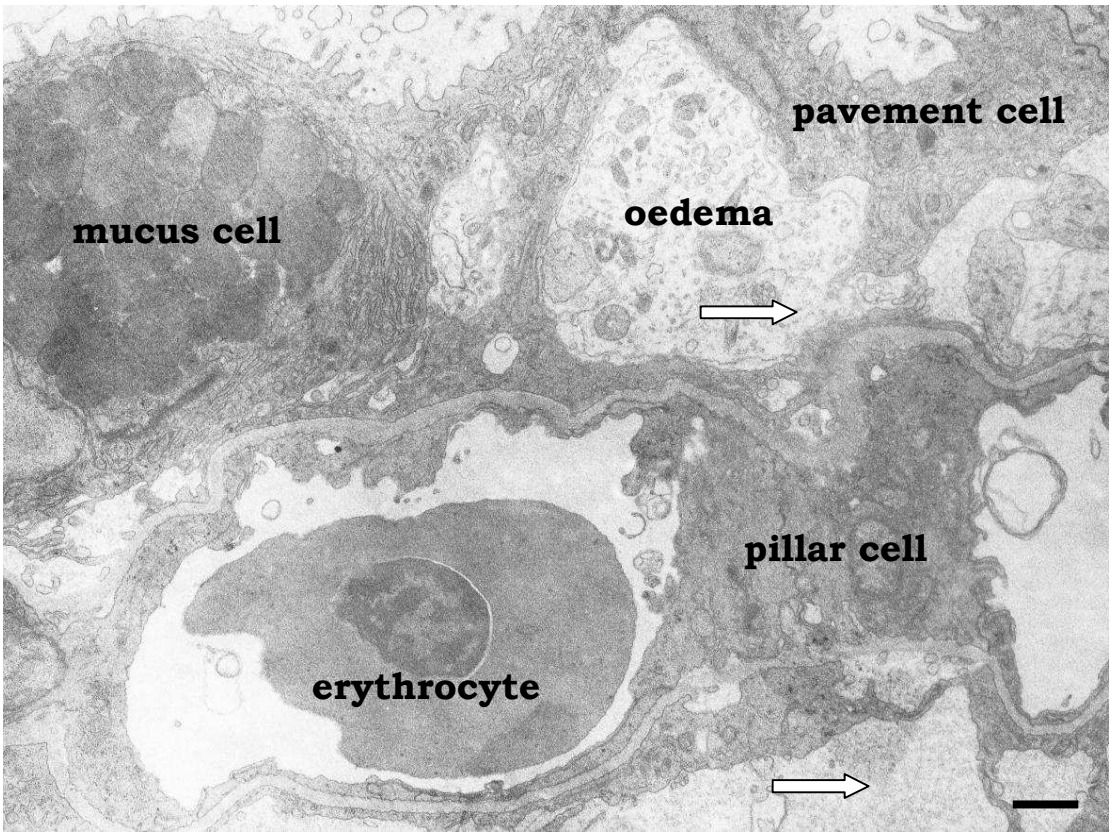

Fig. 20: Severe epithelial lifting (white arrows) with resulting formation of oedema in a secondary lamella of the gill in a rainbow trout exposed to 20 µg /L metoprolol. Scale bar: 1 µm.

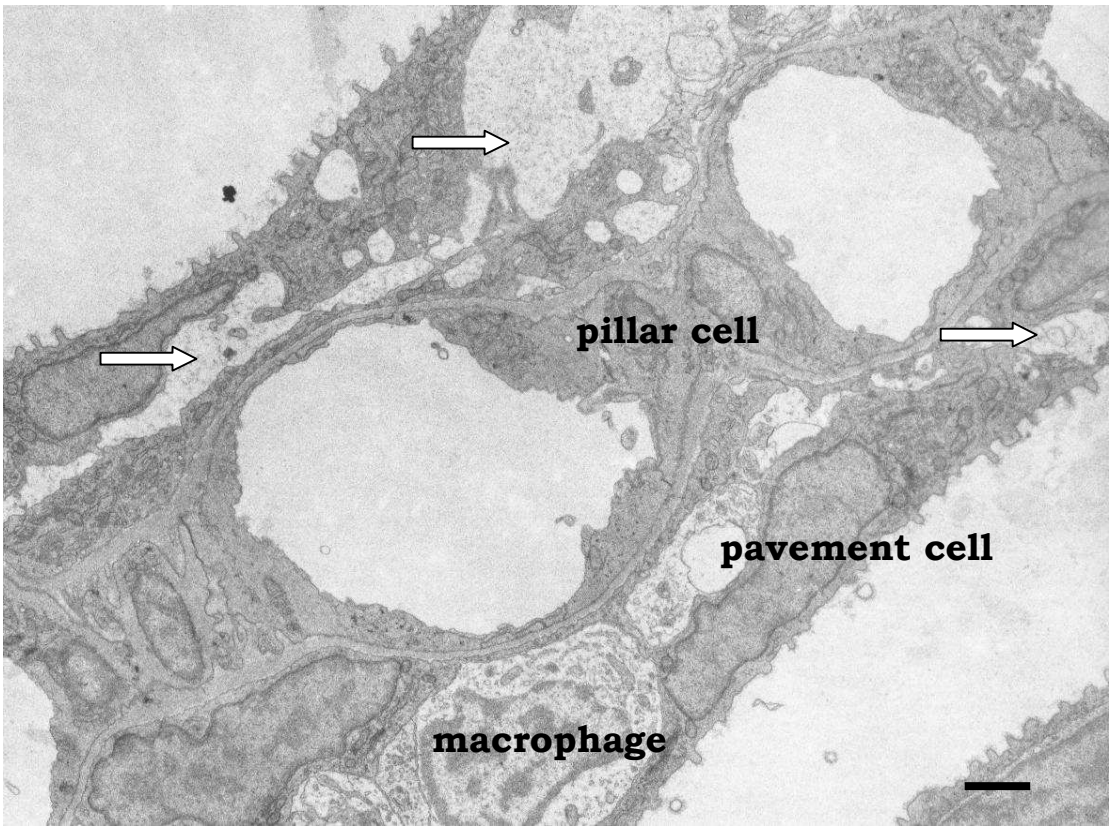

Fig. 21. Epithelial lifting (white arrows) leading to oedema and macrophage infiltration in a secondary lamella of the gill in a rainbow trout exposed to 500 µg /L metoprolol. Scale bar: 1 µm.

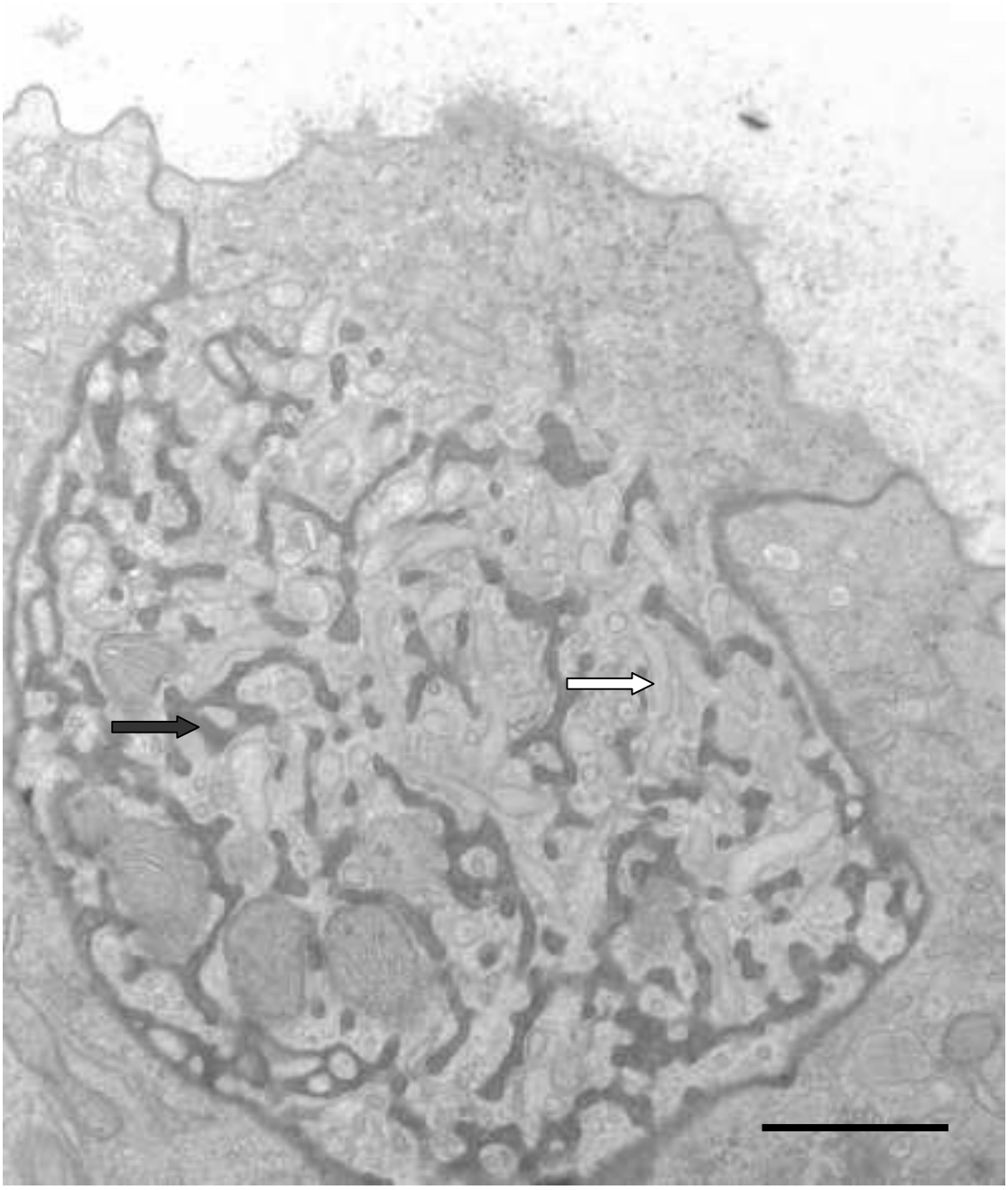

Fig. 22: Chloride cell in the gill of a rainbow trout exposed to 20 µg/L metoprolol with enlarged cisternae of the ER the lumen of which partly appears electron lucent (white arrows) and partly electron-dense (black arrows). Scale bar: 1 µm.

Also in the gills of fish, the most severe lesions occurred in diclofenac-exposed trout. Like the lesions observed in the kidney of these fish, also pillar cell necrosis and the resulting aneurisms as well as the severe epithelial lifting represent cytopathological effects which undoubtedly can be attributed to affect the proper functionality of this organ. Whereas pillar cell necrosis was described as a rather specific reaction to an exposure of fish to several organic pollutants [38], the epithelia lifting is a phenomenon which occurs in response to a wide variety of

chemicals stressors including both metals or a variety of organics [39, 40]. The latter is also true for the hyperplasia and hypertrophy of mucus and chloride cells [41, 42, 43]. However, all these unspecific responses are mainly related to the exposure to substances or environmental conditions which interfere with ion metabolism, as e.g. alterations of the salinity or acidity [42, 43]. In addition, they are discussed to indicate the gills to compensate for an impaired ion reabsorption in the posterior portions of the kidney [44].

Conclusions

The present study on ultrastructural reactions in pharmaceutical-exposed fish showed that the quality and severity of lesions in the three investigated organs was pollutant-specific (Table 1).

The comparison of LOECs for these chronic effects in fish with maximum exposure data in surface waters (Table 2) makes evident that the tested human pharmaceuticals have an effect in non-target organisms even in very low and, with the exclusion of clofibric acid, environmentally relevant concentrations. LOECs or 50% effect concentration (EC50) values obtained from routine test systems and model organisms lie orders of magnitudes higher than the effect data obtained in the present study. For example, the EC50 (*Daphnia magna*, motility) was shown to be 22.43 mg/L for diclofenac [45], and >13.8 mg/L for carbamazepine [45]. The LOEC (early life stage test *Danio rerio*) for diclofenac was reported to be 1000 µg /L [46], or the EC50 (*Daphnia magna*, motility) for metoprolol was 438 mg/L [47]. It is doubtless, that a risk assessment exclusively based on these data would drastically underestimate the risk of chronic pharmaceutical exposure for indigenous fish.

Table 1: Comparison of distinct effects of the four pharmaceuticals tested in liver, kidney and gills of fish (+++ heavy reactions and/or destruction of organ; ++ strong reaction; + moderate reactions; +- slight reactions, but no clear concentration-effect relationships; - no reaction.

	liver	**kidney**	**gills**
diclofenac	++ (collapse of cellular compartmentation, glycogen reduction, membrane material, dilation and vesiculation of ER, increased amount of macrophages)	+++ (glomerulonephritis with thickened basal lamina, shortening of pedicels and retraction from basal lamina, necrosis of endothelial cells, hyaline droplet degeneration)	+++ (epithelial lifting, pillar cell necrosis, hyperplasia and hypertrophy of chloride cells)
carbamazepine	+- (increased amount of macrophages, membrane material)	++ (vesiculation and dilation of ER in PII and DI, enlarged mitochondria in DI, increased amount of macrophages in PII and DI, increased amount of cellular debris in intercellular spaces and secondary lysosomes in basal portions of cells)	+ (epithelial lifting, hyperplasia and hypertrophy of mucus cells)
clofibric acid	+ (dilation of blood vessels, membrane material)	-	++ (epithelial lifting, hyperplasia and hypertrophy of mucus and chloride cells, dilation of ER in chloride cells with electron dense lumen)
metoprolol	++ (collapse of cellular compartmentation, glycogen reduction, membrane material, dilation and vesiculation of ER, increased amount of macrophages, cellular disintegration at the spaces of Disse)	+- (thickening of basal membrane in RC, elongated pinocytotic channels in PI, increased amount of macrophages)	+ (epithelial lifting, hyperplasia and hypertrophy of mucus and chloride cells, dilation of ER in chloride cells with electron dense lumen)

Table 2: Comparison of LOECs for liver, kidney and gill cytopathology (total organ) with maximal concentrations of pharmaceuticals measured in surface waters.

	diclofenac	**carbamazepine**	**clofibric acid**	**metoprolol**
Max. concentration (surface water) [µg/L]	2	1.6	1.1	2.2
LOEC liver (total) [µg/L]	1	> 100	> 100	1
LOEC kidney (total) [µg/L]	1	1	> 100	(1)*
LOEC gills (total) [µg/L]	1	(20)*	5	20

*no clear concentration-effect relationship

Getting aware of this discrepancy, we conclude in agreement with EMEA [2] that more chronic testing rather than only traditional acute toxicity studies is required for pharmaceutical risk assessment We therefore propose that the EU guideline for the testing of pharmaceuticals should be supplemented by test strategies which consider the mode of action of the respective pharmaceuticals and include sensitive parameters in tests with ecologically relevant representatives of potentially affected aquatic environments. This conclusion is in accordance with Fent and colleagues [48] who, in addition, stress the point that in future research not only the ecological relevance of isolated substances but also the ecotoxicological potential of pharmaceutical mixtures should be addressed.

Acknowledgements

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Kapitel 2: Embryo development, stress protein (Hsp70) responses and histopathology in zebrafish (*Danio rerio*) following exposure to nickel chloride, chlorpyrifos and binary mixtures of them

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Abstract

Two different classes of chemicals were tested in a multi level approach in this study: $NiCl_2$ as a representative for heavy metals and chlorpyrifos, a pesticide. Both, the single substances and mixtures of them were investigated for their effects on embryonic development, histological alterations and the stress protein (Hsp70) response in the zebrafish *Danio rerio*. Fish were exposed from fertilisation of eggs up to a maximum of 168h post fertilisation, depending on the investigated endpoint. $NiCl_2$ led to effects in all tests which, however, were less severe at the histopathological level than in developmental (hatching success) and stress protein studies. Chlorpyrifos did not lead to developmental alterations but it was found to induce the Hsp70 response as well as histopathological damages. Mixtures of both substances resulted in similar results as the single substances, the results suggest an independent mode of action of these two substances and additivity of their effects.

Keywords: mixture toxicity, pesticides, heavy metals, histology, Hsp70

submitted to Environmental Toxicology

Introduction

In theory, nickel and chlorpyrifos should target different sites and act in different and independent ways in animals exposed to them. Thus, the toxicity of one of the two substances should not affect the toxicity of the other if they are applied in combination. Nickel, as a heavy metal, should mainly target on active sites of enzymes, whereas chlorpyrifos acts as a specific acetylcholine esterase inhibitor. This study aims to answer the question, whether effects found in zebrafish confirm this theory (no expected synergistic or antagonistic effects of nickel plus chlorpyrifos) or not.

The zebrafish, (*Danio rerio*, Hamilton, 1822), is a widely used test species representative of freshwater fish (Nagel, 2002). Zebrafish can be kept cheaply and easily in the laboratory, their transparent eggs make it easy to investigate the embryonal development from fertilisation up to hatch. *D. rerio* is a very common test species in several biological disciplines like e.g. developmental biology and genetics (Nüsslein-Volhard *et al.*, 2002). In ecotoxicological studies, zebrafish serves as a model freshwater vertebrate species in acute toxicity tests as well as in early live stage tests (Nagel, 2002) where developmental aberrations due to exposure to chemical stressors can be investigated (e.g. Hallare *et al.,* 2004; 2006).

Induction of the 70 kD stress protein family (Hsp70) by various proteotoxic stressors is known to serve as a sensitive biomarker of effect (e.g Eckwert *et al.,* 1997; Nadeau *et al.*, 2001). Heat shock or stress proteins of the 70 kD class are a family of proteins synthesised and accumulated intracellularly in response to a wide variety of biotic and abiotic stressors (Schlesinger, 1990). The advantage of Hsp70 as a biomarker of effect is its capability to integrate overall proteotoxicity exerted by the combined action of all stressors present at the same time.

At the tissue level, histological investigations can provide information on the health of organisms at an early stage of exposure (i.e. before mortality occurs). Histopathological investigations have been established as diagnostic tools for the investigation of adverse effects of chemicals in fish (Myers & Fournie, 2002; Schwaiger *et al.*, 2004; Schwaiger, 2001; Teh *et al.*, 1997; Triebskorn *et al.*, 2004).

Nickel occurs in natural waters predominantly as the ion $Ni(H_2O)_6^{2+}$ (ICPS, 1991), deriving either from natural (WHO, 2007) or from anthropogenic sources like nickel processing industry (ICPS, 1991). Rivers have been shown to carry concentrations from 0.14 µg/L up to 183 mg/L (Finnland, river unaffected by anthropogenic nickel pollution (Mukherjee, 1998), and rivers near a nickel processing industry in Canada (Kasprzak, 1987), respectively).

Chlorpyrifos (O, O,-diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate, CPP) is an organophosphate insecticide, acaricide and miticide used to control foliage and soil-borne insect pests on a variety of food and feed crops (U.S. EPA, 2002). CPP acts as an acetylcholinesterase inhibitor and is primarily a contact poison (Kamrin, 1997). CPP is one of the most widely used organophosphate insecticides, concentrations of CPP found in surface waters reach up to 10.8 µg/L (Marino & Ronco, 2005).
Multiple studies have shown the complex contamination of rivers and streams with numerous chemicals (for reviews see e.g. Konstantinou et al., 2006, Ohe et al., 2004). In this study, not only the effect of the single substances were tested but also combinations of nickel chloride and chlorpyrifos were investigated to get closer to the complex pollution situation in the environment. Furthermore, the main aim was to investigate whether environmental chemicals with different modes of action eventually exert synergistic / antagonistic effects when applied in combination with one another.

Material and methods

Adult wild-type zebrafish (*Danio rerio*, strain WIK, ZFIN ID: ZDB-FISH-010531-2) of both sexes were kept as a breeding stock in the laboratory. They were kept in aerated and filtered aquaria with a minimum of 1 litre water per fish on the average. Culture conditions were 26 ± 1°C at a light regime of a 12:12 hour light:dark photoperiod, the conductivity was maintained at 400 µS/cm^{-1} resulting from a mixture of tap water and deionized water. The adult fish were fed twice a day with dry flake food (Nutrafin Max, Jagen, Germany) and frozen crustaceans or midge larvae from uncontaminated sources, respectively.
The eggs used in the tests were collected using spawn traps which had been placed at the bottom of each aquarium the evening before spawning was required.

Exposure experiments

A negative control containing uncontaminated water (reconstituted water according to ISO (1996) and to OECD (1992) was tested in every experiment. During exposure, about 80% of the solution volume in the Petri dishes were renewed every 48 h.

Experiments with nickel chloride

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) obtained from Carl Roth, Germany, was dissolved in reconstituted water. Concentrations of 0 (control), 0.5, 1, 5, 10 and 15 mg/L Ni (plus 20 and 30 mg/L for histology) resulting from a stock solution of 200 mg/L Ni were tested.

Experiments with chlorpyrifos

Chlorpyrifos obtained from Sigma-Aldrich, Germany was dissolved in reconstituted water at a water temperature of about 40°C, the stock solution was kept at 35°C. Concentrations of 0 (control), 0.1, 1, 10, 100, 300, 600 and 1000 µg/L CPP resulting from a stock solution of 1000 µg/L CPP were tested. A new stock solution was prepared for every exchange of solutions.

Mixture Experiments with nickel chloride and chlorpyrifos

Mixture experiments were conducted using stock solutions like those described above. Mixtures of 100 µg/L CPP + 0.5 mg/L $NiCl_2$, 100 µg/L CPP + 5 mg/L $NiCl_2$, 300 µg/L CPP + 1 mg/L $NiCl_2$, 600 µg/L CPP + 0.5 mg/L $NiCl_2$ and 600 µg/L CPP + 5 mg/L $NiCl_2$, as well as a control containing pure reconstituted water were tested.

Prolonged embryo tests

Prolonged embryo tests were conducted in climatic exposure test cabinets at conditions like in the breeding stock, except for the water used. Exposure of embryos was performed in glass (chlorpyriphos and mixture experiments) or plastic ($NiCl_2$ experiments) Petri dishes containing exposure water and the respective test concentrations of chlorpyriphos and / or nickel chloride. Sixty minutes after triggering egg laying and fertilization by sudden illumination of the aquaria, the spawn traps were removed and the eggs were collected (see Westerfield, 1998). All eggs were transferred immediately into Petri dishes containing the different test solutions. Unfertilized eggs were removed, and the fertilized eggs were placed into new Petri dishes (10 embryos per Petri dish, 4 dishes per tested concentration) containing the respective test solutions. The Petri dishes were covered to avoid evaporation. Embryo development was observed at set time points (Table 1) using a stereomicroscope.

Table 1: Observed endpoints during the embryo test.

Endpoint	**8h**	**12h**	**24h**	**48h**	**60h**	**72h**	**84h**	**96h**
Coagulated eggs / dead	*	*	*	*	*	*	*	*
No epiboly (70%)	*							
Incomplete gastrulation		*						
Exogastrulated embryo		*						
No formation of somites			*					
No detachment of tail			*					
No spontaneous contraction			*					
No formation of the eye			*					
No heart beat				*				
No circulation				*				
Heart rate				*				
No otolith formation					*			
No melanocyte formation					*			
Yolk sac endema					*			
Eye / brain defects					*			
Total number of malformations					*	*	*	*
Number of hatched embryos					*	*	*	*
Edema (heart and head)						*	*	*
Eye defects						*	*	*
Tail deformities						*	*	*
Fin blistering						*	*	*
Weak pigmentation						*	*	*
Helical bodies						*	*	*
Spiral nervous system						*	*	*

Stress protein analysis

The experimental design to obtain embryos for stress protein analysis (Hsp70) was almost identical to that of the prolonged embryo test but required the following modifications. Instead of keeping 10 eggs per Petri dish, 40 eggs per Petri dish (3 dishes per concentration) were used. The tests were extended to 168 h post fertilisation. 10 x 8 embryos from different Petri dishes, respectively, were pooled for the respective concentrations, shock frozen in liquid nitrogen and stored at -20°C for Hsp70 analysis. The pooled larvae were homogenized ultrasonically in 20 µl extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor Sigma P8340, pH 7.5). Subsequently, the homogenate was centrifuged (12 min, 20.000 g at 4°C). The total protein concentration in the

supernatant was determined according to the method of Bradford (1976). Constant amounts of total protein from each sample (20 µg of total protein per lane) were subjected to SDS-PAGE (12% acrylamid-bisacrylamid) for 20 min at 80 V and 120 min at 120 V. The protein was then transferred to nitrocellulose by semi-dry blotting, and these filters were blocked for 2 h in 50% horse serum in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl pH 7.5). After washing in TBS, a monoclonal antibody (mouse anti-human Hsp70; Dianova, Hamburg, Germany, dilution 1:5,000 in 10% horse serum/TBS) was added, and incubated at room temperature overnight. After repeated washing in TBS for 5 min, the nitrocellulose filters were incubated in the secondary antibody (peroxidase-conjugated goat anti-mouse IgG Dianova, Germany, dilution 1:1,000 in 10% horse serum / TBS) at room temperature for 2 h. After repeated washing in TBS for 5 min, the antibody complex was detected by 1 mM 4-chloro(1)naphtol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The grey scale values of the Western blot protein bands were quantified using a densitometric image analysis system (Herolab E.A.S.Y., Germany), and related to an internal *Danio rerio* Hsp70 standard, run in parallel on each gel.

