Toxizität von Umweltchemikalien und deren Mischungen auf ausgewählte aquatische Organismen - Verhalten, Entwicklung und Biochemie -

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Cornelia Kienle
aus Markt Rettenbach
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To see a world in a grain of sand,
And a heaven in a wild flower,
Hold infinity in the palm of your hand,
And eternity in an hour.

(William Blake - Auguries of Innocence)

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Zusammenfassung

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Toxizität von Umweltchemikalien und deren Mischungen auf ausgewählte aquatische Organismen – Verhalten, Entwicklung und Biochemie

2. Einleitung

Grundlagen

Ein erheblicher Anteil der mehr als 100 000 kommerziell produzierten Chemikalien wird beabsichtigt oder unbeabsichtigt in die Umwelt eingetragen und kann teilweise bereits in sehr geringen Konzentrationen nachteilige Auswirkungen auf Organismen, Populationen und Ökosysteme haben. Um die Auswirkungen von Chemikalien auf Ökosysteme beurteilen und diese somit vor negativen Konsequenzen schützen zu können, wurde das Forschungsgebiet der Ökotoxikologie begründet. Nach Fent (2007) beschäftigt sich die Ökotoxikologie als Zweig der Umweltwissenschaften „primär mit der Analyse und dem Verständnis der Auswirkungen von chemischen Stoffen auf die belebte Natur. Dabei werden alle biologischen Ebenen betrachtet“. Zu diesem Zweck wird analysiert, wohin diese Substanzen in der Umwelt gelangen (ihr Schicksal, Expositionsabschätzung) und welche ökologischen Auswirkungen sie dort haben (Effektabschätzung) (Calow, 1993). Somit ist die Ökotoxikologie ein interdisziplinäres Forschungsgebiet in dem Umweltchemie, Toxikologie („die Lehre von den Schadeffekten chemischer Stoffe auf Lebewesen“, (Dekant und Vamvakas, 2005)) und Ökologie (die „Lehre von den Interaktionen der Organismen mit ihrer belebten und unbelebten Umwelt“ (Haeckel, 1866)) verknüpft und integriert werden (Fent, 2007). Als Schadstoffe gelten reine Substanzen oder Gemische (organisch oder anorganisch), die normalerweise nicht in natürlichen Systemen (Organismus, Habitat, Ökosystem) vorkommen oder in solchen Mengen auftreten, dass die natürlichen Konzentrationen überschritten werden (Grue et al., 2002).

Die Abschätzung der Exposition und die Analyse der Effekte auf Organismen sind Bestandteil der Risikoabschätzung von Umweltchemikalien. Hier spielt die neue EU-Chemikalienverordnung REACH (**R**egistration, **E**valuation and **A**uthorisation of **Ch**emicals) eine große Rolle, die am 1. Juni 2007 in Kraft getreten ist und zum Ziel hat,

alle Chemikalien, die in Europa mit einem Produktionsvolumen von mehr als 1 t/a hergestellt und importiert werden, zu registrieren und gefährliche Stoffe bzw. Stoffe mit einem hohen Produktionsvolumen zu evaluieren (>100 t/a) und gegebenenfalls zuzulassen bzw. bei zu großen Bedenken eine Zulassung zu verweigern. Letzteres kann bei besonders gefährlichen Stoffen erreicht werden, die in die Gruppe der persistenten, bioakkumulierenden und toxischen Substanzen (PBT), der kanzerogenen, mutagenen und reproduktionstoxischen Substanzen (CMR) und der stark persistenten und stark bioakkumulierenden Stoffe (vPvB) fallen. Ebenso ist eine Verweigerung der Zulassung eventuell bei Chemikalien, die auf das Hormonsystem wirken (endokrin aktive Substanzen), wie Weichmacher in Kunststoffen (z.B. Phthalate) oder das Kunststoff-Monomer Bisphenol A, die sich bereits in sehr geringen Konzentrationen nachteilig auf die Fortpflanzung von Organismen auswirken können (Lahl und Hawxwell, 2006; Oehlmann et al., 2006), zu erwarten. Ausnahmen bei der Registrierung gelten nur für solche Substanzen, deren Anwendung bereits in der bestehenden Gesetzgebung geregelt ist, z.B. Pflanzenschutzmittel (PSM) in der Pflanzenschutzmittelrichtlinie (91/414/EEC, European Commission, 1991). Schutzziele dieser Verordnungen sind der Schutz der Umwelt vor schädlichen Einflüssen (REACH) bzw. die Vermeidung von nicht vertretbaren Auswirkungen auf den Naturhaushalt (Pflanzenschutzmittelrichtlinie). Pflanzenschutzmittel, wie die in dieser Studie untersuchten Insektizide Diazinon und Chlorpyrifos, werden gezielt und in großen Mengen (in Deutschland: 2,8 kg/ha) in die Umwelt eingebracht, um Schadorganismen zu dezimieren. Trotz Risikoabschätzung überschreiten die gemessenen Werte von Pflanzenschutzmitteln in deutschen Oberflächengewässern teilweise die kurzzeitig als unbedenklich geltenden Konzentrationen erheblich (in einem extremen Fall: Pirimicarb, 27,3 µg/L maximal gemessen, 0,09 µg/L kurzzeitig unbedenklich) (Hommen, 2004), was eine verbesserte Risikoabschätzung und/oder eine verbesserte Kontrolle der Ausbringung für nötig erscheinen lässt. Im Rahmen der Risikobeurteilung dieser Stoffe sind auch verschiedenste Tests zur Beurteilung der Ökotoxizität der einzelnen Substanzen gefordert.