Histopathology

Histopathological analyses were conducted using the larvae from the prolonged embryotests, larvae were kept under the same conditions as in the embryotest until they reached an age of 168 h post fertilisation. Of each exposure group, 10 randomly selected larvae were fixed in Bouin solution (15 portions picric acid / 5 portions formaldehyde / 1 portion pure acetic acid). Prior to fixation, larvae were narcotised by adding one drop of benzocaine solution (1g benzocaine dissolved in 20 ml acetone) to the respective Petri dish. After removal of the picric acid from the samples using 70% ethanol plus 1 drop ammonia (ad 100 ml ethanol, 4 x 15 min.), samples were dehydrated and embedded according to the following procedure: rinsing in 70%, 80%, 90%, 96%, 100% ethanol (3 x 15 min, respectively), transfer to 100% ethanol / synthetic resin (1:1; resin: Technovit 7100, Heraeus Kulzer, Wehrheim, Germany) (120 min.), infiltration in preparation solution (Technovit 7100 plus hardener 1, Heraeus Kulzer, Wehrheim, Germany) overnight. For embedding, larvae were placed with the right side of the body to the bottom of the embedding device. Hardening of samples took place at room temperature within 3-4 hours.

Of each fish, series of 4.5 µm sagittal sections were cut according to the following protocol:

1) Sections were discarded until the eye was visible on a section
2) First section was transferred to the microscopy slide
3) 4 sections were discarded
4) 1 section was transferred to the microscopy slide
5) 4 sections were discarded etc.

This procedure was continued until 15 sections were placed on microscopic slides. All sections were routinely stained with Hematoxilin and Eosin, dehydrated and covered with Eukitt. For each fish, the histology of liver, gut epithelium, pancreas, kidney and skin was qualitatively described and semiquantitatively assessed. For the semi-quantitative assessment, the status of histopathology was first classified into 3 categories (category 1: control status, category 2: status of reaction, category 3: status of destruction) Symptoms characterizing these 3 histological status in the respective organs are summarized in Table 2. In a second step, a modified protocol published by Köhler & Triebskorn (1998) was used in order to weight the occurrence of histopathological effects of category 1-3: When approximately 90%-100% of cells showed the control status while the rest indicated reaction status, histopathology was rated as 1. If approximately 50%-90% of cells displayed control status and the rest appeared in reaction status, histopathology was rated as 2. In case of the appearance of approximately 50-100% of cells were in the reaction status while the rest showed control status, histopathology was rated as 3. When approximately 25%-75% of cells displayed destruction, histopathology was rated as 4. When approximately 75%-100% of cells presented status of destruction, histopathology was rated as 5. First, each of the above- mentioned organs of each fish was individually assessed and a mean assessment value (MAV) was calculated for each organ of each exposure group. Subsequently, the mean of the MAVs recorded for every organ was calculated giving an indication of the histopathological impact on the ‘total’ fish.

Table. 2: Classification of histopathological effects

	Category 1: **Control status**	**Category 2:** **Status of reaction**	**Category 3:** **Status of destruction**
Liver	• cubic cells with round nuclei and flocculent cytoplasm • only isolated macrophages • slightly extended capillary spaces and lumina of the hepatic tubules • in larvae with much yolk: larger hepatocytes with very homogenous cytoplasm and large nuclei, mitosis	• moderate inflammatory reactions with increased number of macrophages • atrophy or hypertrophy of hepatocytes or nuclei • change in density of cytoplasm and / or nuclei • onset of vacuolization • reduction or increase of lipid and/or glycogen storage • dilation of capillaries or lumina of hepatic tubules	• severe inflammatory reactions with high numbers of macrophages • occurrence of necrosis (caryolysis, caryopycnosis) • severe vacuolization of cytoplasm
Gut	• prismatic to highly prismatic cells with basally located nuclei • large supra-nuclear vacuoles in the posterior part of the gut • homogenous cytoplasm with apical microvilli • smooth apical and basal surface	• irregular shape of apical and / or basal surfaces • atrophy or hypertrophy of cells and / or nuclei • altered density of cytoplasm and / or nuclei • alteration of compartmentation • moderate inflammatory reactions • hyperplasia of epithelial and / or mucus cells	• severe inflammatory reactions with high numbers of macrophages • occurrence of necrosis (caryolysis, caryopycnosis) • very large intercellular spaces
pancreas	• exocrine pancreas with cubic cells containing a large number of light enzyme vesicles • endocrine pancreatic cells organized as light islands in the exocrine pancreas	• enlarged intercellular spaces • slight reduction / proliferation of zymogene granules • slight hypertrophy / atrophy of zymogene granules in cells of the exocrine pancreas • altered density of cytoplasm and / or nuclei	• occurrence of necrosis (caryolysis, caryopycnosis) • severe hypertrophy / atrophy of zymogene granules • severe reduction / proliferation of zymogene granules • very large intercellular spaces • severe inflammatory reactions with high numbers of macrophages

Table. 2 (continued): Classification of histopathological effects

kidney	• regularly shaped tubules with cubic to prismatic cells • round, centrally located nuclei • homogenous / slightly flocculent cytoplasm	• altered shape of tubular cells • altered density of cytoplasm • increased protein storage (hyaline droplets) • few macrophages • dilation of tubules • reduction of haemopoietic tissue	• occurrence of necrosis (caryolysis, caryopycnosis) and disintegration of tubules • severe inflammatory reactions with high numbers of macrophages • severe hyalin droplet degeneration
skin (head region)	• thin pavement epithelium (two cell layers) with isolated mucous cells	• hypertrophy / atrophy of epithelial and / or mucous cells • hyperplasia of mucous cells	• occurrence of necrosis and / or heavy inflammation

Statistical analyses

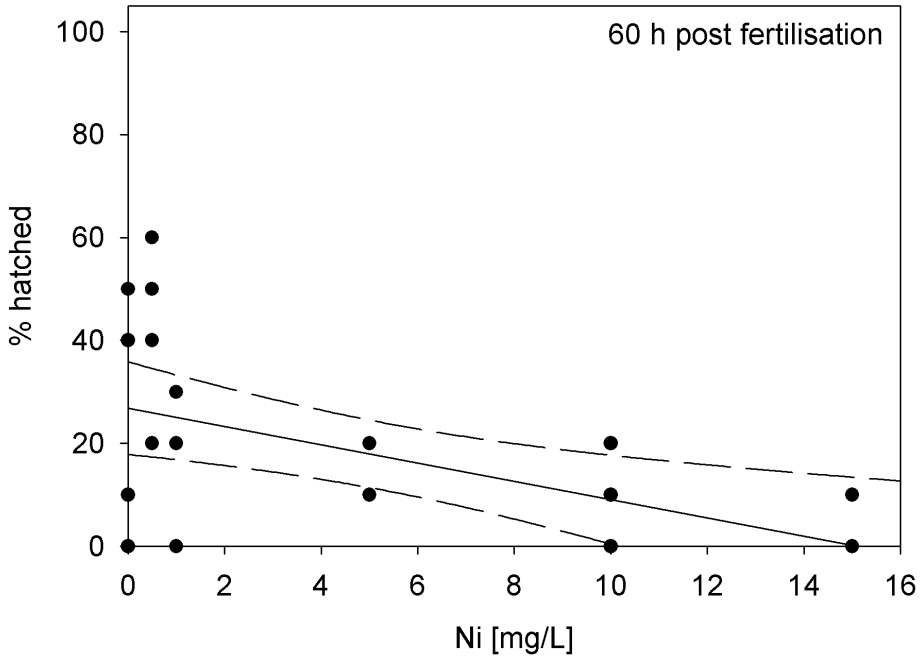
Since data were not normally distributed (checked by Jump 4.0, SAS Institute Inc.), the significance of differences between the respective exposure groups and the control group were tested using the Mann-Whitney-Wilcoxon´s U-test. Significance levels were $p > 0.05$ (not significant), $0.01 < p \leq 0.05$ (weakly significant, *), $0.001 < p \leq 0.01$ (significant, **), and $p < 0.001$ (highly significant, ***). The mixture data were analysed using the "MixToxModules.xls (23.10.2005)" file including concentration addition (CA) and independet action (IA) models (for details see Jonker et al., 2005). The file was obtained from http://www.ceh.ac.uk/sections/er/csvendsen.html. Response surfaces shown were calculated using STATISTICA 5, StatSoft, Inc.

Results

$NiCl_2$

Embryo test: The prolonged embryo test conducted with $NiCl_2$ showed increasing concentrations to lead to delayed hatching success (Fig. 1). Embryos which had not hatched after 96 h post fertilisation usually did not hatch later but died inside the egg. Other investigated endpoints during the embryo test (according to Table 2) did not show any reactions to $NiCl_2$.

a)



b)

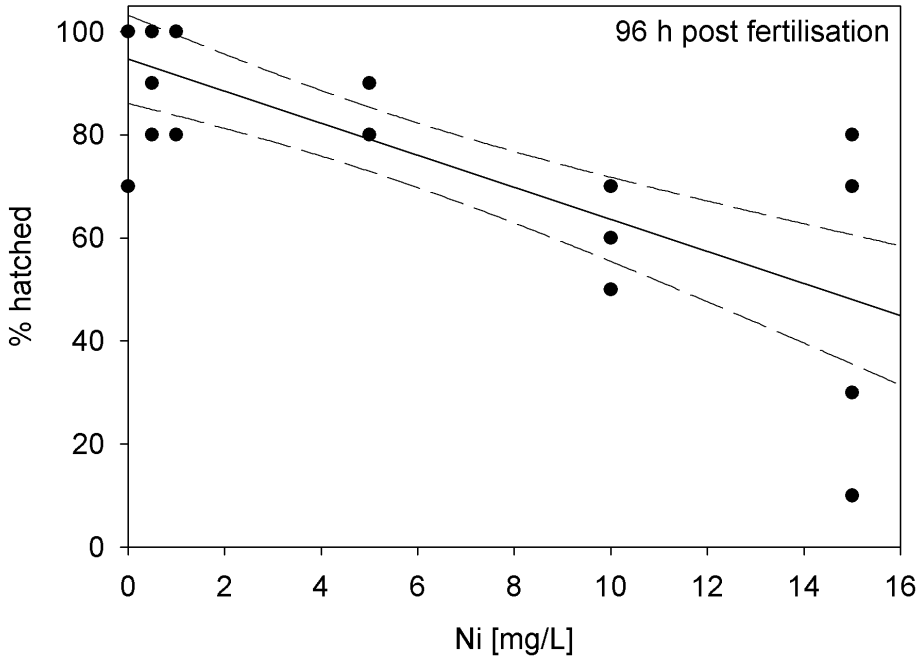


Fig. 1: Hatching success of Danio rerio embryos [% of the initial stock of eggs exposed to NiCl2 at 26°C] . a) 60h post fertilisation, $p<0.01$; b) 96 h post fertilisation. Linear regression analysis and 95% confidence interval, ANOVA: $p<0.001$

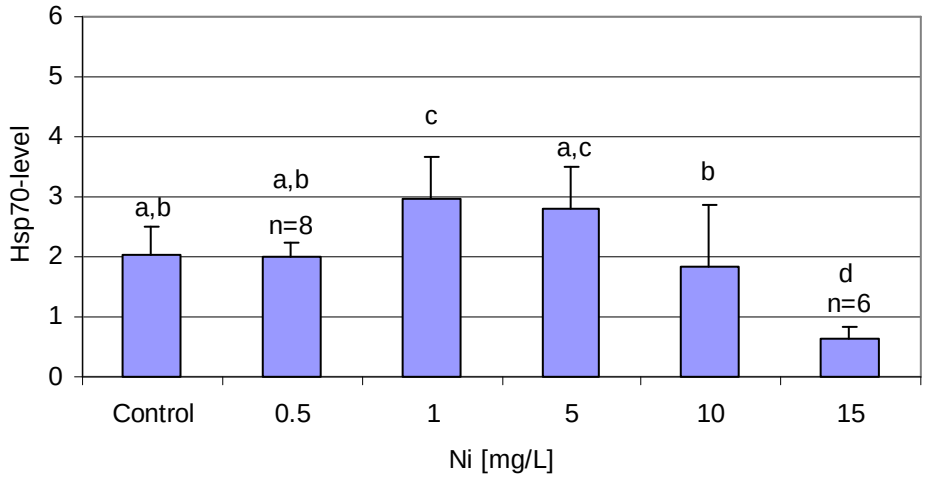


Fig. 2: Hsp70 levels (means + SD) in Danio rerio larvae after exposure to control conditions and five concentrations of NiCl2 at 26°C; n=10 if not explicitely given. Different letters indicate significance at $p \leq 0.05$.

Hsp70: Stress protein (Hsp70) analysis showed a slight increase of Hsp70 levels in larvae exposed to 1 mg/L Ni, Hsp70 levels decreased at higher concentrations of Ni, leading to significantly lower Hsp70 levels (compared to the control group) in larvae exposed to 10 and 15mg/L Ni (Fig. 2).

Histopathology, *qualitative analyses:* Only in the posterior part of the gut, nickel-induced distinct histopathological effects were observed especially in larvae exposed to 20 mg/L and 30 mg/L nickel chloride. These effects include an irregular shape of the apical surface of the gut, an affected cellular compartmentation and the disappearance of centrally located vacuoles. In a few cases, a total disintegration of the epithelium and necrosis became evident.

Neither in the liver, nor in the kidney, pancreas or skin, exposure-specific reactions could be observed. Especially in fish exposed to 15 mg/L nickel chloride, an increased number of macrophages and foci of inflammation were found in the livers. These, however, also occurred in control fish. In a few cases, liver cells of control and exposed larvae were vacuolated and nuclei were heavily enlarged. This might be due to the fact, that in these larvae the yolk sac was not completely reduced. Thus, the developmental processes in the liver related to the reduction of yolk storage were still in progress, and the reaction status of liver cells probably rather indicates the ongoing change in the mode of nutrition of larvae than a reaction to an exposure to nickel chloride.

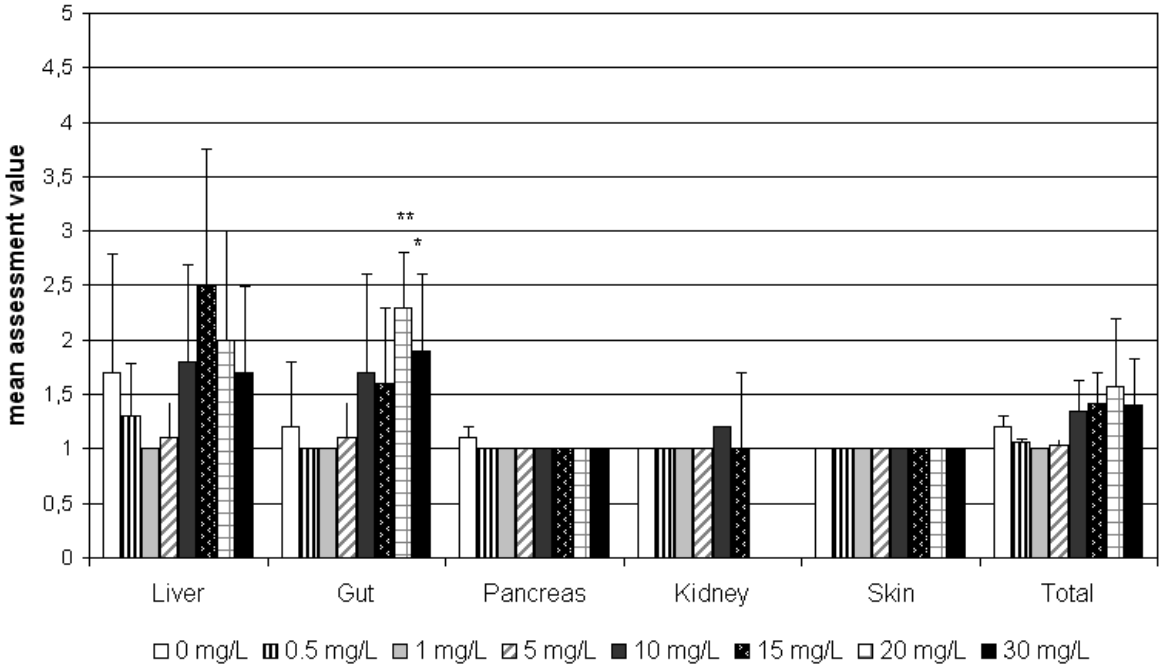


Fig. 3: Semi-quantitative assessment of histopathological effects of NiCl2 in zebrafish larvae. Assessment values (means + SD) obtained for different organs and 'total' fish. *: 0.05 ≥ p ≥ 0.01, **: 0.01 ≥ p > 0.001.

Histopathology, semi-quantification of effects:
Only in the gut, significant differences between the controls, fish exposed to 0.5 mg/L, 1 mg/L and 5 mg/L and those fish exposed to 20 mg/L and 30 mg/L nickel chloride were found. (Fig. 3)

Chlorpyrifos
Embryo test: Prolonged embryo tests with chlorpyrifos did not reveal any effects according to the endpoints described in Table 1. Nevertheless, hatched larvae exposed to 600 and 1000 µg/L exhibited a higher activity and uncontrolled convulsions.
Hsp70: Due to high mortality at 1000 µg/L chlorpyrifos Hsp70 data could not be obtained. At lower concentrations, 100 and 600 µg/L chlorpyrifos led to significantly increased Hsp70 levels (Fig. 4). Even though the Hsp70 level was elevated by 300 µg/L chlorpyrifos as well, significance was lacking.
In contrast to $NiCl_2$ the response to chlorpyrifos showed only the increasing part of the Hsp optimum curve.

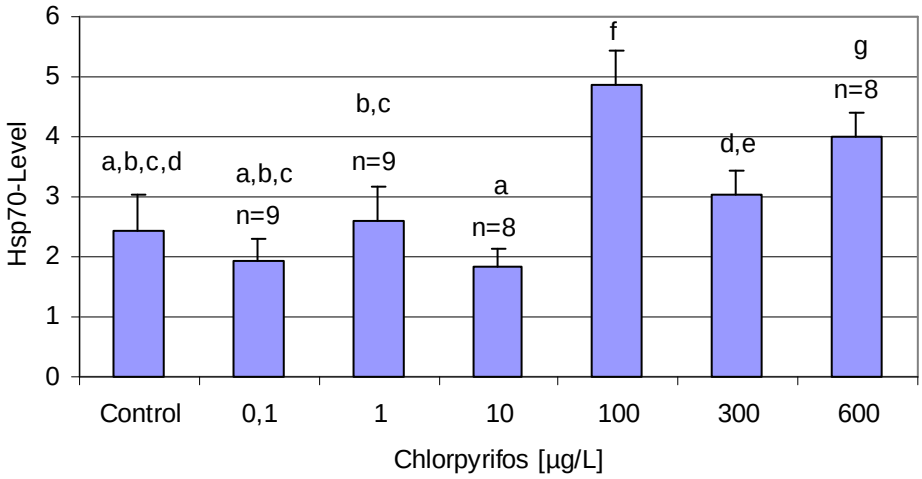
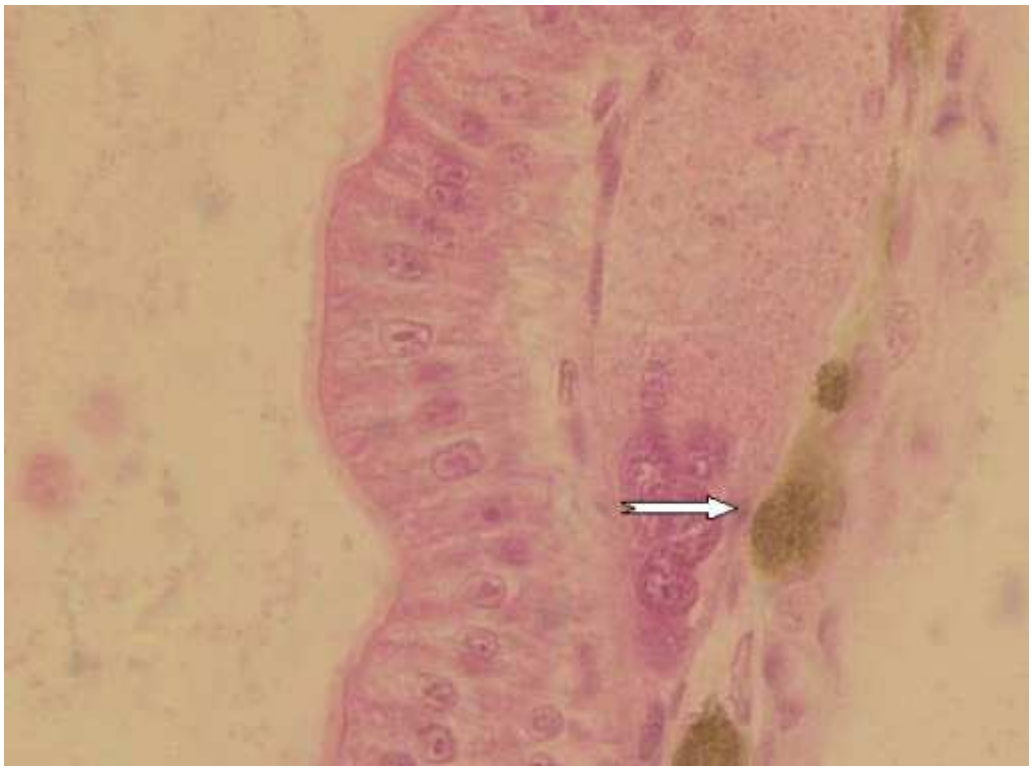


Fig. 4: Hsp70 levels (means + SD) in Danio rerio larvae after exposure to control conditions and six concentrations of chlorpyrifos [µg/L]; n=10 if not explicitly given. Different letters indicate significance at $p \leq 0.05$.

Histopathology, qualitative analyses: In zebrafish larvae exposed to 600 µg/L chlorpyrifos, strong histopathological effects occurred in all organs. These include disintegration of epithelia resulting from necrosis and caryolysis in all investigated organs, macrophage infiltration, inflammation and vacuolization in the liver, disintegration of cellular compartmentation and dilation of intercellular spaces in gut and pancreas. Sample photographs of effects in gut and liver of fish exposed to 600 µg/L chlorpyrifos are shown in Fig. 5 and 6. In many fish, cells of the exocrine pancreas did not contain any or only few zymogene granules and large intercellular spaces occurred between the cells of the pancreas. Kidney cells occasionally showed vacuolization. After exposure to 300 µg/L chlorpyrifos, no necrotic cells were found and only slight reactions were observed in the liver, gut and kidney. These were slight macrophage infiltration, hypertrophy of nuclei, and dilation of intercellular spaces, especially in the gut. In all other exposure groups no histopathological effects were found (Fig. 7).

a)



b)

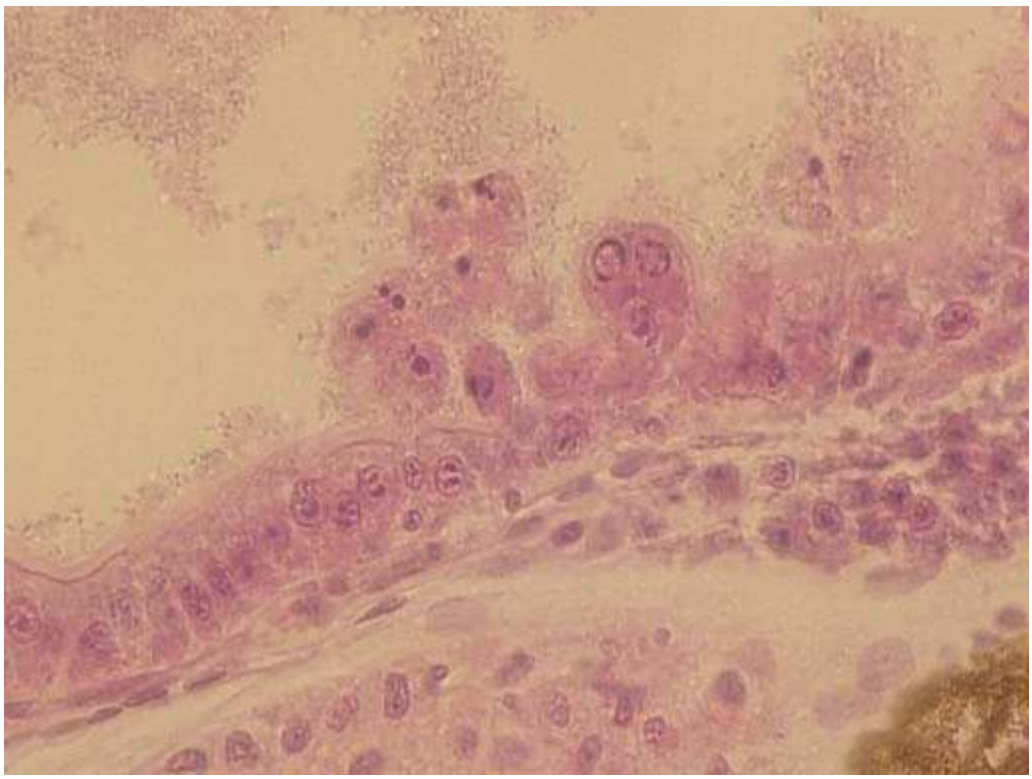
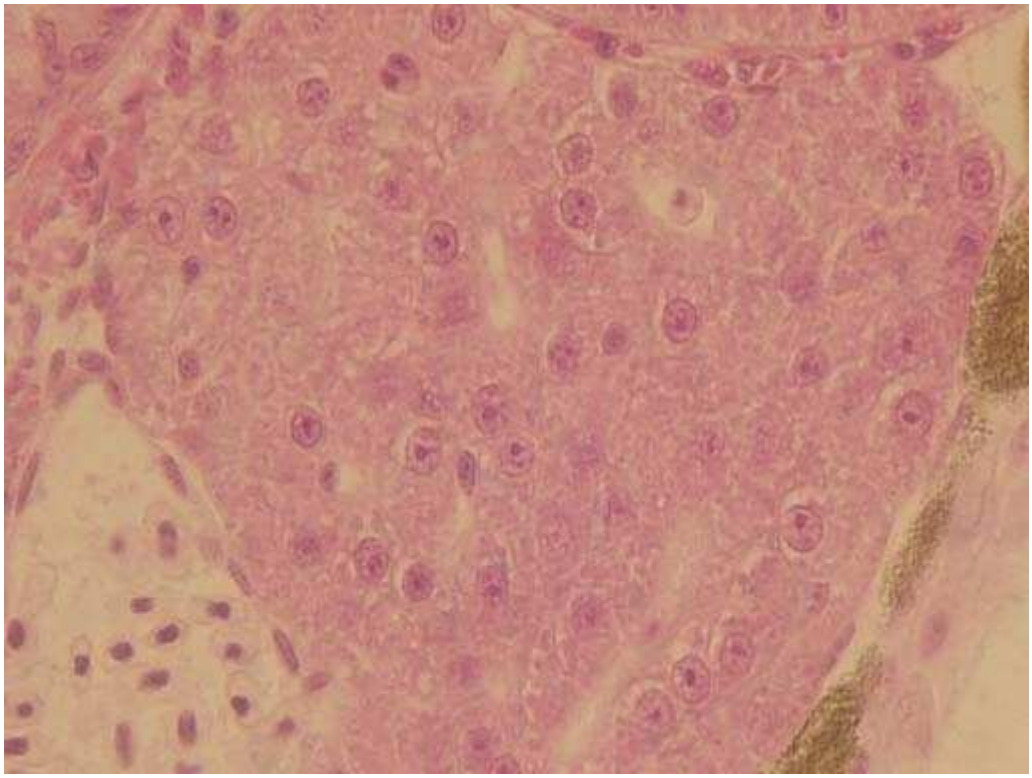


Fig. 5: : a) Gut of CPP-Ni-control fish with yolk residue (arrow). b) Gut of fish exposed to 600 µg/L CPP showing severe cellular lesions. OM (original magnifications) of both photos: x400.

a)



b)

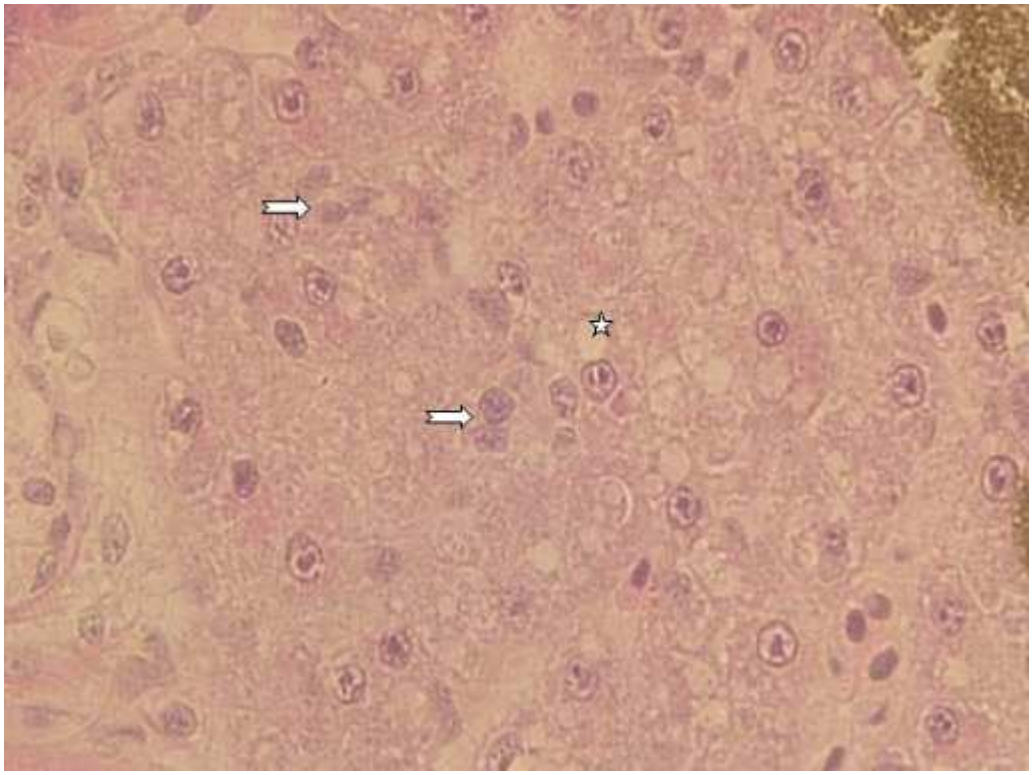


Fig. 6: a) Liver of control fish. b) Liver of fish exposed to 600 µg/L CPP. Vacuolization of liver cells (asterisk) and infiltration with macrophages (arrows). OM (original magnifications) of both photos: x400.

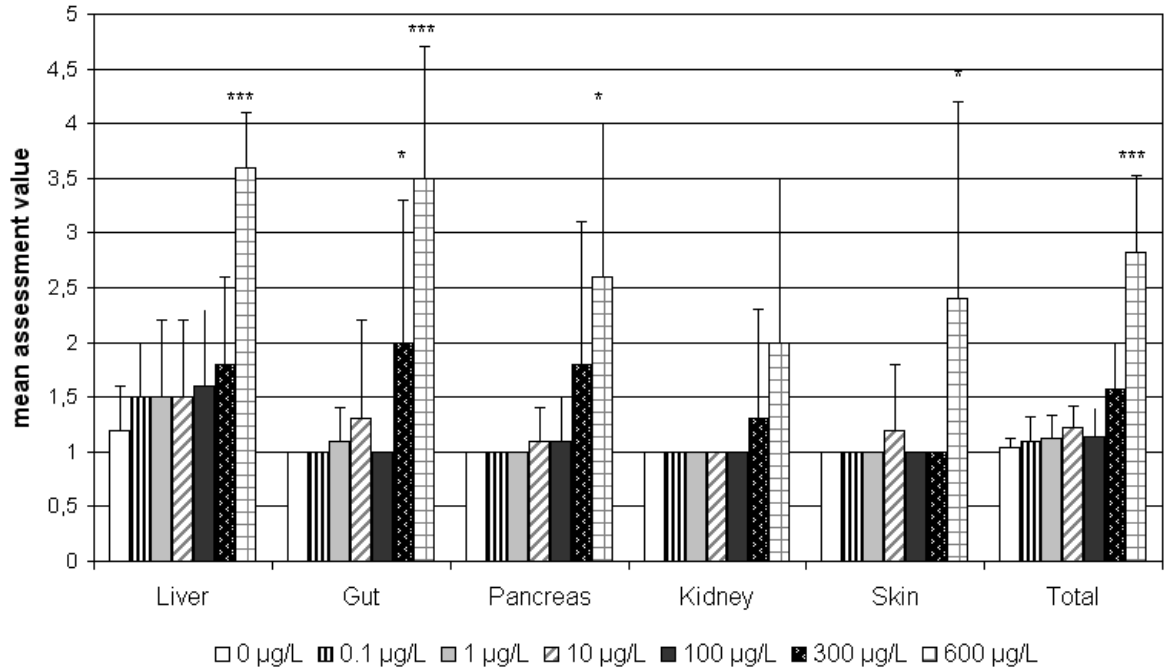


Fig. 7: Semi-quantitative assessment of histopathological effects of chlorpyrifos in zebrafish larvae. Assessment values (means + SD) obtained for different organs and 'total' fish.
*: $0.05 \geq p \geq 0.01$, ***: $p \leq 0.001$.

Histopathology, semi-quantification of effects: The semi-quantitative assessment of histopathology made evident that, after exposure to 600 µg/L chlorpyrifos, cells in all organs were frequently found to represent the status of strong reaction or even destruction (Fig 7). This was especially true for gut and liver cells. In liver, gut, pancreas and skin, data for the 600 µg/L chlorpyrifos exposure were significantly different from data of the other exposure groups (except for 10 µg/L, skin). Assessment values obtained for 300 µg/L chlorpyrifos were higher than those recorded for all organs of the control except for the skin, but significant differences only occurred for the gut. In the kidney obvious differences between the exposure groups were not significant.

Mixtures of $NiCl_2$ and chlorpyrifos

Embryo test: Results obtained for mixture experiments with $NiCl_2$ plus chlorpyrifos also showed a delayed hatching success in mixtures containing higher concentrations of $NiCl_2$, but no further effects according to the endpoints described in Table 2. Hatching success in mixture experiments with $NiCl_2$ and chlorpyrifos is shown in Fig. 8. As displayed in this figure, there is no indication of a synergistic or antagonistic effect of $NiCl_2$ and chlorpyrifos (as expected by the mode of action of both substances) in embryos exposed at 26°C. This is corroborated by the data

analysis using the MixTox model (Jonker et al., 2005) which did not give evidence for a synergistic or antagonistic effect.

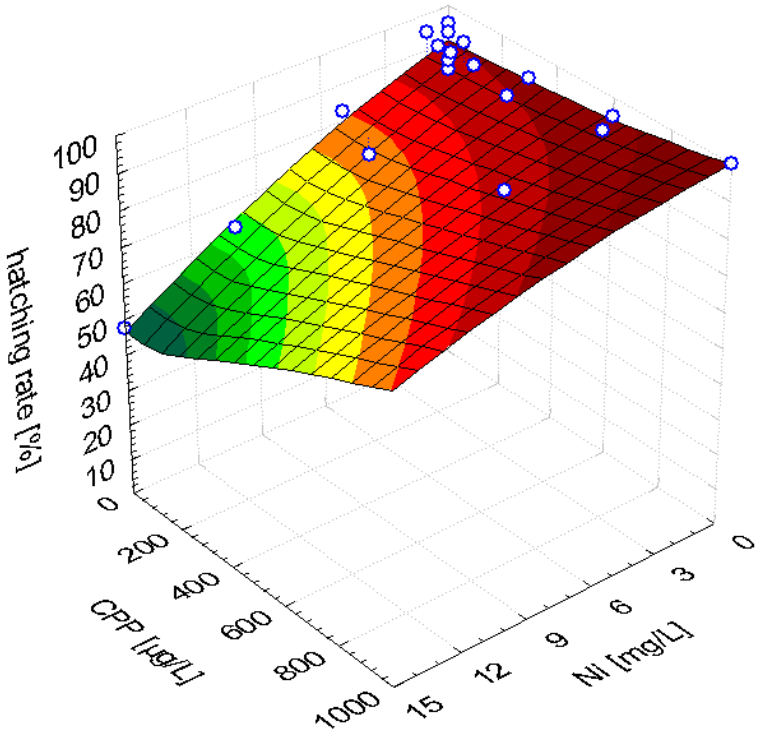


Figure 8. Hatching rate of Danio rerio larvae exposed to NiCl2 and chlorpyrifos (CPP), and binary mixtures of them. Response surface calculated with STATISTICA. The slope of the isoboles neither indicates synergism nor antagonism.

Hsp70: Hsp70 analysis showed increased stress protein levels compared to the control group in groups treated with mixtures containing 100 and 300 µg/L chlorpyrifos as well as in the group treated with 600 µg/ chlorpyrifos plus 0.5 mg/L Ni (Fig. 9). The group treated with 600 µg/ chlorpyrifos plus 5 mg/L Ni showed an Hsp70 level as low as in the control group. If results are arranged on the basis of increasing chlorpyrifos concentrations as in Fig. 9, it seems that the Hsp70 levels are also following an 'optimum curve', indicating that low Hsp70 levels in the group treated with 600 µg/ chlorpyrifos plus 5 mg/L Ni have resulted from an overwhelming of the stress response. Accordingly the Hsp70 response surface is shown in Fig. 10.

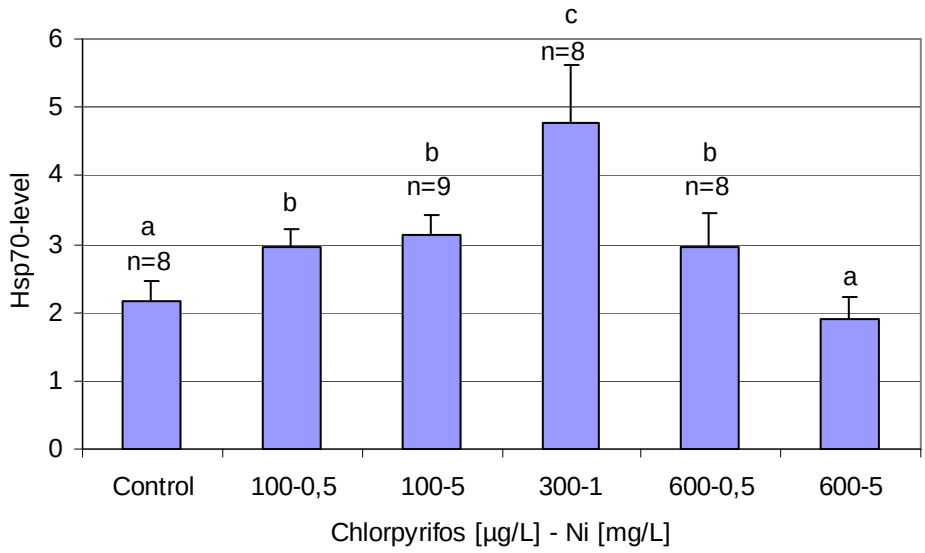


Fig 9: Hsp70 levels (means + SD) in Danio rerio larvae after 1 exposure to control conditions and five mixtures of chlorpyrifos [µg/L] and Ni [mg/L]; n=10 if not explicitely given. Different letters indicate significance at $p < 0.05$.

a) b)

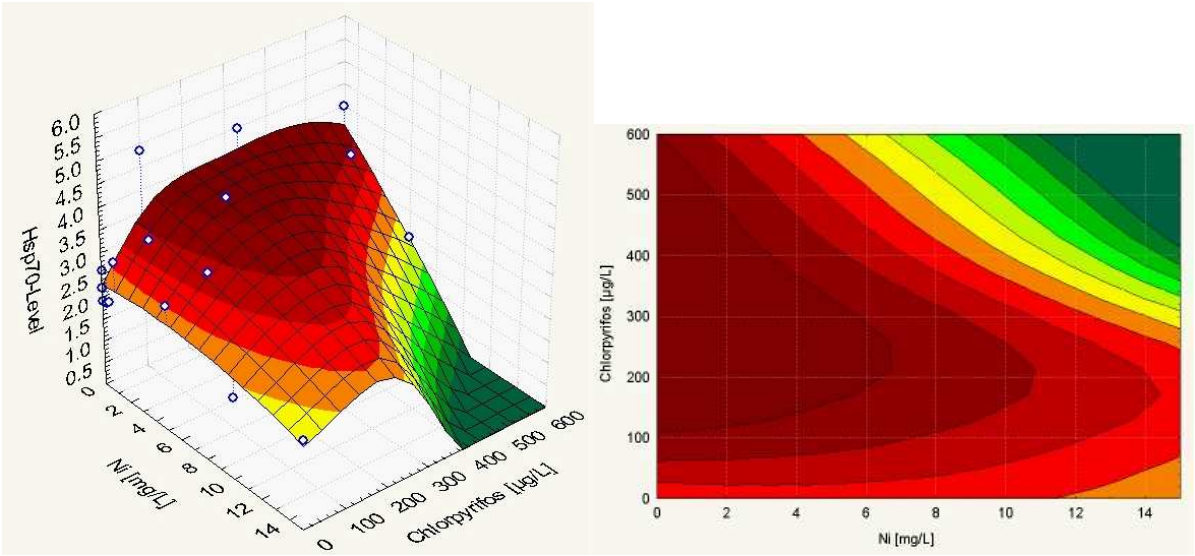


Fig. 10: Response surfaces for Hsp70-Levels resulting from nickel chloride and/plus chlorpyrifos exposures, a) 3d contour plot, b) 2d contour plot. The shape of the isoboles indicate neither synergism nor antagonism.

Histopathology, qualitative analyses: In zebrafish larvae exposed to mixtures of chlorpyrifos and nickel chloride, the two mixtures with 600 µg/L chlorpyrifos resulted in strong histopathological effects affecting all organs. This mainly resembled the reactions observed in fish exposed to chlorpyrifos alone. These reactions include necrosis, caryolysis and hypertrophy of nuclei in gut, liver and pancreas. Macrophage infiltrations and inflammation was found in all organs. In

the liver and kidney, the vacuolization of cells was prominent. A disintegration of the cellular compartmentation and the dilation of intercellular spaces was observed in gut and pancreas. In addition, the reduction of zymogene granules was strongly pronounced in the pancreas and did already occur after exposure to the mixture with 300 µg/l chlorpyrifos. In the skin, cells appeared flattened and the epithelium was compressed. Atrophic and necrotic cells became evident.

Histopathology, semi-quantification of effects: For the two mixtures containing 600 µg/L chlorpyrifos plus nickel chloride, the data were significantly different from control values for all organs except for kidney and skin, in which significant differences occurred only after exposure to the mixture with 5 mg/L nickel chloride (Fig. 11). The assessment value for the 'total' fish as well as the values for the pancreas in the animals exposed to the mixture of 300 µg/L chlorpyrifos plus 1 mg/L nickel chloride were also significantly different from the control data. A response surface for the 'total' values in the mixture experiment is shown in Fig. 12, which indicates, that the observed effects after mixture exposure were solely related to chlorpyrifos.

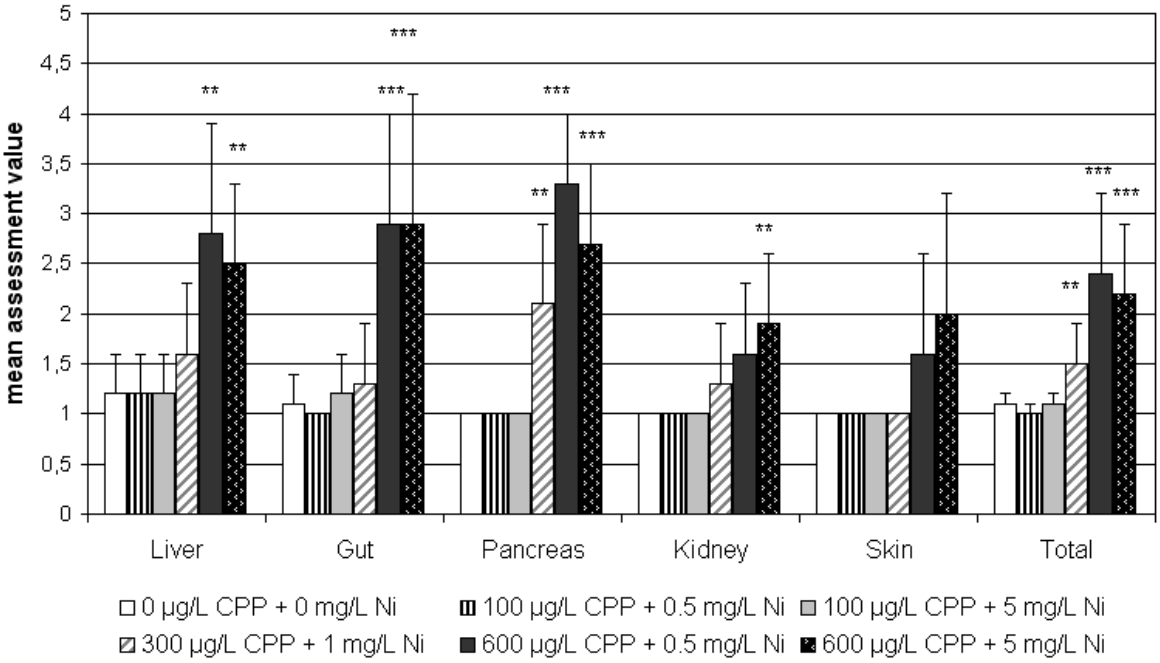


Fig. 11: Semi-quantitative assessment of histopathological effects of different mixtures of NiCl2 and chlorpyrifos (CPP) in zebrafish larvae. Assessment values (means + SD) obtained for different organs and 'total' fish. *: 0.05 ≥ p ≥ 0.01, **: 0.01 ≥ p > 0.001, ***: p ≤ 0.001.

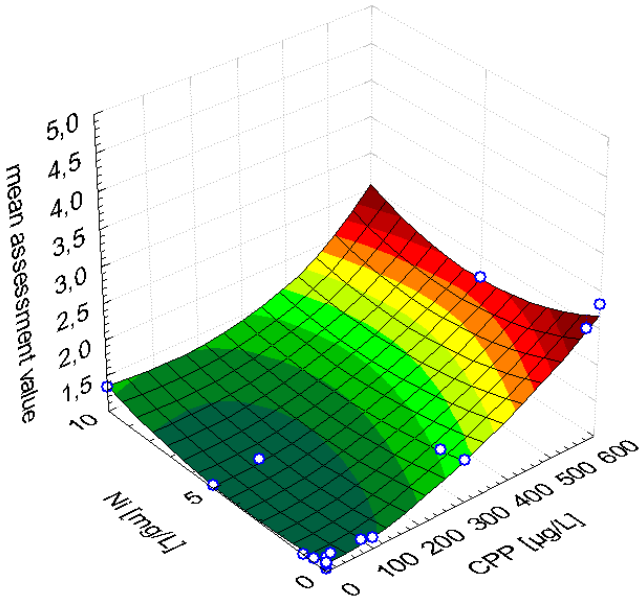


Fig. 12: Response surfaces for 'total' mean assessment values after exposure to NiCl2, chlorpyriphos (CPP) and mixtures of them. The shape of the isoboles indicate neither synergism nor antagonism.

Discussion

Multiple effects of $NiCl_2$, chlorpyrifos and their mixtures were found in zebrafish embryos and larvae. To answer the question whether the two substances act independently, or rather in an antagonistic or synergistic way, effects of single substance exposures and mixture experiments had to be compared. As shown in the Results, the only effect of $NiCl_2$ during the embryo test was a reduced hatching success. Reduced hatching success of embryos exposed to $NiCl_2$ was found in fish species other than *Danio rerio* before (Nebeker *et al.*, 1985, Dave & Xiu, 1991). This effect was also visible in zebrafish exposed to other heavy metals (Hallare *et al.*, 2005). It can be speculated that the reduced hatching success is a result of an interaction of nickel with a metalloprotease called hatching protease (chorionase) (Hagenmaier, 1974). Combinations of $NiCl_2$ with the pesticide chlorpyrifos, which did not affect hatching if applied alone, led to the same results as the single $NiCl_2$ exposure. This speaks for the hypothesis of independently acting substances, since chlorpyrifos was not altering the effects of $NiCl_2$.

The same was true, if the Hsp70 levels in the single substance and mixture experiments were compared. The stress response (Hsp70 level) resulting from exposure to $NiCl_2$ follows an optimum curve as described for other stressors (e.g. by Schill *et al.*, 2003). The reduction of the Hsp70-Level in higher concentrations (10 and 15 mg/L) compared to 1 mg/L indicates rather an overwhelming of the stress protein response by $NiCl_2$ than a recovery of the exposed larvae. In contrast, the

Hsp70 response to chlorpyrifos shows only the 'increasing' part of the Hsp optimum curve, since high concentrations of chlorpyrifos lead to (presumably neurotoxicity-based) death of the larvae before general cythopathology could have decreased the stress protein level. Higher concentrations than 600 µg/L could not be analysed concerning their influence on the Hsp70 level. The shape of the isoboles deriving from the results of the mixture experiments showed that there is no synergistic or antagonistic but an slightly additive effect of $NiCl_2$ and chlorpyrifos (at least for mixtures with higher chlorpyrifos concentrations) as expected by their mode of action.

Histological data revealed the observed symptoms to be only moderate reactions of fish to nickel chloride exposure and did not indicate severe histopathological damage. The only relevant reactions in response to 20 mg/L and 30 mg/L nickel chloride as well as in response to 300µg/L chlorpyriphos were found in the gut, whereas after exposure of larvae to 600 µg/L chlorpyrifos severe cellular damage were found in all organs investigated. The responses of the organs after exposure to the different mixtures of chlorpyrifos and nickel chloride were almost equal to the responses obtained when fish were exposed to chlorpyrifos only. However, whereas the "total" values for 300 µg/L chlorpyrifos were not significantly different from the control values when fish were exposed to the pesticide only, the combination of 300 µg/L chlorpyrifos plus 1 mg/L nickel chloride resulted in significant differences of "total" values and values for the pancreas from the respective controls. In addition, reactions in the kidney and the skin were not significantly different from the controls when fish were exposed to 600 µg/L chlorpyrifos only but were significant when mixtures of chlorpyrifos and nickel chloride were applied. This again speaks for the hypothesis that the two test substances show an independent and additive mode of action.

In the above described tests, $NiCl_2$ and chlorpyrifos acted independently and did not influence each others toxicity in *Danio rerio* embryos and larvae, approving the hypothesis of an independent mode of action of these two substances. Regarding their possible harmfulness if acting in combination in surface waters, the two substances could be assessed as if they were acting alone.