Organismen sind in ihrer Umwelt jedoch nicht nur einzelnen Chemikalien, sondern einer Vielzahl von Stressoren ausgesetzt. Neben biotischen Faktoren, wie Nahrungsverfügbarkeit, Konkurrenz, Reproduktion und Feinddruck, bestimmen

abiotische Faktoren, u.a. Temperatur, Sauerstoff, pH und auch chemischer Stress, wie effektiv ein Organismus physiologisch (und dadurch in seinem Verhalten) in einem bestimmten Habitat funktionieren kann und auch, ob seine *performance* in diesem Habitat optimal ist oder nicht (Walter, 1973). Dies kann für die Zusammensetzung der Artengemeinschaft unter Umständen sehr bedeutsam sein: Können normalerweise unterlegene Konkurrenten einer Art A z.B. anthropogenen Umweltstress besser tolerieren als Art B, so ist davon auszugehen, dass Art A einen Wettbewerbsvorteil unter diesen artifiziellen Bedingungen besitzt, und so können durch unterschiedliche Reaktionen von Populationen oder Arten auf Gradienten eines abiotischen Faktors intra- oder interspezifische Interaktionen in Ökosystemen verändert werden.

In diesem Zusammenhang stellt die Sauerstoffsituation in Gewässern einen wichtigen abiotischen Faktor dar. Durch den vermehrten Ablauf von sauerstoffzehrenden Prozessen (meist verursacht durch erhöhte Atmung von Mikroorganismen) kann ein Defizit an gelöstem Sauerstoff resultieren (Schönborn, 2000). Diese Situation entsteht im Sommer häufig im Hypolimnion eutropher Seen, aber auch in sehr stark organisch verunreinigten Fließgewässern (Schwoerbel, 1992). Sauerstoff gelangt über Diffusion aus der Atmosphäre, durch Photosynthese oder durch den Eintrag von sauerstoffreichem Wasser aus Flüssen ins Hypolimnion und wird durch Atmung und Mineralisation organischer Stoffe, ebenso wie durch Verlust an die Atmosphäre verbraucht (Schwoerbel, 1992). Der Gehalt an gelöstem Sauerstoff ist ein Parameter zur Beurteilung des trophischen und organismischen Zustands eines Gewässers, so kommt es beispielsweise bei einem Sauerstoffgehalt von 3 bis 4 mg/L Wasser (~ 40 bis 50 % Sättigung bei 26 °C) bereits zu einer erheblichen Schädigung der Lebensgemeinschaft im Gewässer, hierbei reagieren Gemeinschaften von Bergbächen am empfindlichsten auf Sauerstoffdefizite: Salmoniden benötigen in der Regel mindestens 6 mg O_2/L H_2O zum Überleben und gehen bei 40-50 % O_2-Sättigung bereits zur Notatmung über, wohingegen Cypriniden noch bei Sauerstoffgehalten bis 1 mg/L (13 % O_2 Sättigung bei 26 °C) überleben können (Schönborn, 2000).

Solche abiotischen Stressoren können die Wirkung von Umweltchemikalien auf Organismen verändern und ähnliche Interaktionen wie Mischungen von Chemikalien ausüben. Hierzu existieren jedoch bisher nur wenige Studien (z.B. Osterauer und Köhler, 2008; Scheil und Köhler, 2009; van der Geest et al., 2002). Neben Interaktionen von