Even though adult fish seem not to be very sensitive to nickel the reduced hatching success already appears in concentrations lower than environmentally relevant concentrations (up to 183 mg/L; Kasprzak, 1987). Also the overwhelming of the stress proteine synthesis speaks for the fact that high nickel concentrations in the environment are harmful to fish. The higher activity and uncontrolled convulsions of hatched larvae exposed to the highest concentrations of chlorpyrifos could be

interpreted as a result of the action of chlorpyrifos as an acetylcholinesterase inhibitor. Nevertheless, chlorpyrifos seems to be not as harmful to fish as to invertebrate pests, the primary target organisms of this compound.
The results of the study suggest that both test substances may not cause acute mortality to zebrafish larvae in environmentally relevant concentrations, either when applied alone or in combination. Nevertheless, they also indicate sublethal effects on the developing fish which, in the long run, may alter life cycle or population parameters in natural populations.

Acknowledgements

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Kapitel 3: Influence of nickel chloride, chlorpyrifos and imidaclopride in combination with different temperatures on the embryogenesis of the zebrafish, *Danio rerio*

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Abstract

Two independent types of stressors, chemicals and high temperatures, which frequently act together in the environment are addressed in this study. Two pesticides (imidacloprid and chlorpyrifos) as well as a heavy metal salt (nickel chloride) were investigated for their toxic effect at different temperatures. Tests focused on the early development of zebrafish (Danio rerio) embryos and larvae (from fertilisation up to 168h post fertilisation) when exposed to the three respective chemicals at an optimum temperature (26°C) and three higher temperatures (up to 33.5°C). The two pesticides did not have a significant impact on the early development of the zebrafish at all temperatures tested; highest concentration of imidacloprid was 50mg/L, highest concentration of chlorpyrifos was 1mg/L. Nickel led to a significant decrease of hatching success at all temperatures, the combination of elevated temperature and nickel exposure revealed a synergistic effect of both stressors.

Keywords: fish; development; heavy metal; pesticide

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submitted to Archives of Environmental Contamination and Toxicology

Introduction

There is a growing public concern about climate change and globally increasing temperatures. Most studies testing the effect of chemicals potentially released into the environment are conducted under laboratory conditions at a given temperature. To obtain additional information on the effects of increased temperatures, in this study, we investigated the influence of elevated temperature in combination with exposure to a heavy metal and two pesticides on zebrafish early development.

The zebrafish, *Danio rerio* (Hamilton, 1822), is a widely used test species representative of freshwater fish (Nagel, 2002). Here we will report on the embryogenesis and early larval development of *D. rerio* embryos exposed to $NiCl_2$ and the pesticides imidachloprid and chlorpyrifos.

Zebrafish can be cheaply and easily held in the laboratory, their transparent eggs make investigation of the embryonal development from fertilisation up to hatching easy. *D. rerio* has been used as a model species in several biological disciplines such as developmental biology and genetics (Nüsslein-Volhard et al., 2002). In ecotoxilogical studies, zebrafish serve as a model freshwater vertebrate in acute toxicity tests as well as in early life stage tests (Nagel, 2002) where developmental aberrations caused by exposure to chemical stressors could be investigated (e.g. Hallare et al., 2004; Hallare et al., 2006).

At pH 5-9 nickel occurs predominantly as the ion $Ni[H_2O]_6^{2+}$ in natural waters (ICPS, 1991) and origins either from natural sources like nickel ore-bearing rocks (WHO, 2007) or from anthropogenic sources like the nickel processing industry (ICPS, 1991). Finnish rivers and streams unaffected by anthropogenic nickel pollution show background nickel concentrations of 0.14 to 4.0 µg/L (Mukherjee, 1998), whereas rivers near a nickel processing industrial site in Canada have been shown to contain up to 183.000 µg/L (Kasprzak, 1987).

Chlorpyrifos (O, O,-diethyl O-3,5,6-trichloro-2-pyridylphosphorothioate) is an organophosphate insecticide, acaricide and miticide used to control foliage and soil-borne insect pests on a variety of food and feed crops (U.S. EPA, 2002). It acts as an acetylcholinesterase inhibitor and is primarily a contact poison (Kamrin, 1997). Chlorpyrifos is one of the most widely used organophosphate insecticides, approximately 5000 tons are applied annually in agricultural settings in the U.S. (U.S. EPA, 2002). Concentrations of chlorpyrifos found in surface waters reach up to 10.8 µg/L (Marino & Ronco, 2005).

Imidacloprid (1-(6-chloro-3-pyridylmethyl)-N-nitro-imidazolidin-2-ylideneamine) is a neurotoxic, neonicotinoid insecticide which is used to control sucking insects on crops. (Tomizawa & Casida, 2005; Tomlin, 1997). Since being introduced to the

insecticide market in 1992, the use of imidacloprid has increased yearly (California Environmental Protection Agency). Concentrations of imidacloprid found in surface waters reach up to 14 µg/L (US Geological Survey, 2003)

Material and methods

Adult zebrafish (*Danio rerio*) of both sexes (strain: WIK, ZFIN ID: ZDB-GENO-010531-2) were kept in the laboratory in aerated and filtered aquaria with a minimum of 1 litre of water per fish on average. Culture conditions were 26 ± 1°C at a 12:12 hour light:dark cycle. The adult fish were fed twice per day with dry flake food and frozen small crustaceans, *Tubifex* or midge larvae, respectively. Fish keeping conditions were the same for all tests.

Prolonged embryo tests

Prolonged embryo tests were conducted at four different water temperatures, namely the standard temperature 26 ± 1°C and three higher temperatures (28 ± 1°C, 30 ± 1°C, 33.5 ± 1°C). Pre-tests aimed at measuring the baseline response of the embryonic development at temperatures differing from the optimal temperature of 26°C were conducted at 23 ± 1°C, 26 ± 1°C and 33 ± 1°C.

The evening before spawning was required, spawn traps covered with stainless steel mesh were placed in the aquaria. A spawning substrate was placed into the spawn traps. 60 minutes after the light was turned on, the spawn traps were removed and the eggs were collected. All eggs were transferred immediately into Petri dishes containing the different test solutions. Then the unfertilized eggs were removed, and the fertilized eggs were placed into new Petri dishes (10 embryos per Petri dish, 4 dishes per concentration) containing the respective test solutions. The tests were performed in climate chambers at a 12:12 hour light:dark cycle, water temperature was maintained at the respective temperatures, the Petri dishes were covered with lids to avoid evaporation. Embryo development was observed using a binocular at specified time points (see table 1) during the next 96h.

As this test procedure was designed for the standard water temperature of 26°C, the time points for endpoint investigations had to be adjusted to the different development rates at higher water temperature. Tests at 30.0°C and 33.5°C were shortened to 72h after fertilisation. Consequently, identical developmental stages were compared.

The water for the exposure of the eggs/embryos was prepared according to ISO-Standard 7346/3, containing 294 mg/L $CaCl_2$, 123.25mg/L $MgSO_4$, 64.75mg/L $NaHCO_3$ and 5.75mg/L KCL, dissolved in aqua bidest.

Chemicals and concentrations used in the tests

Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) obtained from Carl Roth, Germany, was dissolved in exposure water. Nominal concentrations of 0.5, 1, 5, 10 and 15 mg/L Ni resulting from a stock solution of 200 mg/L Ni were tested at 26°C and at 33.5°C, concentrations of 5 and 10 mg/L were tested at 28°C and 30°C additionally. A negative control containing pure exposure water was also tested in every experiment. Solutions in the Petri dishes were renewed every 48 h at 26°C and 28°C and every 36 h at 30°C and 33.5°C. Experiments were performed using plastic Petri dishes.

Imidacloprid obtained from Sigma-Aldrich, Germany, was dissolved in exposure water. Nominal concentrations of 1, 5, 10, 15, 20, 30, 40 and 50 mg/L imidacloprid prepared from a stock solution of 50 mg/L imidachloprid were tested at 26°C. At 28°C, concentrations of 5, 15 and 30 mg/, at 30°C and 33.5°C, concentrations of 5, 10, 25 and 25 mg/L were tested. A negative control containing pure exposure water was also tested in every experiment. Solutions in the Petri dishes were renewed every 48 h at 26°C and at 28°C and every 36 h at 30°C and 33.5°C. A new stock solution was prepared for every exchange of solutions. Experiments were performed using glass Petri dishes.

Chlorpyrifos obtained from Sigma-Aldrich, Germany, was dissolved in exposure water at a water temperature of about 40°C and the solution was stored at 35°C. Nominal concentrations of 0.1, 1, 10, 100, 300, 600 and 1000 µg/L chlorpyrifos resulting from a stock solution of 1000 µg/L chlorpyrifos were tested at 26°C and at 33.5°C, concentrations of 300 and 600 µg/L were tested at 28°C and 30°C additionally. A negative control containing pure exposure water was also tested in every experiment. Solutions in the Petri dishes were renewed every 48 h at 26°C and at 28°C and every 36 h at 30°C and 33.5°C. A new stock solution was prepared for every exchange of solutions. Experiments were performed using glass Petri dishes.

Data analysis (Mixture analysis model)

The data were analysed using the "MixToxModules.xls (23.10.2005)" file including concentration addition (CA) and independet action (IA) models (for details see Jonker et al., 2005). The file was taken from http://www.ceh.ac.uk/sections/er/csvendsen.html. Response surfaces shown were calculated using STATISTICA 5, StatSoft, Inc.

Results and Discussion

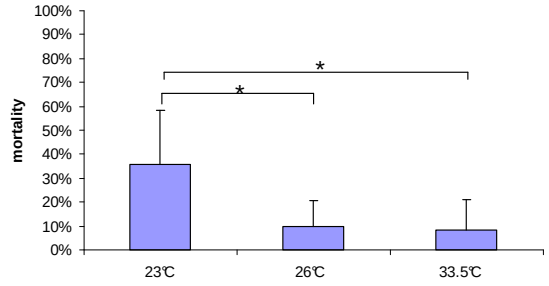
The pre-tests revealed temperatures of 23°C to lead to significantly higher mortality compared to higher temperatures ($p<0.05$, Wilcoxon-test) as shown in Fig. 1a. The higher mortality of the zebrafish embryos kept at 23°C (Fig. 1a) is a result of reduced hatching success (Fig. 1c), indicating that mortality occurred while the embryos were still located inside the egg but should have hatched already, judging by their development stage.

A second endpoint that was affected by temperature was the occurrence of yolk sac edema. This parameter also was only found to be elevated at 23°C (Fig. 1b). The occurence of yolk sac edema was significantly higher in zebrafish embryos exposed to this temperature in comparison with 33.5°C ($p<0.01$, Wilcoxon-test). Due to a high standard deviation, occurrence of yolk sac edema at 26°C was not significantly different to the percentages recorded for 23°C or for 33.5°C but, indeed, very low.

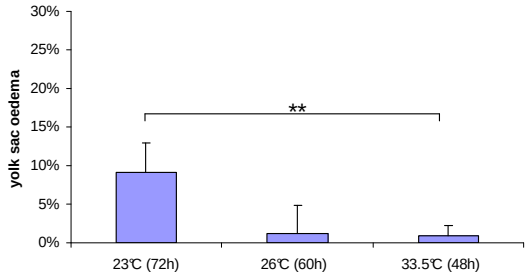
Another endpoint which was affected by temperature was hatching time. Due to the faster development of embryos kept at higher temperatures the larvae hatched earlier in these treatments. Because of high embryo mortality (inside the egg) at 23°C, the hatching success was relatively low in this group (Fig. 1c).

To avoid higher mortality and the occurrence of a high rate of edema due to low temperature stress, and in accordance with the goal of showing potential effects of higher temperatures in the environment it was decided to conduct the following tests using the standard temperature as well as the highest temperature investigated in the pre-test. In addition, two intermediate temperatures (28°C and 30°C) were tested in the main experiments.

a)



b)



c)

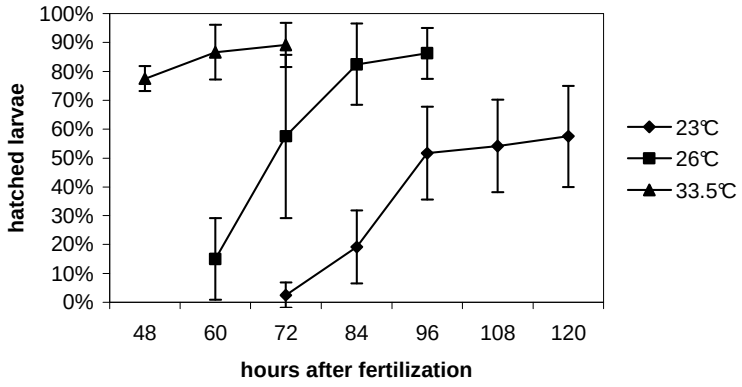


Fig.1: a: Mortality during the pre-tests at three different temperatures, means of 3 repeated experiments, + sd, *: $p<0.05$. b: Percentage of yolk sac edema during the tests at three different temperatures but identical develomental stages (time point of observation given in parentheses), means of 3 repeated experiments, + sd, **: $p<0.01$. c: Hatched larvae in % of the initial stock of eggs per temperature, means ± sd.

The prolonged embryo test conducted with $NiCl_2$ showed increasing concentrations of the agent to lead to delayed hatching success at all investigated temperatures. An example of the reduced hatching success (compared to control conditions) at the optimum temperature of 26°C and at the highest temperature tested is shown in Fig. 2 and 3, at two time points respectively.

Embryos developing at 26°C or higher temperatures which had not hatched after 96 h post fertilisation usually did not hatch later, but died inside the egg within the following days. Fig. 4 displays the response surface of the hatching rate vs $NiCl_2$ concentrations and different temperatures.

a) b)

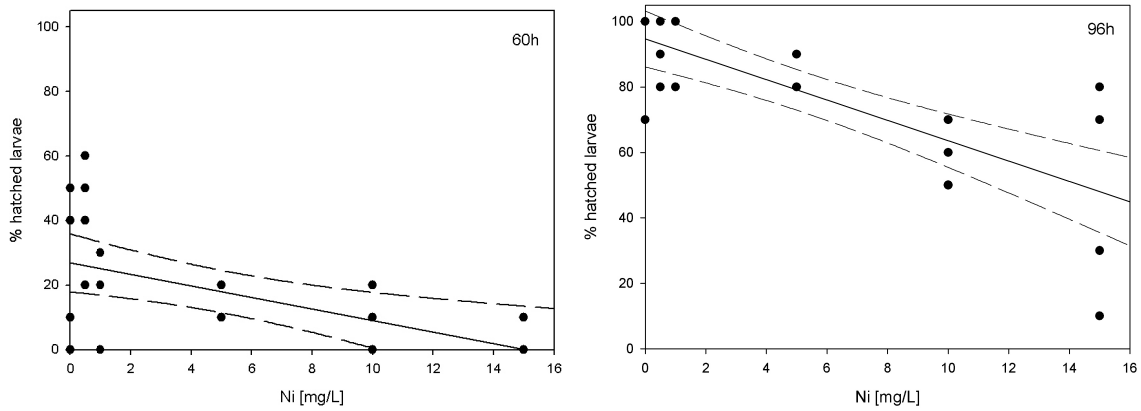


Fig. 2: Hatching success of *Danio rerio* embryos in % of the initial stock of eggs exposed to NiCl2 at 26°C. a: 60h post fertilisation, Linear regression analysis and 95% confidence intervals, ANOVA p<0.01 b: 96 h post fertilisation, ANOVA: p<0.001.

The delay in hatching increased with rising temperature, predominantly when the higher temperatures were combined with high nickel concentrations. Analysis of combined $NiCl_2$ exposure and temperature stress revealed a synergistic effect (p=0.0025) of the two stressors.

Other investigated endpoints during the embryo test (according to Table 1) did not show any reaction to $NiCl_2$.

b) b)

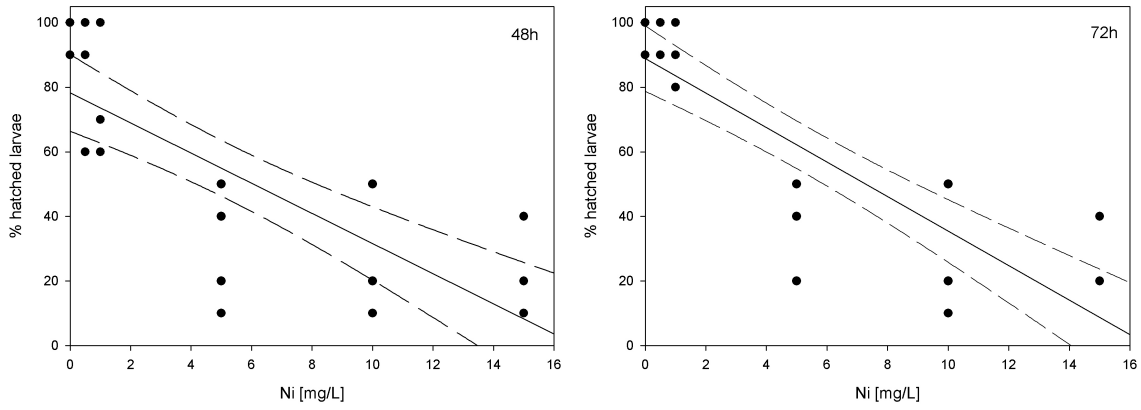


Fig. 3: Hatching success in % of *Danio rerio* embryos of the initial stock of eggs exposed to NiCl2 at 33.5°C. a: 48h post fertilisation, Linear regression analysis and 95% confidence intervals, ANOVA: p<0.001 b: 72 h post fertilisation, ANOVA: p<0.001.

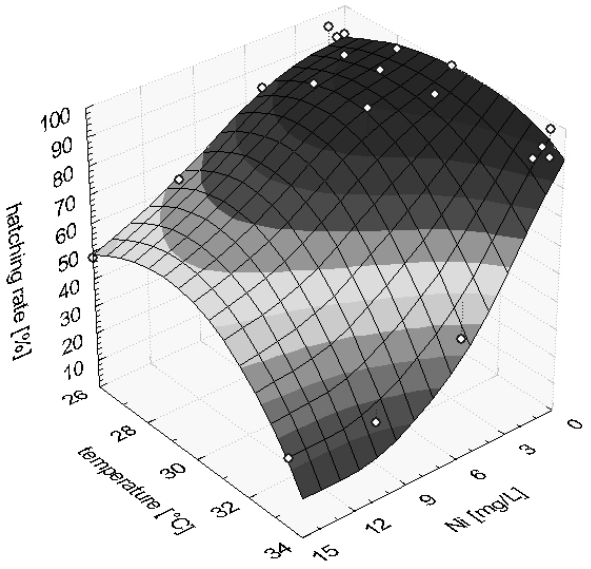


Figure 4: Hatching rate (percent of hatched larvae related to initial 40 eggs) of Danio rerio larvae exposed to NiCl2 at four different temperatures

The effect of reduced hatching success of embryos exposed to $NiCl_2$ resembles results of earlier experiments which showed this effect as a result of exposure to nickel (Dave & Xiu, 1991; Gauthier et al., 2006) as well as to other heavy metals (Hallare et al., 2005). Reduced hatching success might be a result of an interaction of nickel with the hatching protease (chorionase) (Hagenmaier, 1974), a metalloprotease. Even if adult zebrafish seem to be rather insensitive to nickel, (only moderate histopathological effects if exposed for 5d to 20 mg/L $NiCl_2$, data not shown) the reduced hatching success already appears at concentrations lower than environmentally relevant concentrations (up to 183 mg/L; Kasprzak, 1987). Regarding the predicted elevation of global temperatures the extreme delay in hatching success at higher temperatures is alarming. This should also be taken into account for risk assessment for Ni and other heavy metals.

Prolonged embryo tests with chlorpyrifos as well as tests with imidacloprid did not reveal any effects according to the endpoints described in Table 1 regardless of temperatures. Nevertheless, hatched larvae exposed to 600 and 1000 µg/L chlorpyriphos showed higher activity and uncontrolled convulsions. These symptoms are most likely a result of the acetylcholinesterase inhibitor property of chlorpyrifos. Nevertheless, chlorpyrifos seems to be not as disruptive of the early development of zebrafish as of that of invertebrates against which it is targeted. However, Levin et al. (2003, 2004) showed that exposure to concentrations of 100 ng/L chlorpyrifos during early development could impair the swimming

behaviour of older larvae and that 10 to 100 ng/ml of chlorpyrifos could cause significant spatial discrimination impairments in zebrafish when they are adults.

Imidacloprid is a representative of the group of neonicotinoids, which generally have a low toxicity to mammals, birds, and fish (Tomizawa & Casida, 2005). This is the result of the specificity of the drug binding at the nicotinic acetylcholine receptors. It was shown that neonicotinoids designed to bind to insect nicotinic acetylcholine receptors do not bind as well to vertebrate nicotinic acetylcholine receptors. (Tomizawa & Casida, 2005)
Concerning imidacloprid in our tests, the absence of detrimental effects may also be due to the fact that the embryos remained in the egg for approximately ¾ of the test duration and were protected by the chorion. This protective effect is likely, because Jemec et al. (2007) mention an unpublished LC_{50} (96h) of 10 mg/L for adult zebrafish, which is lower than the highest concentration testet in our study. Other studies (e.g. Sanchez-Bayo & Goka, 2005) found stress symptoms like massive parasite infestation in fish exposed to imidaclopride in their environment.

In summary, in the present work we were able to show that elevated temperatures could increase the toxicity of $NiCl_2$ on *Danio rerio* embryos. Two investigated insecticides, chlorpyrifos and imidacloprid showed no or only little effect on the early development of *D. rerio*.

Acknowledgments

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Kapitel 4: Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae

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Abstract

In this study the effects of 3,4-dichloroaniline (3,4-DCA), a decomposition product of the herbicides propanil and diuron (and other pesticides), and diazinon, a neurotoxic insecticide, on early life stages of zebrafish *Danio rerio* were assessed. The toxicity of these substances with different modes of action (acetylcholine esterase inhibitor *vs.* unspecific membrane irritant) was tested for single substances as well as in binary mixtures. To study effects on different biological organisation levels (from the molecular up to the whole organism level) the molecular stress response regarding Hsp70, the embryonic and larval development and the locomotor activity were investigated as integrative biomarkers.

In single substance tests 3,4-dichloroaniline affected locomotor activity, deformations, and mortality at ≥0.5 mg/L during the 11 d subchronic tests. Diazinon effects on those parameters were obvious at ≥2 mg/L, except for the deformation rate (11 d: 1 mg/L). In equitoxic mixtures of both substances concentration additivity was observed for deformation rate and mortality (11 d). An increase in the Hsp70 content occurred in zebrafish exposed to 0,25 mg 3,4-DCA/L as well as to 0.05 mg diazinon/L; in mixtures concentration additivity could be shown.

The investigated endpoints varied in respect to their sensitivity. Accordingly, for an integrated understanding of the effects of chemicals and their mixtures on fish, a battery of different test methods should be applied.

Keywords: fish, multi-level approach, pesticides, stress proteins, behaviour, DarT

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submitted to Aquatic Toxicology

1. Introduction

In the environment organisms, usually are not exposed to single chemicals, but rather to mixtures of pollutants. The behaviour of chemicals in mixtures is strongly influenced by their toxic mode of action. If two or more chemicals have different target sites, their effect can usually be treated independently. Mixtures of chemicals with a common target site and the same mode of action act according to concentration or dose additivity. However, if the mixture components interact with each other, they might cause antagonistic or synergistic effects. (Escher and Hermens 2002).

Zebrafish (*Danio rerio*, Hamilton 1822, Pisces, Cypriniformes) are popular test organisms in developmental biology and genetics (Kimmel 1989, Nüsslein-Volhard 1994) as well as in ecotoxicology (e.g. Bachmann 2002, Hallare et al. 2004, Osterauer and Köhler 2008). The early life-stage test (ELS) with *Danio rerio* (DarT) has been established by Nagel (2002) to substitute the fish acute toxicity test (OECD 1992). It has gained increasing attention in the last years because of the higher sensitivity of embryos and larvae compared to adult fish (Hoang et al. 2004).

In the present study different test parameters were chosen to analyse the impact of two independently acting substances. To gain information about the sensitivity of a broad range of test parameters, early life stage and subchronic developmental tests, behavioural tests as well as stress protein analyses have been performed with zebrafish. In this multi-level approach, the two following substances with different modes of action were chosen:

3,4-dichloroaniline (3,4-DCA), acting as a non-specific membrane irritant or metabolic inhibitor, is an intermediate product in the synthesis of 3,4-dichlorophenylisocyanate, the herbicide propanil (and other pesticides) and an azo dye for polyester fabrics. In Western Europe, 12,000 tonnes of 3,4-DCA was produced in 1991 Currently, there is no direct use of 3,4-DCA without chemical transformation (EU 2006).In the environment, 3,4-DCA is mainly a result from biotransformation of certain crop protecting agents originally produced from 3,4-DCA and is, therefore, mainly released in agricultural soils (BUA 1994). 3,4-DCA is highly soluble in water (580 mg/l at 20°C, with no hydrolysis and an estimated half-life of 18 days (IHCP 2006)). In surface waters, concentrations ranging from < 0.05 – 1.5 µg/L were found (EU 2006, Planas et al. 2006). 96-hour LC_{50} values for fish were 1.94 mg/l for rainbow trout (Hodson 1985) and 8.5 mg/L for zebrafish (Becker et al. 1990). In chronic tests, including early-life-stage and life-cycle tests, the threshold concentrations (LOEC) for the effect of 3,4-dichloroaniline on body length, body weight, deformation, mortality and reproduction, following 4 to a

maximum of 16 weeks of exposure, were 0.2 mg/l as tested with four fish species, among them the rainbow trout. According to Allner (1997), 3,4-DCA is rapidly taken up by fish and metabolised to 3,4-dichloroacetanilide. Also back-metabolisation to 3,4-DCA was observed in this study.

Diazinon is a non-systemic organophosphate insecticide extensively used for pest control e.g. against a variety of sucking and leaf-eating insects in home gardens and farmland, and in veterinary treatments. The substance is available in a variety of formulations, e.g. dust, granules, seed dressings, wettable powder or emulsifiable solution formulations (Kamrin 1997). In the US $6.1x10^6$ kg were produced in 1999 (PAN 2000). Diazinon excerts its target effect by inhibiting the enzyme acetylcholine esterase which inactivates the neurotransmitter acetylcholine (Pesando et al. 2003). 96 h LC50 values range from 0.32-0.35 µg/L for *Ceriodaphnia dubia* (Bailey et al. 1997), 1.35 mg/L for *Oncorhynchus mykiss* (Meier et al. 1979), 1.53 mg/L for larval *Cyprinus carpio* (Aydin and Köprücü 2005) and 2.21 – 8 mg/L for adult *Danio rerio* (Ansari et al. 1987, Keizer et al. 1991) up to 10.3 mg/L for adult fathead minnow (*Pimephales promelas*) (Meier et al. 1979). Environmental concentrations of 1.5 µg/L have been found in urban waterways in California (Bailey et al. 2000). Diazinon is soluble in water up to a concentration of 40 mg/L (at 20 °C). The breakdown rate in water is dependent on the respective acidity: the half-life of diazinon ranges from 12 h (at high acidic levels) to 6 months (in a neutral solution) (Kamrin 1997).

The aim of the present study was to assess the toxicity of 3,4-dichloroaniline (3,4-DCA) and diazinon as single substances and in binary mixtures on different biological organisation levels of embryos and larvae of zebrafish *Danio rerio* in a multi-level approach.

The following hypotheses were tested for juvenile zebrafish:

1. Endpoints at lower levels of biological organisation (molecules) should exhibit higher sensitivity to 3,4-dichloroaniline, diazinon and mixtures of them than those on higher levels (organisms).
2. 3,4-Dichloroaniline and diazinon should act independently in equitoxic mixtures.
3. The acetylcholine esterase inhibitor diazinon should lead to more severe effects than the unspecific toxicant 3,4-dichloroaniline.

2. Materials and methods

2.1 Maintenance of test animals and acquisition of eggs

Adult male and female zebrafish (*Danio rerio*, strain: WIK, ZFIN ID: ZDB-GENO-010531-2) were kept in the laboratory in aerated and filtered aquaria with a minimum of 1 litre of water per fish on average. Fish keeping conditions were a temperature of 26 ± 1°C at a 12:12 hour light:dark cycle. A conductivity of 400 µS was gained by mixing tap water with deionisised water. Adult fish were fed twice a day with dry flake food and frozen small crustaceans (Bosmidae, *Moina* sp.), *Tubifex* or midge larvae, respectively. For the acquisition of eggs, spawn traps with spawning substrate were placed in the aquaria the evening before spawning was required. Sixty minutes after beginning of spawning (triggered by sudden illumination of the aquaria in the morning), the spawn traps were removed and the eggs were collected. This procedure was the same for all tests.

2.2 Test substances

3,4-Dichloroaniline (3,4-DCA, techn., Fluka, Steinheim, Germany) was dissolved in reconstituted water (OECD 1992) to a stock solution of 50 mg/L while constantly stirring. The test solutions were prepared directly before use from the stock solution. The test concentrations for the respective tests are given in Table 1. For the prolonged embryo test, 0.5, 0.7, 1, 1.5 and 2 mg/L 3,4-DCA were tested. For the Hsp70 analysis 0.05, 0.1, 0.15, 0.2 and 0.25 mg/L 3,4-DCA were tested. The subchronic test comprised six concentrations (0.005, 0.01, 0.1, 0.25, 0.5 and 1 mg/L 3,4-DCA).

Table 1: Test concentrations for the prolonged embryo test, the subchronic test and the Hsp70 analysis (single substance and mixture tests)

embryo test		subchronic behaviour test		Hsp70 analysis	
3,4 DCA [mg/L]	Diazinon [mg/L]	3,4 DCA [mg/L]	Diazinon [mg/L]	3,4 DCA [mg/L]	Diazinon[mg/L]
0	0	0	0	0	0
0,5	0	0,005	0	0,05	0
0,7	0	0,01	0	0,1	0
1	0	0,1	0	0,15	0
1,5	0	0,25	0	0,2	0
2	0	0,5	0	0,25	0
0	0,1	1	0	0	0,05
0	0,5	0	0,01	0	0,1
0	2	0	0,1	0	0,2
0	3	0	0,25	0	0,5
0	5	0	0,5	0	1
0,667	1,333	0	1	0,083	0,033
1,333	0,667	0	2	0,167	0,017
0,333	0,667	0	5	0,042	0,017
0,667	0,333	0,167	0,333	0,083	0,008
1	2	0,333	0,667	0,125	0,05
		0,083	0,667		
		0,5	1		
		0,25	2		
		0,167	1,333		

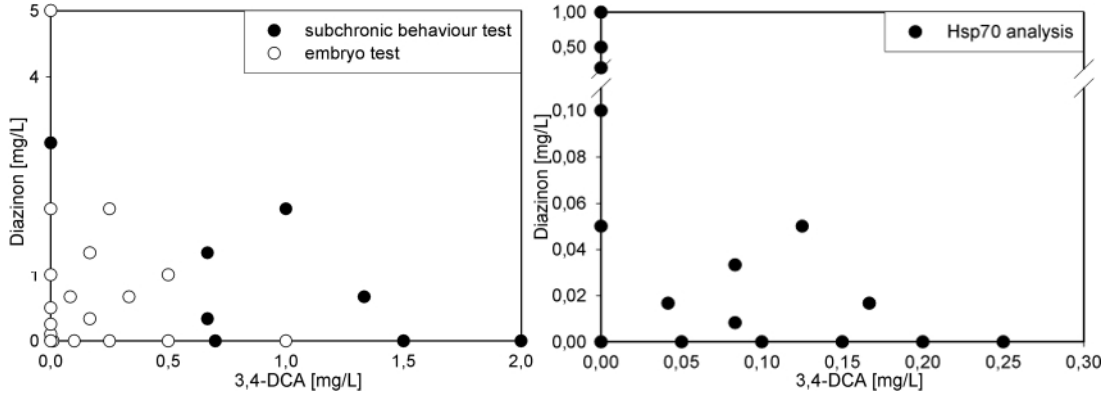

Fig. 1 Test design for the mixture experiments with 3,4-dichloroaniline [mg/L] and diazinon [mg/L].

Diazinon (Pestanal, analytical standard, Sigma-Aldrich, Seelze, Germany) was dissolved in reconstituted water (OECD 1992) in order to prepare a stock solution of 10 or 20 mg/L while constantly stirring. Test solutions were prepared from this stock solution directly before use. The test concentrations for the respective tests are given in Table 1. The prolonged embryo test comprised 0.1, 0.5, 1, 2 and 3 mg/L diazinon and, for the biochemical investigations, 0.05, 0.1, 0.21, 0.5 and 1 mg/L diazinon.were tested. Diazinon concentrations of 0.01, 0.1, 0.25, 0.5, 1, 2 and 5 mg/L Diazinon were examined for the subchronic test.

The test design for the mixture experiments is given in Fig. 1 and Table 1. All mixtures were selected according to the results of the single substance tests. For every test and its parameters, individual calculation of mixtures was based on the LOECs (= 1 toxic unit, 1 TU) obtained in the respective single substance tests. In the mixture experiment combinations of the two substances were equal to either 0.5, 1, or 1.5 TU. In all tests, a negative control with pure reconstituted water was run in parallel.

2.3 Prolonged embryo test

Prolonged embryo tests were conducted at 26 ± 1°C, according to the protocols of Nagel (2002) and OECD (1992). After the collection of spawned eggs, all eggs were transferred immediately into Petri dishes containing the different test solutions. Then the unfertilized eggs were removed, and the fertilized eggs were placed into new Petri dishes (10 embryos per Petri dish, 4 dishes per concentration) containing the respective test solutions. The tests were performed in climate chambers at a 12:12 hour light:dark cycle, water temperature was maintained 26 ± 1°C, the Petri dishes were covered with lids to avoid evaporation. Embryo development was observed using a binocular at specified time points during the next 96h.

2.4 Subchronic test

The subchronic test was conducted according to the VMD Guidance Note “Ecotoxicity testing of medicines intended for use in fish farming” (VMD 1996). The zebrafish were exposed to 3,4-DCA, diazinon, or binary mixtures of both from the time of fertilization (see 2.3) onwards up to an age of eleven days in glass Petri dishes with 30 fertilized eggs each and three replicates per concentration. Several endpoints were recorded daily in the course of the experiment, such as hatching rate (up to an age of 96 h), deformations and mortality. From each replicate, four larvae were randomly removed at regular intervals (5, 8, and 11 days after fertilization) for behavioural measurements, which were performed in the same

toxicant concentrations as used for the subchronic exposure. Measurement of the locomotor activity of the larvae was performed with the Multispecies Freshwater Biomonitor® (LimCo International, Germany, see Section 2.5). No food was provided during the experiments.

2.5 Behaviour measurements using the MFB

The Multispecies Freshwater Biomonitor® is an online biomonitor for quantitative and continuous recording of the behaviour pattern of animals (Gerhardt et al. 1994). The activity of the animals is measured in flow-through sensor chambers with quadropole impedance conversion as measuring principle connected to a measuring unit and a personal computer with specific software for data evaluation (Gerhardt 2000). Different types of behaviours e.g. locomotion and ventilation can be differentiated (Gerhardt et al. 1994).

Chambers with a size of 4 cm in length and a diameter of 1 cm allowed free movement of the fish (size of fish larvae: ~ 3.8 mm in length, ~ 0.5 - 1 mm in diameter) as mentioned in an earlier study (Kienle et al. 2008). For behaviour measurements in the subchronic test, the measurement chambers were placed into glass aquaria (15*20*20cm) filled with 1.5L of the respective solution. Those were arranged in duplicate in a surrounding black basin (to prevent disturbance from movement along the aquaria) containing temperature adjusted water (26 ± 1°C) and illuminated from above during the measurements (58 Watt neon light, distance to chambers: 145 cm). Only healthy larvae were transferred carefully into the chambers (one larva per chamber), the lid closed and the remaining air bubbles in the chambers removed with a Pasteur pipette. Subsequently, the chambers were placed horizontally on the bottom of the test aquarium. Following an acclimation time of 10 min. the measurement was started and the behaviour of 11 - 12 larvae per treatment was continuously recorded for a duration of 2 h in intervals of 10 min with a duration of 4 min each.

2.6 Hsp70 Analysis

To obtain embryos for stress protein (Hsp70) analysis 40 eggs per Petri dish (3 dishes per concentration) were exposed in the way described for the prolonged embryo test. The tests lasted 168 h. Ten times 8 embryos from different Petri dishes, respectively, were pooled for the respective concentrations (n=10), shock frozen in liquid nitrogen and stored at -20°C. The pooled larvae were ultrasonically homogenized in 20 µl extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor Sigma P8340, pH 7.5).

Subsequently, the homogenate was centrifuged (12 min, 20.000 g at 4°C). The total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant amounts of total protein from each sample (20 µg of total protein per lane) were subjected to SDS-PAGE followed by Western blotting, staining of the Hsp70 protein bands, and their densitometrical quantification (for a detailed protocol see Scheil et al., 2008).

2.7 Data analysis

Nonparametric methods were chosen for statistical evaluation as the data were only partially normally distributed (Shapiro-Wilk test, JMP 4.0, SAS systems, USA). The data of all tests were analysed for significance with a Friedman's ANOVA (Statistica 5.0, StatSoft, USA), followed by a Wilcoxon two group test (JMP 4.0, SAS systems, USA) in order to detect differences between control and exposure treatments (signficance levels ***: $p \leq 0.001$, **: $0.001 < p \leq 0.01$, *:$0.01 \leq p < 0.05$). Values for lethal concentrations (LCs) were calculated with Table Curve™ 2D 5.1 non-linear analysis software (SYSTAT software Inc., USA). For behaviour measurements, means of locomotor activities (% time spent on locomotion) for each larva were calculated separately for the first and the second hour, to take into account possible early warning reactions and the decrease of activity over time. Tor statistical evaluation, the data on "percentage time spent on locomotion" were arcsine transformed from proportional values. Calculation of the response surfaces for mixture data of 3,4-DCA and diazinon was performed with Statistica 5.0 (StatSoft, USA). Types of mixture responses were calculated using the MixTox Model (Jonker et al. 2005).

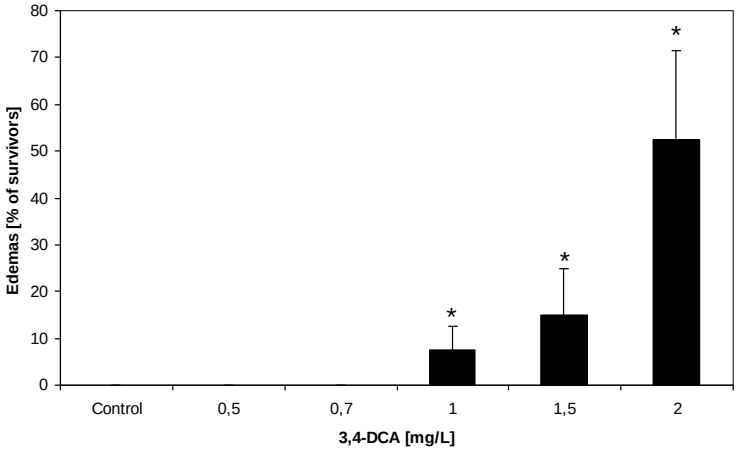

Fig. 2 Percentages of larvae exposed to 3,4 dichloraniline which showed edemas 96h post fertilisation. *: significantly differen to the control, $p<0.05$. n=10, bars represent means ± SD.

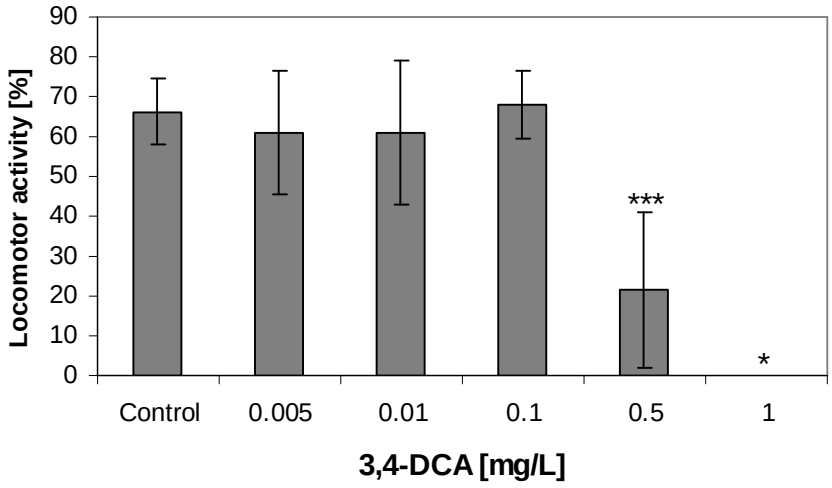

Fig. 3 Locomotor activity (percent of total time spent in locomotion) of five- and eight-days-old D. rerio larvae exposed to different 3,4-dichloroaniline concentrations [mg/L].

3. Results

3,4-Dichloroaniline

During the prolonged embryo test, edemas occurred in significant amounts. A significant increase of heart and yolks sac edemas was found in fish exposed to 1 mg/L 3,4-DCA and higher concentrations (Fig. 2) at 96h post fertilisation. Mortality was not significantly increased in any test concentration during the 96h embryo test. When the test duration was extended to 11 days (subchronic test with behaviour measurements), deformities were significantly increased in zebrafish exposed to 0.5 and 1 mg 3,4-DCA/L from an age of 5 and 4 days onwards as well as in ≥ 9-days-old larvae at 250 µg/L. Among these mostly oedema (98.3 and 100%, respectively at day 9) and an abnormal bending of the spine (65.7 and 88.9%, respectively at day 9) occurred. Furthermore mortality increased at 0.5 and 1 mg/L from an age of 7 and 6 days onwards, respectively, as well as in ≥ 5-days-old larvae at 5 µg/L (Fig. 4), resulting in an LC_{50} of 0.388 mg/L 3,4-DCA at 11 days. A significant reduction in locomotor activity at an age of 5 days was measurable at 0.5 and 1 mg 3,4-DCA/L (Fig. 3) A significant increase of the Hsp70 level was observed if the embryos and larvae were exposed to 250 µg/L 3,4-DCA for 168h. Hsp70 levels of all investigated groups are displayed in Fig. 5.

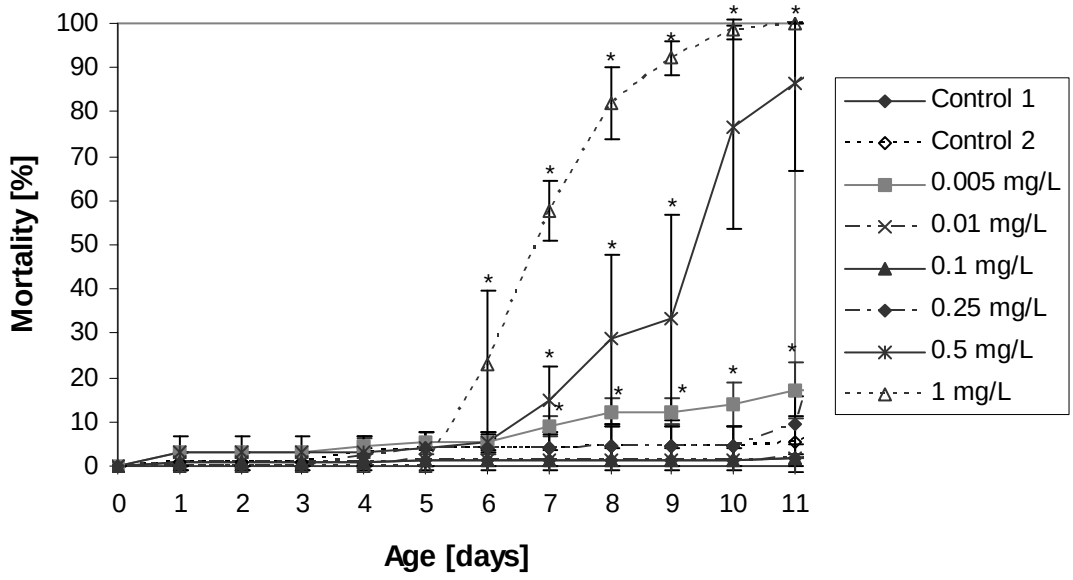

Fig. 4 Cumulative mortality [%] of D. rerio larvae exposed to different 3,4-dichloroaniline concentrations [mg/L] (means ± SD; number of larvae per replicate: 30 (days 0 - 5); 26 (days 6 - 8); 22 (days 9 - 11), 3 replicates for each experiment). *: Significantly different to control treatment at $p < 0.05$.

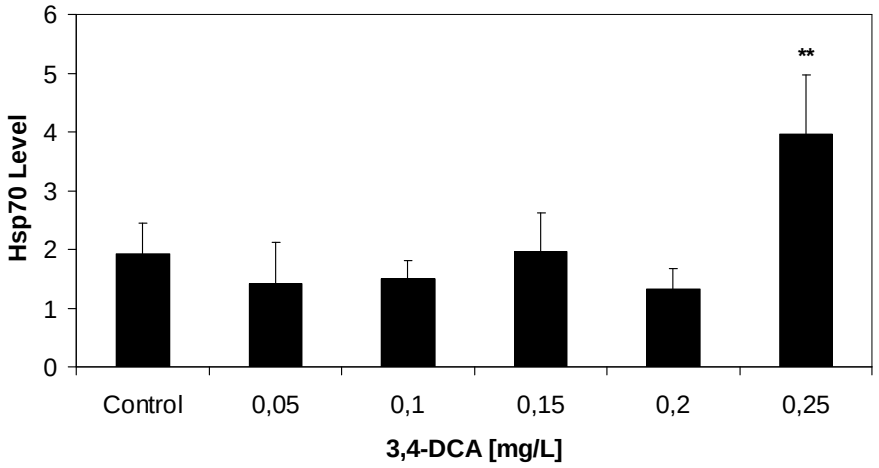

Fig. 5 Hsp70 levels of zebrafish larvae exposed to 3,4-dichloroaniline (n=10, means ± SD). **: significantly different to the control at $p<0.01$.

Diazinon

A significant decrease in the heart rate occurred during the prolonged embryo test at 2 and 3 mg/L diazinon at an age of 48 h. The hatching rate was impaired at 3 mg/L. At 2 mg/L a significant increased deformation rate, mostly edema and spine deformations, was observed; at 3 mg/L mortality was increased as well. Even after 1 day of exposure, 5 mg/L induced deformities, edema and an abnormal bending of

the spine in 100% of the larvae. Mortality in 1- and 2-days-old *D. rerio* was increased significantly at 5 and 2 mg/L diazinon, respectively, and this effect remained unchanged until the end of the exposure (Fig. 7). Diazinon in a concentration of 2 mg/L decreased the locomotor activity in 5- and 8-days-old *D. rerio* larvae (Fig. 6). The Hsp70 level of 7 days old fish was found to be elevated at diazinon concentrations of 50 µg/L diazinon or higher (Fig. 8).

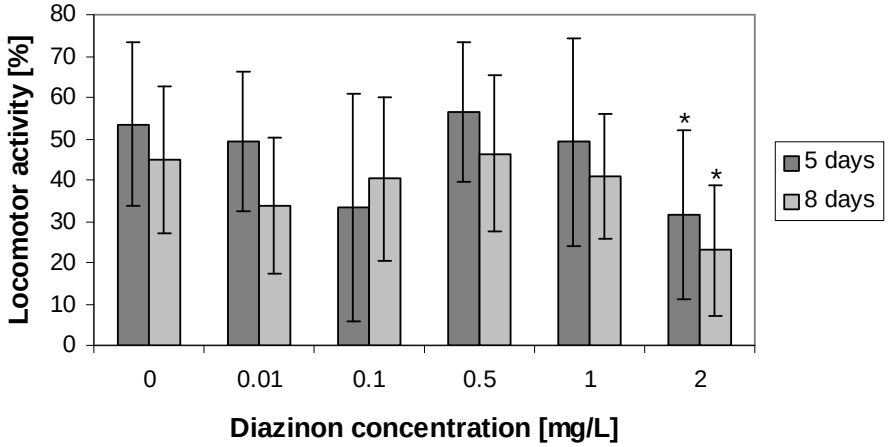

Fig. 6 Locomotor activity (% time spent on locomotion) of five and eight days old D. rerio larvae exposed to different diazinon concentrations [mg/L] (n = 10-12, means ± SD)

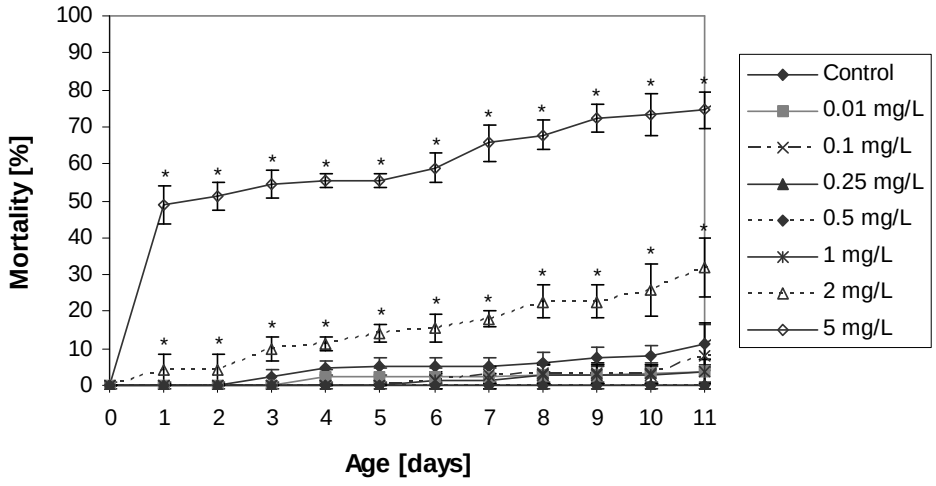

Fig. 7 Cumulative mortality [%] of D. rerio larvae exposed to different diazinon concentrations [mg/L] (means ± SD; number of larvae per replicate: 30 (days 0 - 5); 26 (days 6 - 8); 22 (days 9 - 11), 3 replicates for each experiment). *: Significantly different to control treatment at $p < 0.05$.

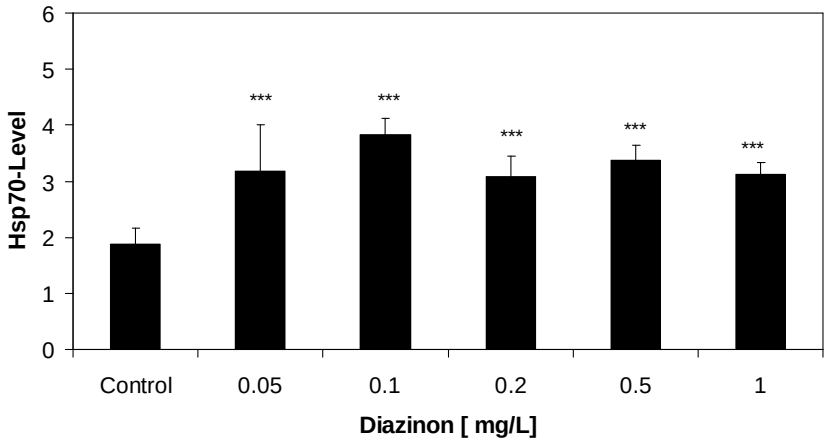

Fig 8 Hsp70 levels of zebrafish larvae exposed to 3,4-dichloroaniline (n=10, means ± SD).
***: Significantly different compared to the control at $p<0.001$.

Binary mixtures of 3,4-dichloroaniline and diazinon

In contrast to the single substance tests, edemas occured at the end of the prolonged embryo test (96h) only. In single substance test, edema occured early if animals were exposed to diazinon (72h post fertilisation, most animals with oedema died during the following 24h) and later if exposed to 3,4-DCA (96h post fertilisation, see Fig. 2). Due to the different time points of occurence of oedema, an integrating figure including single substance test results as well as mixture test results is not shown. If tests were extended to subchronic tests, concentration additivity was observed for the parameters locomotor activity (5 days) (Fig. 9a), deformation rate (10 days) (Fig. 9c) and mortality (10 days) (Fig. 9d). The exposure to mixtures of 3,4-DCA and diazinon led to increased Hsp70 levels in all groups. As shown in Fig. 9b also an additive effect of the two substances was observed. An overview over all LOECs obtained for the different endpoints is given in Table 2.

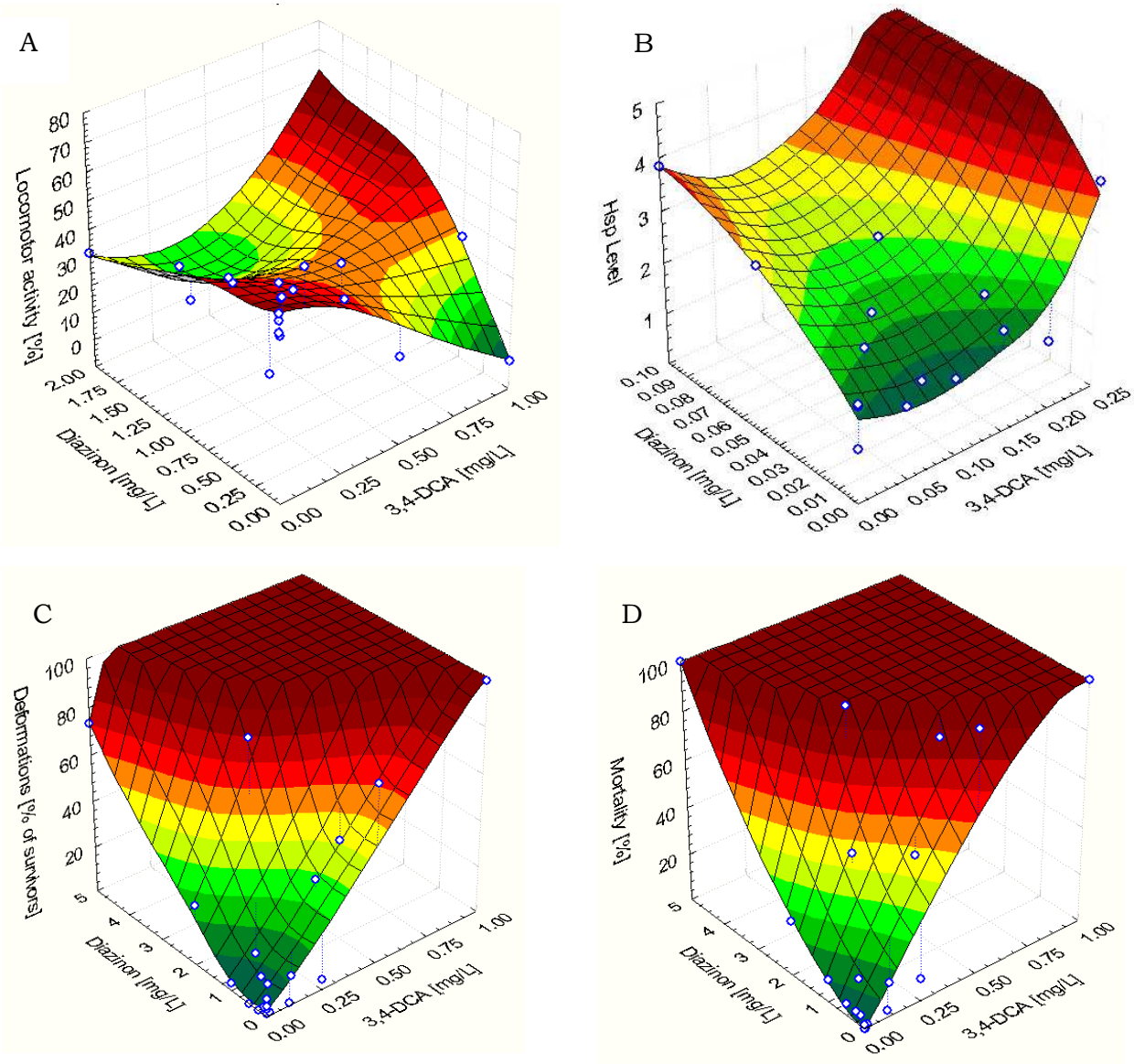

Fig. 9 a) Locomotor activity (percent of total time spent in locomotion) of 5-days-old D. rerio larvae, (b) Hsp70 levels (b) of 7 days old D. rerio larvae, (c) deformations [%] and (d) mortality [%] of 10 days old D. rerio larvae exposed to different 3,4-dichloroaniline and diazinon concentrations [mg/L], single and in binary mixtures (surface plots with isobolic lines calculated on the basis of means).

Table 2: Comparison of the LOECs of the exposure of zebrafish embryos and larvae to 3,4-dichloroaniline and diazinon. Abbreviations: C.A. Concentration addition.

		LOEC			
Test method	**Parameter**	**3,4-Dichloroanilin [mg/L]**	**Diazinon [mg/L]**	**Mixtures**	**Reference**
Prolonged embryo test	**Hatching rate**	>2	2	C.A.	Osterauer and Köhler 2008 Present study
	Heart rate	>2	2		
	Deformations	1	2		
	Mortality after 96 h	>2	2		
	Behavioural anomalities	>2	-		
Sub-chronic test	**Locomotor activity**	0.5 (5d)	2 (5 d)	C.A. (10 d)	Osterauer and Köhler 2008 Present study
	Deformations	0.25	1 (11 d)		
	Mortality after 10 d	0.5	2 (≥1 d)	C.A. (10 d)	
Stress protein investigations	**Hsp70-Level** (significantly elevated)	0.25	0.05	C.A.	Present study

4. Discussion

In the present study, a pesticide and a pesticide degradation product were investigated concerning their ecotoxicological impact on a broad range of biological endpoints. Both substances have been assessed in previous studies, mainly in toxicity tests with mortality as the only endpoint. Nevertheless, data on mixture toxicity of these two substances are lacking. Futhrermore, onformation on substance-induced reactions in a specified zebrafish strain on different biological levels is scarce. Most publications deal either with single substances and / or are lacking information about the fish strains used: typically, in the literature, fish are just referred to be obtained from 'a local hatchery' with no further information.

Dichloroaniline is known to be toxic to fish, 96 h LC_{50} values for fish range from 1.94 mg/l for rainbow trout (*Oncorhynchus mykiss*) (Hodson 1985) to 8.5 mg/L for *Danio rerio* (Becker et al., 1990). On the basis of a ring test with some deviating results Nagel et al. (1991) estimated LOECs of 0.1-0.2 mg/L for survival rates in zebrafish exposed to 3,4-DCA for one week. Diazinon is also toxic for freshwater fish and aquatic invertebrates. 96 h LC_{50} values range from 0.32-0.35 µg/L for *Ceriodaphnia dubia* (Bailey et al. 1997) to 26.7 mg/L in Common carp (*Cyprinus carpio*). (Svoboda et al. 2001). Compared to other fish species, *D. rerio* is moderately sensitive to acute diazinon exposure, the estimated LC_{50} is 8 mg/L (Keizer et al. 1991). Mixture toxicity tests with these two substances have not been done before.

Concerning the single substance tests with 3,4-DCA, the first reactions were found in zebrafish exposed to 0.25 mg/L. Tests with diazinon revealed first reactions at 0.05 mg/L (see Table 2 for both). Both LOECs are much higher than concentrations reported for environmental samples (max. 1.5 µg/L for 3,4-DCA (Planas et al. 2006) and diazinon (Bailey et al. 2000). Nevertheless, both substances are highly soluble in water and may occasionally occur in spatial hotspots. Also, chronic exposure to low concentrations may lead to similar effects as short-time exposure to higher concentrations of these substances. Taking this into account, our results described above have to be seen as relevant for wildlife, at least for regions with natural water temperatures comparable to those in the tests. But even for cold waterbodies our results should be considered relevant: assuming that degradation of pesticides in cold water takes longer than in warmer water, low concentrations of pesticides may act over a longer time. In addition, cold water fish may be more sensitive to pesticide exposure. 96h LC_{50} values are 4.5 to 6 times higher in zebrafish than in rainbow trout, for example (Keizer et al. 1979, Meier et al. 1979, Hodson 1985, Becker 1990).

As a molecular response mechanism to stress, the Hsp70 response is a biomarker on a low level of biological organisation. Both substances led to a stress protein reaction, indicating proteotoxic stress. In this context, a similar induction of Hsp70 was excerted by diazinon concentrations which were about 10 times lower than the corresponding 3,4-DCA concentrations. Taking into account that Hertl and Nagel (1993) found bioconcentration factors of 86 in four days old zebrafish larvae exposed to 3,4-DCA, this massive difference of proteotoxicity caused by the two substances is remarkable.
Data recorded in the prolonged embryo test as well as during the subchronic test (edemas) are in accordance with histopathological results which also indicated the higher toxicity of 3,4-DCA (R. Triebskorn, unpublished), even though all other endpoints (besides the occurrence of edemas and mortality during the subchronic tests) showed reactions to diazinon exposure exclusively (for details see Osterauer & Köhler 2008).
The investigated behavioural endpoints were less sensitive than the biochemical parameter Hsp70, but responded at five days already (*vs.* 7 days in hsp70 analysis). In the single substance test with 3,4-DCA, locomotor activity was first affected at higher concentration than the other monitored parameters, but for diazinon behavioural measurements were as sensitive as the other investigated endpoints. In other studies with the acetylcholine esterase inhibitor chlorpyrifos, locomotor activity has been shown to be a very sensitive parameter in zebrafish (Kienle et. al 2008 in prep.). Diazinon has already been shown to impair zebrafish larval behaviour and also adult medaka (*Oryzias latipes*) showed behavioural changes when exposed to 0.1 mg/L diazinon (Wall 2000, Chon et al. 2005). However, no information on behavioural effects to fish concerning 3,4-DCA and mixtures of diazinon and 3,4-DCA were available prior to this study.

With respect to the hypotheses mentioned in the introduction, hypothesis 1 ("Endpoints at lower levels of biological organisation (molecules) should exhibit higher sensitivity to 3,4-dichloroaniline, diazinon and mixtures of them than those on higher levels (organisms).") has been verified for the single substances. Hypothesis 3 ("The acetylcholine esterase inhibitor diazinon should lead to more severe effects than the unspecific toxicant 3,4-dichloroaniline.") was found to be true for some endpoints only, but, as predicted, the most severe effects (hatching rate, mortality during the first 96 post fertilisation) exclusively occurred after exposure to diazinon.

Hypothesis 2 (“3,4-Dichloroaniline and diazinon should act independently in equitoxic mixtures.”), dealing with the binary mixtures was proven as well. For all endpoints, no synergistic or antagonistic effects were found, but rather concentration addition was observed. Mechanistically, it is therefore proposed that both substances do not interact with one another but act independently.

To conclude, both substances as well as the binary mixtures led to severe impairments in Danio rerio embryos and larvae. A multi level approach was effectively used to demonstrate that different endpoints can react with different sensitivity, depending on the chemical. Due to uncertainties in predicting the endpoint which may be influenced by a certain substance, it seems useful to investigate a reasonable number of as much endpoints of different character at different biological organisation levels in such an approach.

Acknowledgements

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Kapitel 5: Developmental toxicity in zebrafish embryos (*Danio rerio*) exposed to textile effluents

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Abstract

An effluent that was collected from the discharge pipe of a textile dyeing mill in Dar es Salaam, Tanzania, was used in laboratory experiments to investigate the toxicity of the effluent for developing fish. An aliquot of the raw effluent was fractionated into samples of differing polarity by counter-current chromatography. The assumption was made that the separation by counter-current chromatography corresponds to the separation of compounds in the field following the interaction of the mill effluent with mangrove sediments. The toxicity of the untreated effluent and the most polar environmentally relevant fractions was tested using early life stage tests with zebrafish embryos (*Danio rerio* Hamilton). The raw effluent delayed gastrulation, decreased heart rate, decreased hatching rates and elevated mortality rates at dilutions lower than 1:50, while exposure to the polar fractions only resulted in a decrease in heart rate at the lowest dilution. The results of this investigation revealed that the highest embryotoxicity is exerted by the apolar fractions of the effluent.

Keywords: Textile effluent, early life stage, *Danio rerio*, Tanzania

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submitted to Environmental Monitoring and Assessment

Introduction

In the production processes of textile industries vast amounts of water drawn from nearby rivers are used and later released as waste water. Upon release, the water is polluted with the waste products from dyeing and finishing processes. As a result of this pollution, the coloured water requires treatment to minimise toxic effects to the environment, as well as aesthetic effects. Worldwide much research has focussed on the removal of dyes and toxic constituents from textile effluents and a variety of treatment processes for detoxification and decolourisation are currently known [1-3]. The treatment processes are, however, not always applied to sufficient extent. As a consequence heavily stained waters and toxic effects due to the release of textile effluents do still occur [4]. Textile effluents have been shown to cause physiological disturbance in fishes which may be expressed as inhibition of ATPase activity in the liver, brain, and muscle [5], induction of CYP1A in the liver [6], and DNA damage [4]. In the mangroves of the Mtoni estuary near Dar es Salaam, Tanzania, we have observed reduced growth, decreased longevity, and frequent occurrence of anophthalmia in a resident population of mudskippers, Periophthalmus argentilineatus Valenciennes. These adverse developments were associated with exposure to waste water released by a textile dyeing mill, indicating that pollutants may have interfered with embryonic development [7].

The embryonic and early larval stages of fishes are the life stages that are most sensitive to environmental pollutants. The high sensitivity follows from the high metabolic rate of fish during these life stages and the fact that epithelia and organs are not yet completely differentiated [8]. Because of their sensitivity early life stage fishes are often used in experimental exposure tests to determine and quantify sublethal and lethal effects of xenobiotics. In the present study the toxicity of different fractions of an effluent from the textile dyeing mill in Dar es Salaam was tested in the laboratory on zebrafish (*Danio rerio* Hamilton) embryos to investigate whether the released textile effluent could be the cause of the defects that were previously observed in the mudskipper [7]. The use of an early life stage-test (ELS) with zebrafish offers major advantage over the use of mudskippers, because the ELS-test with zebrafish is a widely used [9-12] and standardised test (DIN 38415-6). Moreover zebrafish eggs are readily available, and since many research efforts have focussed on the zebrafish, the development and physiology of the zebrafish embryos are well known [13,14]. In contrast, the knowledge of the early life stages of mudskippers is very limited and collection of eggs and observation of early development are complicated by the fact that mudskippers breed in burrows well

below the surface of the mangrove sediments [15] and reproduction in captivity is very difficult [16,17].

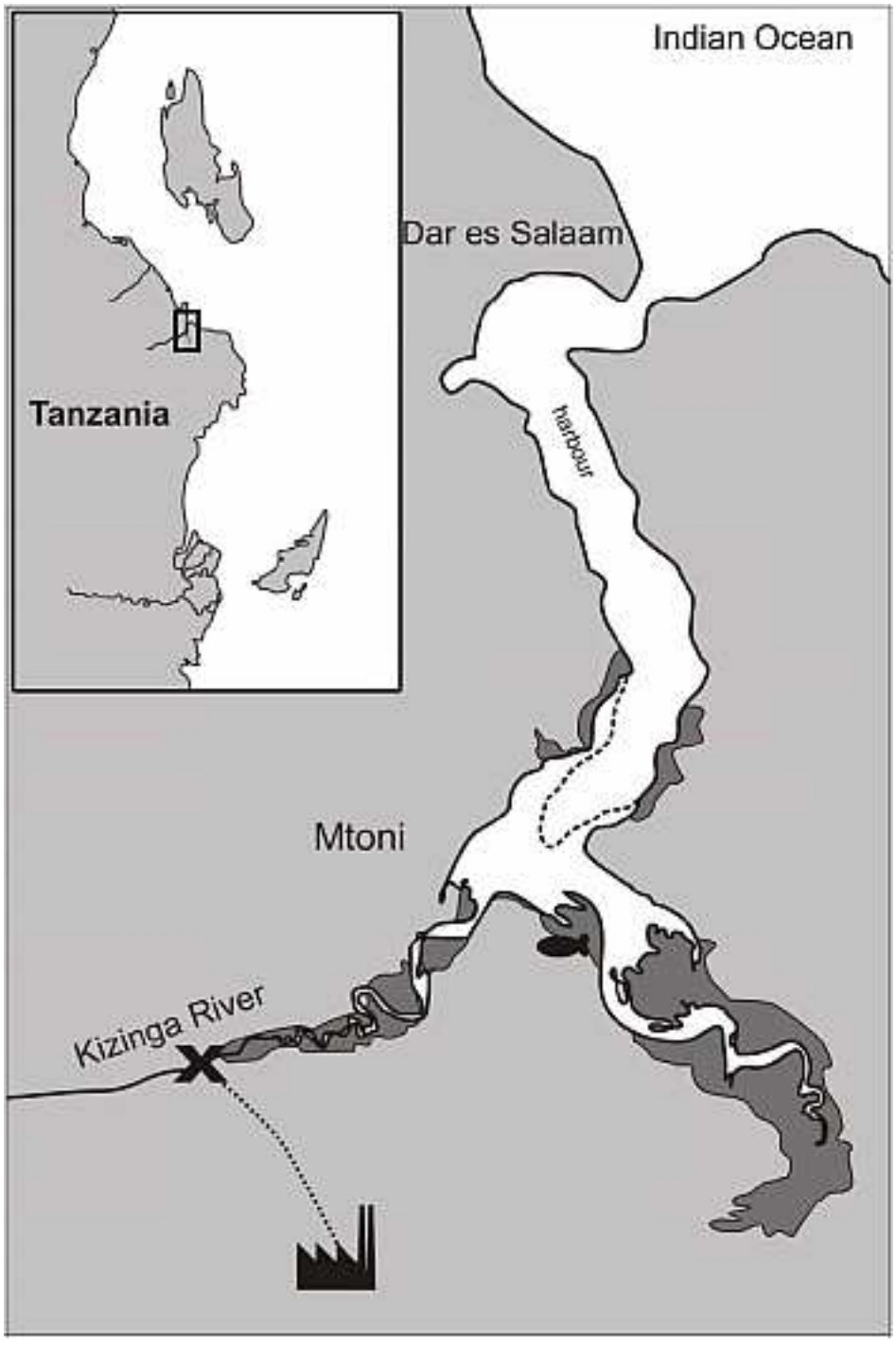

Fig. 1. Map of the Mtoni estuary near Dar es Salaam, Tanzania. The cross marks the point of effluent release; the fish symbol indicates the location of the mudskipper population; the densely shaded areas represent the mangrove stands; the dashed line indicates the low-tide mark.

Materials and Methods

Collection of effluent and field survey

The textile dyeing mill in the Mtoni suburb of Dar es Salaam, Tanzania (Fig. 1) is located on a hill near the banks of the Kizinga River. The discharge pipe of the mill runs downhill and releases waste water into the river 300 meters before it enters a mangrove stand and widens into an estuarine basin. The mangrove stand stretches for two km towards the coast after which the estuary deepens and widens to form a shallow bay. The mudskipper population in which adverse effects of pollution were observed [7] lives in the mangroves in proximity of the bay, at the far end of the mangroves relative to the textile mill.

To assess potential effects of the textile mill effluent on the mangrove fauna a field survey was made. During this survey an effort was made to screen the mangrove stand over its entire length to locate traces of inhabitation of different sections of the mangrove by mudskippers and intertidal crabs (Uca sp.). To further investigate the presence of macrofauna samples of the top 50 centimetres of sediment were collected and sieved. No efforts were made to quantify mudskippers, Uca crabs or other macroinvertebrates since the abiotic factors in the mangroves influence their distribution and burrowing activities encumber quantification.

Effluents from the textile dyeing factory in Mtoni were collected straight from the discharge pipe in polypropylene bottles (Nalgene, Rochester, USA). The bottles were immediately sealed upon collection, frozen at -20°C, and transported to The Netherlands.

Processing of effluent

The contents of one bottle were used for chemical processing at the Department of Organic Chemistry of the University of Nijmegen. The compounds of the textile mill effluent were separated by counter-current chromatography using a two-phase solvent system of butanol/acetic acid/water (4:1:5, v/v/v). After 80 cycles of the counter-current chromatography, seven separate fractions could be isolated on the basis of colouration (fractions 1 to 7). The first fraction contained stationary polar compounds that are not transported by polar solvents or water. The second fraction contained polar hydrophilic compounds that are carried by the solvent. Because of their hydrophilic properties the compounds of the second fraction were considered environmentally relevant in the field since they might be transported throughout the mangroves downstream of the point of emission ,. The second fraction was separated further on the counter-current chromatograph during 120 additional

cycles resulting in 6 fractions (fractions 2A-F). All isolated fractions of both columns were concentrated by evaporation and the concentrated liquids were freeze dried into solid products. The freeze dried products of the two most polar fractions that were isolated during the second series of cycles, fractions 2A and 2B, were transported frozen to the University of Tübingen in Germany together with a bottle filled with the untreated raw effluent.

Animals

Zebrafish embryo tests were conducted at the Animal Physiological Ecology Section of the University of Tübingen, Germany. Adult zebrafish were kept in the laboratory in aerated and filtered aquaria with a minimum of 1 L water per fish, at 26 ± 1 °C and a 12:12 h light:dark cycle. The adult fish were fed twice daily with TetraMin dry flake food (Tetra, Melle, Germany) and either Artemia nauplii or with red mosquito larvae from uncontaminated sources. The eggs used in the test were collected from 6 aquaria, each containing approximately 30 fish with unknown sex ratio. To collect the eggs spawn traps were used which had been placed at the bottom of each aquarium the evening of the day before spawning was required. Spawning was triggered once the light was turned on and was completed within 30 min. Eggs from all aquaria were pooled and randomly distributed to the respective treatments. All eggs were transferred immediately into glass Petri dishes containing the different test solutions. Then the unfertilized eggs were removed, and the fertilized eggs were placed into new glass Petri dishes (10 embryos per Petri dish, 4 dishes per concentration) containing the respective test solutions. The water for the exposure of the eggs/embryos was prepared according to ISO-Standard 7346/3, and contained 294 mg/L $CaCl_2$, 123.25 mg/L $_{MgSO4}$, 64.75 mg/L $NaHCO_3$ and 5.75 mg/L KCL, dissolved in aqua bidest. The water was aerated to oxygen saturation before addition of the test substances. The tests were performed in climate chambers at a 12:12 h light:dark cycle, water temperature was maintained at 26 ± 1 °C. The Petri dishes were covered to avoid evaporation.

Exposure to whole effluent

To prepare the test solutions the untreated raw textile dyeing effluent was thawed and diluted with the exposure water to different concentrations (1:5, 1:10, 1:30, 1:50, 1:100, 1:300, 1:500 and 1:1000). In addition to these dilutions of the effluent a negative control containing pure exposure water was used. Embryo development was monitored in all exposure groups at 0, 8, 12, 24, 48, 60, 72, 84, and 96 hrs after fertilization. Endpoints used for assessing the effects of the textile dyeing

effluent included 70% epiboly (i.e. blastoderm enveloping 70% of the yolk sphere [13]), egg and embryo mortality, gastrulation, somite formation, movement, tail detachment, pigmentation, heartbeat and circulation, and hatching time and success (Table 1). Malformations and delays in development were also noted and described for the developing eggs from both control and treated groups, using a stereomicroscope.

Exposure to effluent fractions

The two freeze dried fractions of the textile dyeing effluent were mixed and reconstituted to the original volume of 200 ml with the water that was prepared for the exposure. The reconstituted fractions of the effluent were further diluted to 1:30, 1:50, 1:100, 1:300, 1:500 and 1:1000. The small quantities of effluent fractions that were available were insufficient to enable testing at dilutions 1:10 and 1:5. The tests with the fractions of the textile dyeing effluent were conducted as described for the tests with the raw effluent.

Statistical analysis

Exposure effects on the delay of epiboly, heart rate, and mortality of the zebrafish embryos were tested using one-way analysis of variance (ANOVA; SPSS: General Linear Model 1) in combination with Games-Howell post-hoc tests. Before performing the ANOVA thehomogeneity of variance was tested with a Levene's test. The data for epiboly and mortality for the group exposed to the raw effluent were log-transformed to correct for inhomogeneity of variance. Effects of the textile effluent on hatching rates were analysed with one-way repeated measures ANOVA followed by Games-Howell post-hoc tests (SPSS: General Linear Model 3). All statistical tests were performed using SPSS version 11.5. Significant differences were accepted at the $p \leq 0.05$ level.

Table 1. Investigated endpoints during zebrafish egg development (modified after OECD 210 and DIN 38415-6).

Endpoint	**8h**	**12h**	**24h**	**48h**	**60h**	**72h**	**84h**	**96h**
Coagulated eggs / dead embryos	*	*	*	*	*	*	*	*
No epiboly (70%)	*							
Delayed gastrulation		*						
Exogastrulated embryo		*						
No formation of somites			*					
No detachment of tail			*					
No spontaneous contraction			*					
No formation of the eyes			*					
No heart beat				*				
No blood circulation				*				
Heart rate				*				
No otolith formation					*			
No melanocyte formation					*			
Yolk sac oedema					*			
Brain defects					*			
Number of hatched embryos					*	*	*	*
Eye defects					*	*	*	*
Edema (heart and head)						*	*	*
Tail deformities						*	*	*
Fin blistering						*	*	*
Weak pigmentation						*	*	*
Helical bodies						*	*	*
Spiral nervous system						*	*	*

Results

Field observations at collection site

The raw textile effluent was turbid and dark blue in colour and had a pH of 10. The effluent did not contain particulate matter. Simple dilution did not result in colour change.

In the immediate surroundings of the discharge pipe the effluent stained the sediments heavily, which resulted in an indigo colouration. Just before the mangrove stand, about 200 m from the discharge pipe, the sediments and the water

were dark green in colour, whereas effluents produced a rusty brown colour in the upstream section of the mangroves, where both sediments and water appeared to be devoid of fauna. Living fauna was only encountered 1 km downstream into the mangroves where the turbidity of the water had decreased and the water had a dark red colour but the sediments were unstained. At the transition from mangrove to the open bay the water was red-brown in colour. The colour faded upon mixing with tidal waters in the bay.

Chemical processing

The chemical separation of the raw textile effluent on the basis of polarity resulted in 7 fractions, of which fraction 2 was further separated into 6 sub-fractions. The colours of the various fractions resembled the colours that were observed in the Kizinga River and the adjacent mangrove forest. Blue and green colours dominated in the apolar fractions (butanol > water), while red and brown colours were found in the more polar fractions (butanol < water).

Exposure to test solutions

The number of embryos that had not reached 70% epiboly after 8 h was overall positively correlated with the concentration of raw effluent ($p < 0.05$; Fig. 2a). Games-Howell post-hoc test did however not reveal significant differences between groups exposed to different dilutions of the raw effluent. There was no effect of dilution on epiboly after exposure to the polar fraction of the effluent (Fig. 2b).

Exposure to the raw effluent resulted in an increase in mortality rates before hatching which was significantly correlated with dilution ($p < 0.001$; Fig. 3a). Exposure to the raw effluent caused 100% mortality within 12 hours in all groups at concentrations of 1:5, 1:10, and 1:30. Exposure to the polar fractions of the effluent did not result in an increase in mortality rates (Fig. 3b).

The heart rate of zebrafish embryos at 48 h after fertilization was significantly correlated with dilution of the raw textile effluent ($p < 0.001$; Fig. 4a) as well as with dilution of the polar fraction of the effluent ($p < 0.05$; Fig. 4b). Lower heart rates were found after exposure to more concentrated solutions.

Hatching rate of zebrafish embryos exposed to raw textile effluents was significantly affected by dilution of the effluent ($p < 0.05$; Fig. 5a). Delay in hatching increased with increasing concentrations of the raw effluent. The polar fraction did not affect the hatching rate of zebrafish embryos (Fig. 5b).

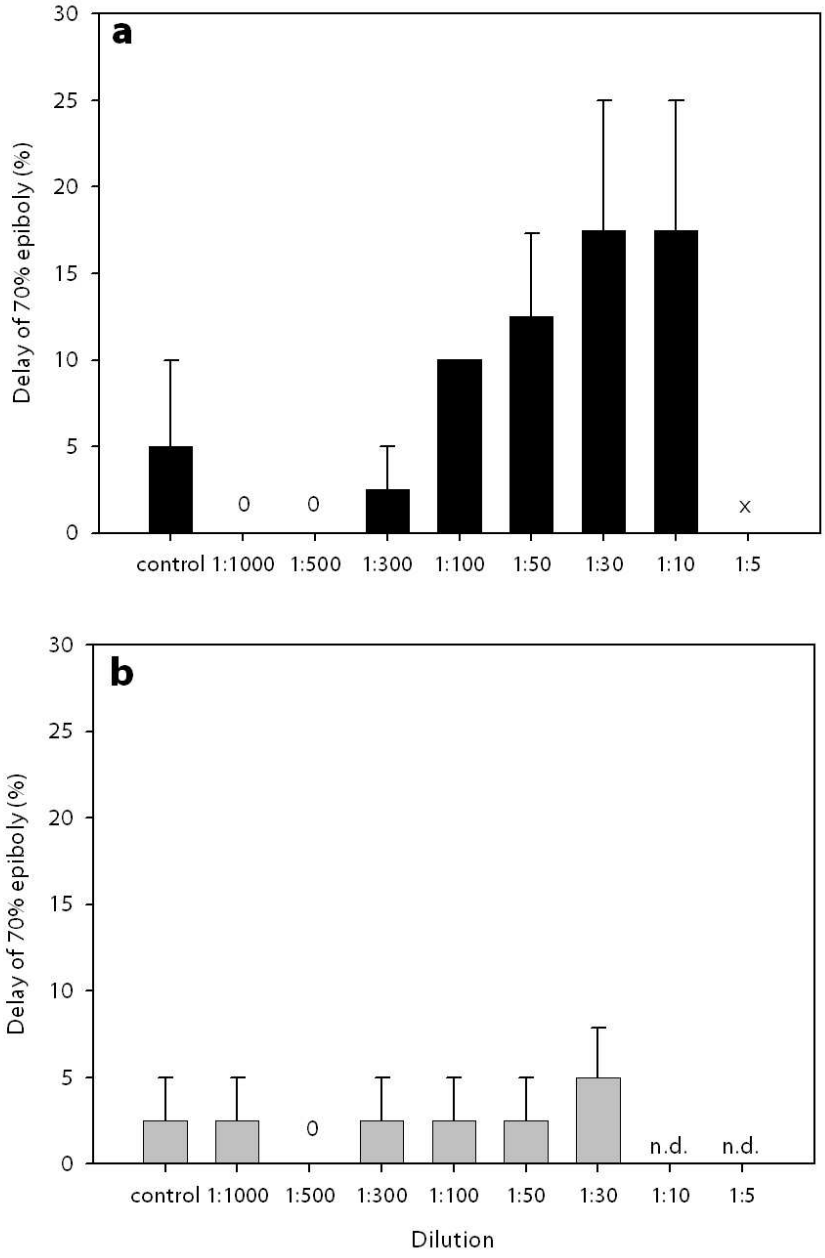

Fig. 2. Percentage of zebrafish embryos in which epiboly had not reached 70% after 8 hours of exposure to raw textile effluent (a) and polar fractions of the effluent (b) at different dilutions. Each exposure group encompased 40 embryos. Cross indicates that no heart was available due to 100% mortality before 8 hours had elapsed. N.D. indicates that epiboly was not determined. Error bars represent standard errors.

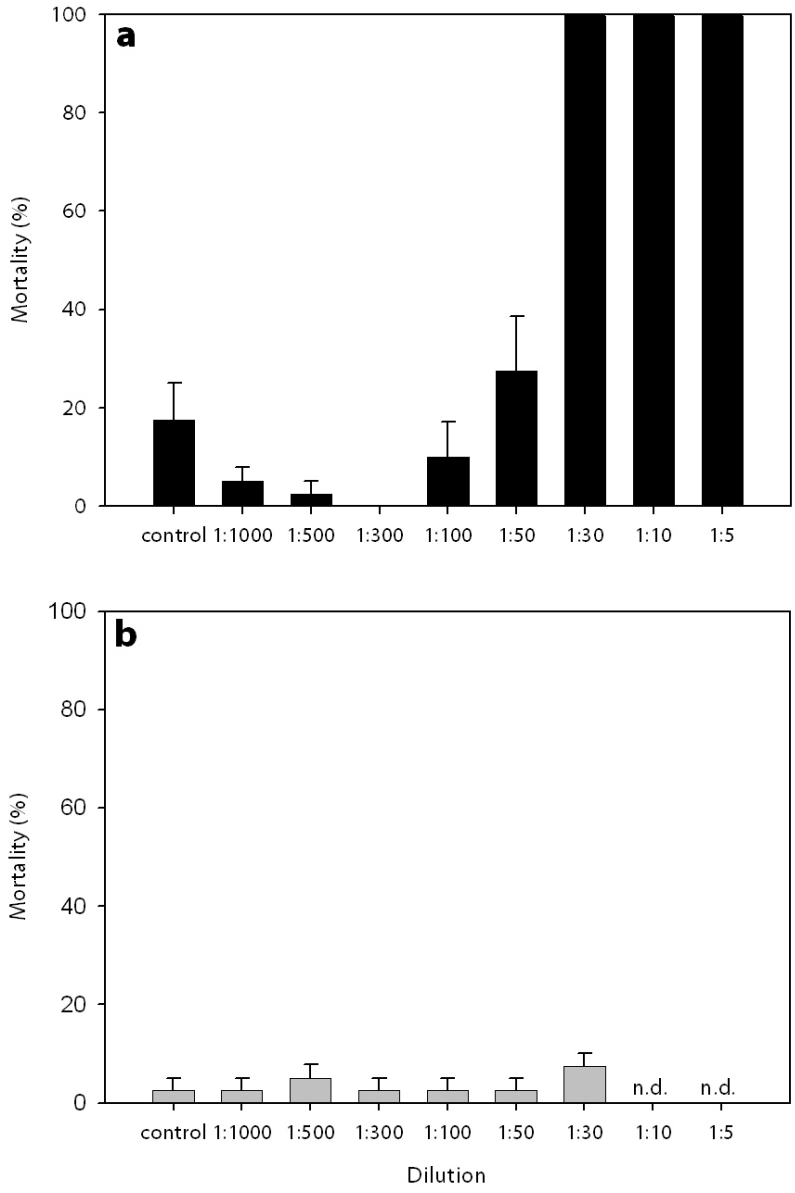

Fig. 3. Mortality in zebrafish embryos subjected to different concentrations of raw textile effluent (a) and polar fractions of the effluent (b) at 96 hours after fertilisation. Each exposure group encompased 40 embryos. N.D. indicates that mortality was not determined. Error bars represent standard errors.

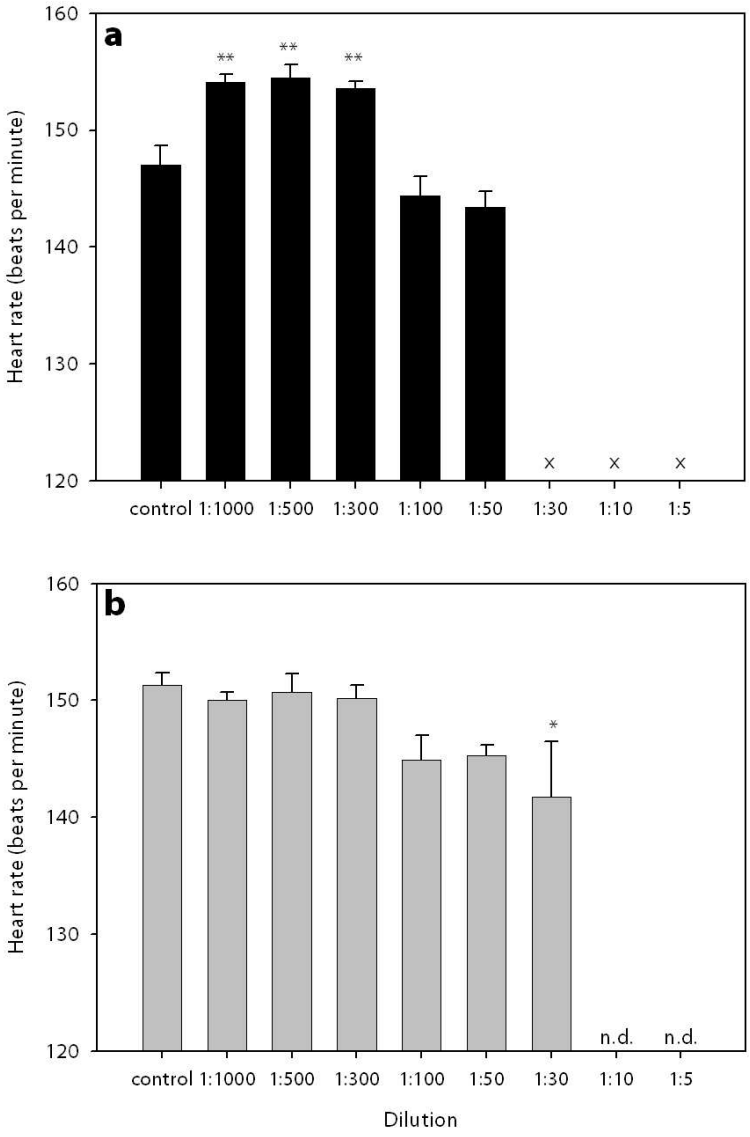

Fig. 4. Heart rate of zebrafish embryos subjected to different dilutions of raw textile effluent (a) and polar fractions of the effluent (b) at 48 hours after fertilisation. Cross indicates that no heart was available due to 100% mortality before 48 hours had elapsed. Each exposure group encompased 40 embryos. N.D. indicates that heart rate was not determined. Error bars represent standard errors. Significant differences from the control are indicated with asterisks over the bars: * $p < 0.05$, *** $p < 0.001$ (One-way ANOVA, Games-Howell Post Hoc test).

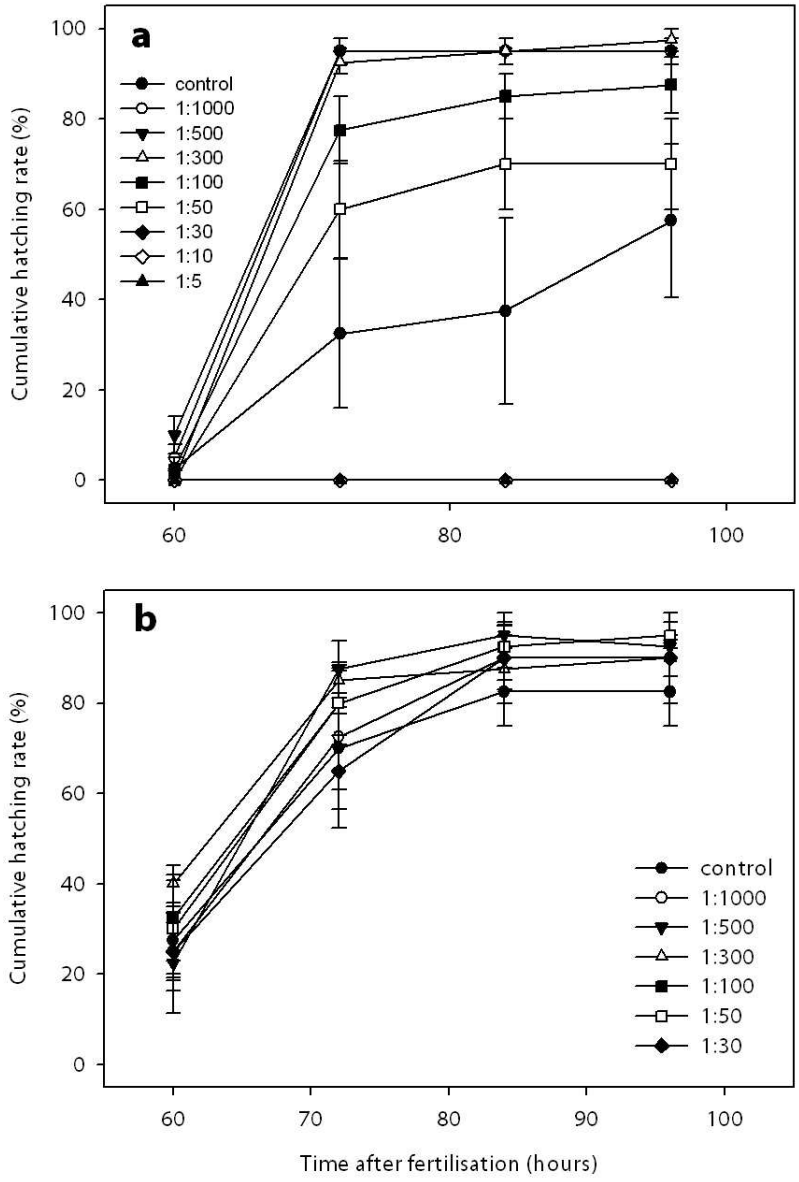

Fig. 5. Hatching rate of developing zebrafish subjected to different concentrations of raw textile effluent (a) and the polar fractions of textile effluent (b) at various time points after fertilisation. Each exposure group encompased 40 embryos. Error bars represent standard errors.

Other detectable developmental differences between control and exposed groups were occasionally observed, but their distribution over the various groups appeared to be random (Table 2).

Table 2. Total number of aberrations observed in 4 replicate groups of 10 zebrafish embryos exposed to different concentrations of raw effluent or polar effluent after 96 hours. Horizontal bars indicate that the specific endpoint could not be defined due to 100% mortality.

	Raw effluent									Polar effluent fractions						
Endpoint	**Control**	**1:1000**	**1:500**	**1:300**	**1:100**	**1:50**	**1:30**	**1:10**	**1:5**	**Control**	**1:1000**	**1:500**	**1:300**	**1:100**	**1:50**	**1:30**
Delayed gastrulation	2	3		1	2	2	-	-	-	2	4			1	2	3
Exogastrulated embryo							-	-	-							
No formation of somites							-	-	-							
No detachment of tail							-	-	-							
No spontaneous contraction							-	-	-							
No formation of the eyes							-	-	-							
No heart beat							-	-	-					1		
No blood circulation					1		-	-	-					1		
No otolith formation							-	-	-							
No melanocyte formation							-	-	-							
Yolk sac oedema							-	-	-			1				
Brain defects							-	-	-							
Eye defects							-	-	-							
Edema (heart and head)				2	1		-	-	-			1				1
Tail deformities							-	-	-							
Fin blistering							-	-	-							
Weak pigmentation							-	-	-					1		1
Helical bodies							-	-	-							
Spiral nervous system							-	-	-							

Discussion

The range of colours that were observed in the field suggests that the effluent from the textile dyeing mill at Mtoni in Dar es Salaam, Tanzania, is a chemical mixture that is separated into different fractions after release into the environment, probably due to interaction with mangrove sediments. Mangrove sediments are known to trap metals from overlaying waters by complexation with sulfides, particulate organic carbon, or iron oxyhydroxides, depending on prevailing physico-chemical conditions [18], while the large quantities of organic matter in the sediments provide extensive binding surfaces to organic pollutants.

In the laboratory, chromatography revealed that the textile effluent was composed of a number of components that could be separated on the basis of polarity (i.e. hydrophobicity), and colour. The colours of the various fractions and their following order appeared to correspond with the colouration that was found in the water and sediments of the mangroves downstream from the discharge pipe of the textile factory. The apolar fractions found after chromatography had a blue-green colour that was similar to the colour of the sediments in the direct vicinity of the effluent pipe, while the red-brown colours of the hydrophilic fractions that were obtained after chromatography corresponded with the colour of the water in River Kizinga at the point of mixing with the water from the bay. Even though there is no certainty that the compounds that were found in the laboratory are identical to the compounds found in the field, the results of chromatography strongly indicate that the separation that occurs in the field is also based on polarity of the constituents of the textile mill effluent.

Toxicity tests

In the toxicity tests it was apparent that the toxic effect exerted by the textile effluent on the zebrafish embryos was strongly dependent on the fractions that were present in the effluent. The raw effluent affected the time of completion of 70% epiboly, heart rate, time of hatching, hatching rate as well as survival of the developing zebrafish. These results showed that the textile effluent had profound adverse effects on the development of the zebrafish embryos from the early stages of development up to the moment of hatching. The polar fractions of the effluent only affected the heart rate. Hence the early life stage tests showed that the toxicity of raw effluents is much higher than the toxicity of the isolated polar fractions, which suggests that the toxicity of the effluent is mainly derived from relatively apolar, i.e. hydrophobic, fractions.

The concentration of the exposure media appeared to be a major determinant for the impact that the textile effluent had on the development and survival of the zebrafish embryos. The lowest observed effect concentration was 1:100 for the raw effluent and 1:30 for the polar fraction of the effluent. The highest effects were observed in exposures to the raw effluent at concentrations of 1:30, 1:10, and 1:5. Other studies on the toxicity of textile dye mill effluents for fish have reported the occurrence of similar effects and similar correlations with effluent dilution. Sakthivel and Sampath [19] found reductions in growth rate and an increase in mortality rates in juvenile carp, *Cyprinus carpio*, that increased with concentration of the effluent. Mortality rates were reported to reach 100% after exposure to an effluent concentration of 15%. The same mortality rate was found in zebrafish embryos in the present study at effluent dilutions of 1:30. In another study on juvenile carp, textile dye effluents were found to be able to exert genotoxic effects on fish tissues [4]. Direct exposure to textile effluents induced enzyme activity (EROD, GST, UDP-GT) in adult tilapia, *Oreochromis niloticus*, at distances of 0.6, 4, and 8 km from the point of effluent discharge [6]. Chhaya et al. [20] found that textile dye effluents significantly altered acid phosphatase activity in adult mudskippers (*Periophthalmus dipes*) after exposure to concentrations of 0.1, 0.5 and 1%.

Mtoni mangroves

After release of the raw textile effluent into the Mtoni mangroves near Dar es Salaam, apolar constituents from the effluent will bind rapidly to organic matter and sediments to avoid the polar water layer. Consequently, these substances are mostly restricted to the upstream mangrove sections. More polar fractions remain longer in the water layer but may be lost to the sediments further downstream, at a distance depending on their polarity. The most hydrophilic components will remain in the water layer throughout transport through the mangroves and will be carried down to the opening into the bay. From our experiments it follows that this gradient in polar fractions in the water layer can result in a gradient in toxicity, because the apolar fractions exerted a higher toxicity than the more polar fractions. As the textile effluent passes through mangrove sections further downstream, the polarity and subsequently the toxicity of the effluent fractions in the water layer will decrease. In the direct vicinity of the discharge pipe both polar and apolar fractions will be present in the water, therefore toxic effects will be most extensive in this mangrove section. The apolar fractions of the textile effluent are likely to have a similar (acute) toxic effect in the field as they had on the zebrafish embryos at lower dilutions under laboratory conditions, and may account for the apparent, total

absence of fauna from upstream mangrove sections. In an earlier study we reported the occurrence of abnormal eye development, decreased growth and decreased longevity in the natural population of mudskippers in the Mtoni mangroves [7]. Other studies reported the occurrence of similar adverse effects in fish after exposure to textile dyes [21,22] or textile dye effluents [19]. The present study suggests that the mudskippers are primarily exposed to polar fractions of the effluents at the location where they reside. However, according to this hypothesis, the low toxicity of the polar fractions of the textile dye effluent in the tests with the zebrafish embryos appears to contradict the observed effects in the mudskippers of Mtoni. Several reasons for this discrepancy can be proposed: (1) the concentrations of waste products of the mill in the mangrove environment may vary strongly over space and time following variations in the physical and chemical conditions, as well as variations in release due to alterations in industrial processes; (2) the apolar fractions may have traveled further in the field than expected based on the laboratory tests; (3) the observed effects may be the result of accumulation of compounds from the textile effluent resulting in elevated concentrations (e.g. heavy metals); (4) observed effects in the field are due to chronic exposure, while the embryo tests with polar fractions were aimed at detecting more acute effects.

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Kapitel 6: Monitoring pollution in river Mureş, Romania, Part III: Biochemical effect markers in fish and integrative reflection

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Abstract

Along a downstream stretch of River Mureş, Romania, adult males of two feral fish species, European chub (*Leuciscus cephalus*) and sneep (*Chondrostoma nasus*) were sampled at four sites with different levels of contamination. Fish were analysed for the biochemical markers hsp70 (in liver and gills) and hepatic EROD activity, as well as several biometrical parameters (age, length, wet weight, condition factor). None of the biochemical markers correlated with any biometrical parameter, thus biomarker reactions were related to site-specific criteria. While the hepatic hsp70 level did not differ among the sites, significant elevation of the hsp70 level in the gills revealed proteotoxic damage in chub at the most upstream site, where we recorded the highest heavy metal contamination of the investigated stretch, and in both chub and sneep at the site right downstream of the city of Arad. In both species, significantly elevated hepatic EROD activity downstream of Arad indicated that fish from these sites are also exposed to organic chemicals. The results were indicative of impaired fish health at least at three of the four investigated sites. The approach to relate biomarker responses to analytical data on pollution was shown to fit well the recent EU demands on further enhanced efforts in the monitoring of Romanian water quality.

Keywords: biomarker, chub, cytochrome P450, Danube tributary, hsp70, monitoring, sneep

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1. Introduction

In order to set discharge effluent and surface water quality standards, chemical criteria have originally been developed and applied to natural water bodies for centuries. Relying on chemical criteria alone for assessing the status of surface water integrity can, in many instances inaccurately portray the biological and ecological condition of aquatic ecosystems (Adams 2002). This is shown by a comparison of the indicative potential of biological and chemical criteria in more than 600 river and stream segments (Yoder and Rankin 1998). Within this context, chemical criteria indicate contamination but cannot show biological or environmental damage. With a great variety of biological assessment tools now available, an improved understanding of contaminant effects on ecosystem structure and function, and an increased ability to interpret biological data is possible. The use of physiological, cellular and biochemical effects, so-called biomarkers, has become attractive and useful for assessing the effects of environmental stressors on the sub-lethal level of biological systems.

Chemical analyses on sediments of the downstream part of River Mureş, Romania, in a stretch from the Carpathian Mountains through the Plain of Arad to the Romanian-Hungarian border showed high concentrations of cadmium (up to 8.7 mg/kg) and copper (up to 49.2 mg/kg) which surpassed the (exposure defined) quality standards from a number of industrialized countries, most likely due to the mining and metallurgical activities at many tributaries to the Mureş (Sandu et al. 2006, this issue). In addition to the highest measured metal levels, pollution by untreated faecal waste was proven in the stretch of the river course between the Carpathian Mountains and the city of Arad. Despite contamination, however, the species number and biomass of invertebrates and planktonic algae in the river were not affected and the structure of the planktonic communities seemed to be slightly affected at a single site only (Sandu et al. 2006, this issue).

In the present study, we applied biomarkers to assess whether chemically detected pollution of the River Mureş has resulted in effects on fish health. Effects were assessed in organs that are sites of primary attack and accumulation, the gills and the liver. Apart from documenting metal accumulation in fish liver and concomitant histopathological changes in both fish gills and liver which were found all along the downstream stretch of the river Mureş (Triebskorn et al. 2006, this issue), we used the following two biochemical markers for health assessment in two feral fish species, the carnivorous European chub (*Leuciscus cephalus*) and the planctivorous sneep (*Chondrostoma nasus*):

(1) Stress proteins. The best investigated stress protein family, hsp70, is commonly used as a marker which effectively integrates overall adverse effects on protein integrity, hence measures proteotoxicity. Its induction by heavy metals in a variety of species has been shown in numerous studies (for reviews see Schramm et al. 1999, Kammenga et al. 2000).
(2) Hepatic cytochrome P450. The induction of CYP1A, a group of isoforms among more than 900 gene products identified throughout phylogeny, is accepted as a measure of bioavailable arylhydrocarbon receptor (AhR) ligands, such as dioxins, polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and structurally similar compounds (Schlenk and Di Giulio 2002). This marker has been correlated with liver lesions and immune suppression in fish (Collier et al. 1998, Reichert et al. 1998).

2. Material and Methods

2.1 Sites and sampling

Four sampling sites along a downstream stretch of the River Mureş were investigated (in upstream to downstream order): site 1 (Zam), 107 km upstream of the city of Arad; site 2 (Mândruloc), 15 km upstream of Arad; site 3 (Bodrogu Vechi), right downstream the influx of the municipal wastewater treatment plant of Arad; and site 4 (Pecica), 21 km downstream of Arad and right downstream of an industrialized area (for a map see Sandu et al., 2006, this issue). At each sampling site, 8-10 individuals of *C. nasus* and 6-10 individuals of *L. cephalus* were caught in the open water body by means of electro-fishing from boats during three subsequent days (May 25-27, 2004), in the order site 4 to site 1 opposite to the direction of the water flow. Due to different habitat preferences of male and female fish during spawning, all captured fish were adult males. Fish were anaesthetized with 0.05% ethyl-4-aminobenzoate (benzocaine) for one minute, killed, measured for length (L [in cm]) and wet weight (wt [in g]), and dissected in the field. The liver was excised immediately and cut into pieces of which two portions were frozen in liquid nitrogen each for stress protein and EROD analysis. The other pieces were used for histopathology and metal analysis (see Triebskorn et al., 2006, this issue). Subsequently, the gills were removed and the right branches frozen in liquid nitrogen for stress protein analyses. From each fish, a couple of scales were removed for age analysis. The individual condition factor (cf) was calculated according to the equation

$cf = wt \cdot L^{-3} \cdot 10^2$ (Fulton, 1902)

2.2 Stress protein analysis

Gill and liver samples were homogenized on ice in a buffer (80 mM potassium acetate, 5mM magnesium acetate, 20 mM Hepes, pH 7.5) and analyzed by a highly reproducible Western blotting technique (methodological variability between identical samples on different gels ± 2.7%, Köhler et al. 2005) and subsequent image analysis. Total protein concentration in the supernatant was determined according to the method of Bradford (1976). Constant protein weights (10 mg of total protein/lane) were analyzed by minigel SDS-PAGE (12% acrylamide, 0.12% bisacrylamide (w/v), 15 min at 80 V, 90 min at 120 V). Every gel contained a standard extract from zebrafish (*Danio rerio*) in order to ensure methodological reproducibility. Protein was transferred to nitrocellulose by semi-dry blotting and the filter blocked for 2 h in 50% horse serum in TBS (50 mM Tris pH 5.7, 150 mM NaCl). After washing in TBS, monoclonal antibody (mouse anti-human hsp70; Dianova, FRG, dilution 1:5,000 in 10% horse serum/TBS) was added and incubated at room temperature (22°C) overnight. After repeated washing in TBS for 2 min, the nitrocellulose filter was incubated in secondary antibody goat anti-mouse IgG (H+L) coupled to peroxidase (Dianova, FRG, dilution 1:1,000 in 10% horse serum/TBS) at room temperature (22°C) for 2 h. After subsequent TBS washing, the antibody complex was detected by 1 mM 4-chloro(1)naphthol and 0.015% H_2O_2 in 30 mM Tris pH 8.5 containing 6% methanol. The optical volumes (average grey scale value x area) of the Western blot protein bands were measured after background subtraction with a densitometric image analysis system (Herolab E.A.S.Y.). Optical volumes were normalized using the respective *D. rerio* standard on the respective blots as a reference. The methodological variability of this protocol has been shown to be ±2.7% from the mean (Köhler et al., 2005).

2.3 Cytochrome P450 analysis

Liver samples were weighed and homogenized in 2ml ice-cold homogenization buffer (2 M sucrose, 20 mM Mops, 1% EDTA/ethanol, 0.2 mM phenylmethylsulfonylfluorid, 1 mM ε-amino capronic acid, 0.3 M mercaptoethanol, 0.02 mM dithiotreitol) with three strokes of a Potter-Elvehjem homogenizer at 300 rpm. The homogenate was centrifuged for 20 min at 10,000 x g and 4°C and the supernatants were again centrifuged in an ultracentrifuge (Beckman Optima) for 60 min at 100,000 g. After centrifugation, the supernatant was removed and the microsomal pellet was solubilized in 100 µL of homogenization buffer. The microsomal fraction was directly assayed for CYP1A by measuring the catalytic 7-

ethoxyresorufin-O-deethylase (EROD) activity. Subsequently, the microsomes were frozen at –80°C until protein determination.

The catalytic activity of CYP1A was detected fluorometrically by measuring the conversion of 7-ethoxyresorufin-O-deethylase into the fluorescent product, resorufin (Burke and Mayer 1974). EROD activity was determined in a kinetic microplate assay using 96well plates and a fluorescent plate reader (Victor2, Wallac, Perkin-Elmer, Freiburg, FRG). The reaction mixture contained ethoxyresorufin dissolved in methanol at a final concentration of 0.5 µM in phosphate buffered saline (PBS – 0.08 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.15 M KCl, pH 7.8) and the reaction was started by the addition of NADPH (47 µM final concentration). Resorufin fluorescence was detected at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The amount of resorufin produced was calculated using a resorufin standard curve. Enzyme activity was expressed in pmol resorufin/mg protein/min.

Protein content of the microsomal cell fraction was measured spectrophotometrically using the Bio-Rad DC protein assay kit which is based on the method of Lowry et al. (1951). Bovine serum albumin was used as a standard.

2.4 Statistical analysis

Data were tested for normal distribution using the Shapiro-Wilk W test. Since data were not normally distributed, significance of differences between two respective test groups was tested by the non-parametric Mann Whitney Wilcoxon test. Levels of significance were set to $p \leq 0.01$ (**), and $0.01 < p \leq 0.05$ (*). For correlation analysis, the recorded data for hsp70 in gills, hsp70 in liver, EROD in liver, histopathology of the liver and the gills (both taken from Triebskorn et al. 2006, this issue), individual age, wet weight, length, and condition factor were subjected to linear and polynomial (2^{nd} and 3^{rd} degree) regression analysis. 95% confidence intervals for each regression curve and significance at the $p = 0.05$ level (ANOVA) were calculated. All statistical analysis was conducted with SAS JMP 4.0.0.

3. Results

All morphometrical parameters (length, weight, condition factor) and age were found to correlate significantly with one another in both fish species (*L. cephalus* weight vs. condition factor with $p = 0.002$, all other combinations with $p < 0.0001$). In contrast, none of the investigated biochemical markers correlated with age, length, weight, or condition factor of fish (data not shown) and it was therefore concluded that biochemical markers reflected the environmental conditions at the respective

sites. Consequently, all biochemical data were analysed in respect to the variable 'site'.

Stress protein (hsp70) induction indicated proteotoxic action of environmental threats in the gills of both fish species. In gills of *L. cephalus*, significantly elevated hsp70 levels were found in individuals from sites 1 and 3. Also *C. nasus* gills showed hsp70 to be significantly induced at site 3 (Fig. 1). In contrast, the stress protein levels in the liver did not reveal any differences between the four sites, neither in *L. cephalus* nor in *C. nasus* (Fig. 2).

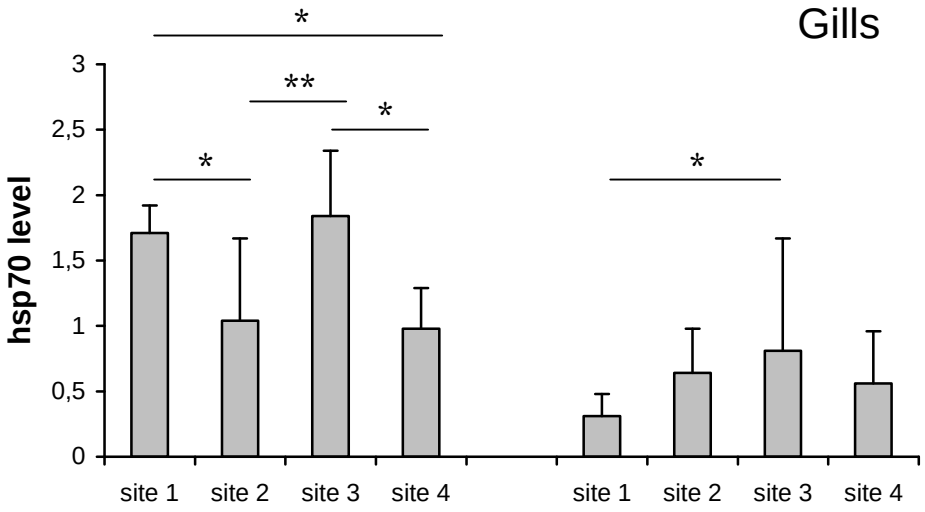

Figure 1: Hsp70 levels (optical volume relative to a standard) in the gills of *L. cephalus* and *C. nasus* sampled at the four sites at River Mureş. Means and SD. Significance at $p \leq 0.01$ (**) and $0.01 < p \leq 0.05$ (*).

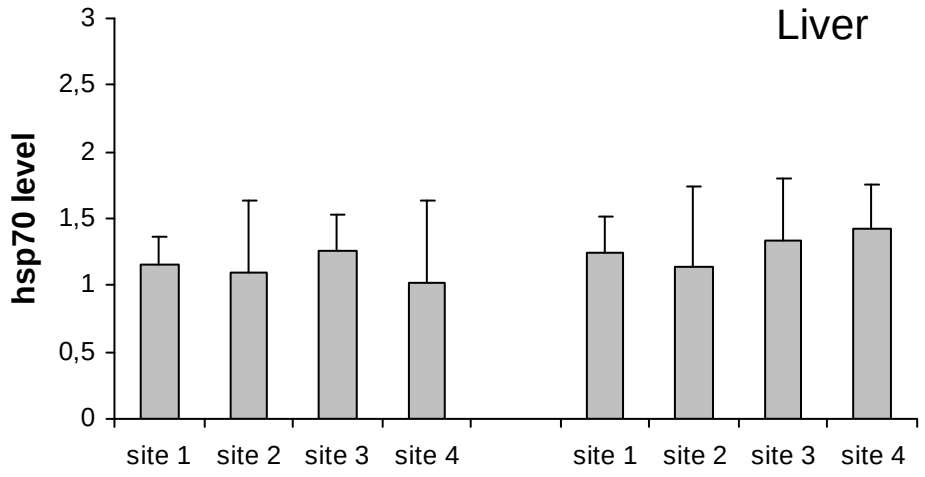

Figure 2: Hsp70 levels (optical volume relative to a standard) in the liver of *L. cephalus* and *C. nasus* sampled at the four sites at River Mureş. Means and SD. No correlation was found.

Hepatic cytochrome P450 activity, measured as EROD activity and indicative of water pollution with organic compounds like PAHs or coplanar PCBs, was slightly higher in *C. nasus* than in *L. cephalus* at all sites. A significant elevation in EROD activity could be found in fish downstream of the city of Arad, precisely in *L. cephalus* at site 3 and in *C. nasus* at site 4 (Fig. 3).

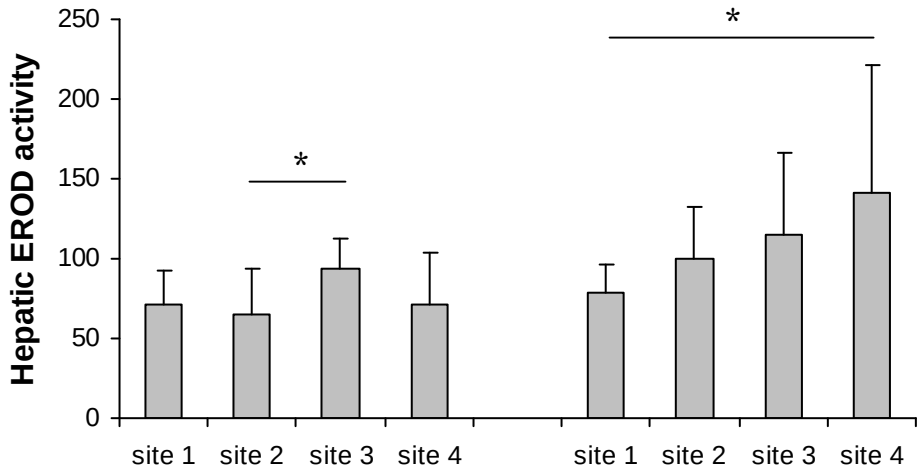

Figure 3: EROD activity (pmol . min-1 per mg microsomal protein) in the liver of L. cephalus and C. nasus sampled at the four sites at River Mureş. Means and SD. Significance at $0.01 < p \leq 0.05$ (*).

4. Discussion

Biochemical biomarkers such as stress proteins and cytochrome P450-associated enzyme activity are commonly accepted as sensitive indicators of toxic impact since these molecular responses are typically the first line of defense following exposure to xenobiotics. However, they have been shown to be also extremely variable and plastic among individuals in a given population (Schlenk and DiGiulio 2002). Nevertheless, both defense systems, the hsp70 stress response and the cytochrome P450-dependent biotransformation system, have been shown to comprise suitable ecotoxicological markers whenever a sufficient number of individuals is analyzed. The CYP1A catalytic activity, measured by means of the EROD assay is accepted to indicate exposure to important organic environmental contaminants such as PAHs, coplanar polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and –furanes (PCDDs, PCDFs), and a number of pesticides (Stegeman and Hahn 1994, Van Veld et al. 1997, Whyte et al. 2000, Navas et al. 2003). On the other hand, the level of the stress protein hsp70 is a typical effect marker, integrating overall proteotoxic impact of stressors regardless of their nature (Schramm et al. 1999,

Kammenga et al. 2000). Despite the particular advantages of these markers, it has become common sense that modern ecotoxicological monitoring programmes must combine a selection of markers at different levels of biological organization, chemical analytics, conventional limnochemistry, and community-level indicators (Triebskorn et al. 2001), together with an array of physiological covariates (Hodson 2002). This approach has been realized in the present Mureş River study.

Even though our results represent a temporal snapshot of summer 2004 only, we have also included measures which integrate over a longer period of time. Thus, on the basis of our data, we are able to draw up a series of conclusions on the quality of the downstream part of the River Mureş.

(1) Fish health. Significantly elevated stress protein levels in the gills of *L. cephalus* indicated acute effects at sites 1 and 3, the most upstream site and the site right downstream the city of Arad. Also the sneep, *C. nasus*, showed its highest hsp70 levels in its gills at site 3 and a remarkably low hsp70 level at site 1. The latter likely has to be attributed to an inhibition of the stress response due to pathologic damage of the hsp system as reported e.g. by Köhler et al. (2001) after exposure to high concentrations of pollutants. On the basis of stress protein analysis, proteotoxic action of environmental compounds must be considered at least for these two sites. As well, histopathology indicated significantly impaired integrity of the gills for the sites 1 and 2, and also considerable, though not significant impairment at site 3. It is known that gills are particularly sensitive to metals (Mallat 1985) since they are the target organ for their uptake. Chemical analytics have shown extreme contamination of these sites with cadmium and copper (up to 8.7 and 49.2 mg per kg sediment, respectively; Sandu et al. 2006, this issue) and, thus, it is likely that proteotoxicity and subsequent disintegration of cellular structures in the gills of fish were exerted by these metals. Nevertheless, fish seemed to be able to cope with this burden to some extent and to accumulate these two metals in their livers as symbolized by the exceptionally high concentrations measured in this organ (Triebskorn et al. 2006, this issue). This sequestration over time seems to level out the differences in concentrations in the outer environment at the different sites for some part, since all fish showed equally high stress protein levels in their livers. Therefore, it has to be assumed that hepatic metal accumulation took place in a way which largely removes the stored metals from physiological acute impact, e.g. by protein-mediated sequestration or precipitation as insoluble salts. Even though background data for hepatic hsp70 are lacking

for European chub and sneep, absolute values corresponded to the highest gill hsp70 levels measured in this study. Liver histopathology also revealed strong impairment of health, predominantly at site 1 but also at sites 2 and 3 (Triebskorn et al. 2006, this issue). As well as biochemical markers, also the histopathology data taken from Triebskorn et al. (2006, this issue) did not correlate with morphometrical parameters with the exception of gill pathology in *L. cephalus* which slightly correlated in a negative way with the individual age ($p = 0.034$).

(2) Exposure. As mentioned earlier, massive metal contamination presumably deriving from mining activities and metallurgical processing in Western Transsylvania (Sandu et al. 2006, this issue) is ecotoxicologically crucial for the investigated stretch of the River Mureş. The most relevant metals were cadmium and copper the concentration of which in the sediment surpassed the quality criteria of a number of Western states. Both metals were found to accumulate in the liver of abundant fish (Triebskorn et al. 2006, this issue). Moreover, significantly elevated hepatic EROD activities indicated the presence of aromatic organics in fish caught downstream the city of Arad. However, the absolute values measured for EROD activity were not extremely high and, thus, the influence of PAHs, PCBs and other CYP1A-inducing organics seemed to be limited as indicated by their low concentrations in the sediment (Sandu et al. 2006, this issue). The differences in EROD activity between the two fish species may be a consequence of their feeding behaviour: *L. cephalus*, which feeds mainly on plankton in the water column, seems to be less affected by organic pollution than *C. nasus,* which feeds on periphyton and detritus. Due to their high affinity to organic matter, organic pollutants tend to associate with other suspended particles settling to the sediment and, thus, the accumulation of organic pollutants in sediment makes benthic species feeding on contaminated algae or detritus more vulnerable than pelagic ones. At sites 1 and 2, microbiological analyses revealed an additional impact of faecal waste being acutely released into the Mureş (Sandu et al. 2006, this issue) which may have substantially contributed to the fact that the fish caught at site 1 in early summer of 2004 were not suitable for human consumption (Mureş Sampling Consortium, personal experience).

(3) Community integrity. Community-level indicators measure the state of an ecosystem. These indicators are of highest ecological relevance but rather insensitive compared to subindividual markers, and responses to pollution

occur either very slowly or are camouflaged by background noise. Even though community indices have little plausibility for cause-effect mechanisms, they may provide evidence of cause through association with a sampling location. In this monitoring, the diversity of plankton was affected by a recent flood two weeks before sampling, and the structural parameters did not vary significantly among the investigated sites. Still, a slight decrease of diversity index was recorded at site 1 (Sandu et al. 2006, this issue) which spatially corresponds to the highest concentrations of metals. Since logistic constraints only allowed to sample the sites once, the sampling design was particularly critical for a community survey in a variable environment and, thus, the existing dataset seems not to be robust enough for a final conclusion.

5. Conclusions and outlook

Applying an approach that integrates indicators and markers of different character at different levels of biological organization, we showed that a single cross-survey of even larger stream stretches can provide a refined view of the situation in the aquatic environment. This approach turned out to be especially suitable for regions where pollution can be anticipated but ecotoxicological data are scarce. This is particularly true for the countries of Eastern Europe, as the economy and hence the pollution both quantitatively and qualitatively is very much different from the industrialized countries in Western Europe and North America. It its recent report on the structural development of future member states, the European Commission has included environmental pollution in the list of areas of serious concern for Romania (European Commission, 2005). This report stated that “the capacity to issue integrated permits of a sufficient quality (…) for all industrial installations (…) represents a major challenge and requires serious efforts”. Furthermore, “serious concerns exist in relation to industrial pollution. Considerable efforts are required to ensure that relevant permits are issued at local and regional level. (…) The monitoring of water quality requires further enhanced efforts.” Not much work is being done to detect pollution effects in the catchment area of the Lower Danube by applying biomarkers and using fish as biomonitors as suggested earlier by Burkhardt-Holm and Bloesch (2000). To the best of our knowledge, a single biomarker study has been conducted in the Danube tributary, River Drava (Croatia) which revealed an inhibition on acetylcholine esterase and an increase in EROD activity in the Prussian and the common carp (*Carassius auratus gibelio* and

Cyprinus carpio) indicating pollution by organophosphates and polyaromatic and/or polychlorinated hydrocarbons (Jaric and Stepic 2005). Also in the Mureş case, our results indicated a situation of concern, at least in view to impaired fish health. In this situation, the advantage of biomarkers as sensitive early-warning sentinels becomes clear, particularly since they are able to integrate the effects of the entirety of contaminants, not only of those, respectively, selected for chemical analysis. Conventional limnochemical analyses and macrozoobenthos faunistics, commonly used in water quality assessment, were not relevant in this case, stressing the importance of applying combined approaches like the present one. In combination with the results presented by Sandu et al. (2006, this issue) and Triebskorn et al. (2006, this issue) this study could reveal both the character of (a number of) discharged substances and resulting effects, exhibiting ways to terminate further pollution in view to a restoration of the system. On the basis of our entire study, not only on the basis of the biomarkers presented in this paper, we propose that the metal pollution of the River Mureş derives from the adjacent mining and metallurgical activity and that its ecological impact probably is much more severe in those upstream tributaries which directly pass the industrial areas. The spatial limitation of sampling and the fact that our initiative was the first of its kind in Romania, however, characterizes this monitoring as a pilot study. Nevertheless, it should be considered as a starting point for larger surveys of the Danube River system including its tributaries in Eastern Europe. Profound ecotoxicological information on the situation in the downstream part and the catchment area of one of Europe´s largest streams is urgently needed.

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Scheil V, Köhler H-R (eingereicht): Influence of nickel chloride, chlorpyrifos and imidaclopride in combination with different temperatures on the embryogenesis of the zebrafish, *Danio rerio*. Archives of Environmental Contamination and Toxicology

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Buchbeitrag

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Tagungsbeiträge

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Poster und Abstract: Scheil, V, Triebskorn, R, Köhler, H-R: Stress protein (HSP70) responses and reactions of hepatopancreatic cells in *Gammarus fossarum* after short-term exposure to the UV-filter 3-benzylidene camphor

SETAC Europe 16th Annual Meeting, Den Haag (2006)

Poster: Scheil, V, Triebskorn, R, Köhler, H-R: Influence of nickel chloride, chlorpyrifos and binary mixtures of them on *Danio rerio* embryos and larvae

Platform presentation: Triebskorn, R, Scheil, V, Schwaiger, J: Drugs for fish: How effective are human pharmaceuticals in aquatic organisms?

SETAC Europe 17th Annual Meeting, Porto (2007)

Poster: Scheil,V, Osterauer, R, Triebskorn, R, Köhler, H-R:Influence ofthree pesticides, a decomposition product of a pesticide and twobinary mixtures of them on the stress protein level of *Danio rerio* larvae

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