Einzelchemikalien mit abiotischen Faktoren treten häufig auch Mischungen von Chemikalien in der Umwelt auf, wenn z.B. Tankmischungen von PSMs ausgebracht werden, bei Verwendung von Formulierungshilfen oder bei Hintergrundbelastung durch bereits vorhandene Stoffe. Das Verhalten von Chemikalien in Mischungen wird stark von ihrem toxikologischen Wirkmechanismus beeinflusst. Haben zwei oder mehr Chemikalien unterschiedliche Wirkorte, kann ihre Wirkung im Allgemeinen als unabhängig betrachtet werden (Konzept der unabhängigen Wirkung, *independent action*, IA). Bei Chemikalienmischungen mit einem gemeinsamen Wirkort und dem gleichen Wirkmechanismus addieren sich die Wirkungen (Konzept der Konzentrationsadditivität, *concentration addition*, CA) (Escher und Hermens, 2002; Faust et al., 1996). Interagieren die Komponenten einer Mischung miteinander, können sie antagonistische, d.h. schwächere Effekte als durch das Konzept der unabhängigen Wirkung vorhergesagt oder synergistische Effekte, d.h. stärkere Auswirkungen als durch das Konzept der Konzentrations-Additivität vorhergesagt, hervorrufen (Escher and Hermens, 2002).

Die Auswirkungen von Stressoren in der Umwelt erstrecken sich auf verschiedene biologische Organisationsebenen, von Molekülen, Zellen, Organen und einzelnen Organismen bis hin zu Populationen, Biozönosen und Ökosystemen. Zur Quantifizierung dieser Effekte auf Organismen werden Biotests durchgeführt. Ein Biotest ist eine Analysemethode, die lebende Organismen in definierter Art und Anzahl einsetzt, um deren Reaktion auf eine Exposition zu messen (Fent, 2007). Die Richtlinien von internationalen Standardisierungsorganisationen (ISO, OECD) dienen als Anleitung für die Durchführung und Auswertung dieser Tests (z.B. OECD, 1992b). Ein Organismus, der Informationen über die Umweltbedingungen seines Habitats durch sein Vorhandensein, seine Abwesenheit oder sein Verhalten gibt, wird als Bioindikator bezeichnet (van Gestel und van Brummelen, 1996). Molekulare, biochemische, zelluläre und physiologische Antworten oder Reaktionen eines Organismus auf Umweltveränderungen, gelten als Biomarker (van Gestel und van Brummelen, 1996). Hierbei wird zwischen Biomarkern unterschieden, bei denen eine eindeutige Beziehung zwischen Exposition und Biomarkerantwort besteht (*biomarker of exposure*), wodurch Aussagen über die Qualität und/oder die Quantität der Exposition gemacht werden können. Beispiele hierfür sind die Analyse von Metallothioneinen (metallbindenen

Proteinen), bei denen die Menge an nachgewiesenem mt-Protein zusammen mit dem Sättigungsgrad von mt durch gebundene zweiwertige Metallionen indiziert, inwieweit Organismen diesen Metallen ausgesetzt waren (Köhler und Triebskorn, 2004). Die Hemmung der Aktivität des Enzyms Acetylcholinesterase kann ebenfalls als Expositionsbiomarker klassifiziert werden, da dadurch eine Exposition gegenüber neurotoxischen Insektiziden angezeigt wird (Walker, 1995). *Biomarkers of effect* hingegen indizieren „einen Stresseffekt, der durch die Gesamtheit aller aktuell wirkenden Einflüsse bedingt ist“ und erlauben so Aussagen über den Gesundheitszustand von Organismen. Ein klassisches Beispiel hierfür ist die Induktion von Stressproteinen (Hitzeschockproteine, Hsps), die verstärkt produziert werden, wenn der Organismus proteotoxisch wirkendem Stress (ungeachtet dessen chemischer oder physikalischer Natur) ausgesetzt ist. Durch Effektmarker wird eine entsprechende Wirkung der Gesamtheit aller aktuell auf den Organismus wirkenden Einflüsse quantifiziert (Köhler und Triebskorn, 2004).

Die Parameter, die in Biotests gemessen werden, wie z.B. Enzymaktivität, Mortalität, Reproduktion, bezeichnet man als Endpunkte (Fent, 2007). Toxizitätsparameter hierbei sind die NOEC (*no observed effect concentration*), d.h. die höchste getestete Konzentration, bei der keine signifikanten Auswirkungen auf Überleben oder andere Effekte auftreten (OECD, 1984) und die LOEC (*lowest observed effect concentration*), als niedrigste Konzentration bei der im Vergleich zur Kontrolle ein signifikanter Effekt auftritt (OECD, 1992b). Eine LC_{50} bzw. LC_x ist die letale Konzentration, bei der im Biotest innerhalb einer definierten Expositionszeit 50 % bzw. x % der Organismen gestorben sind (OECD, 1992a), wohingegen die EC_{50} bzw. EC_x die Effektkonzentration darstellt, bei der 50 % bzw. x % der Organismen in der Expositionszeit einen definierten Effekt zeigen, z.B. Hemmung der Mobilität im Daphnientest (OECD, 2004).

Oft reagieren Organismen auf eine Exposition gegenüber Schadstoffen unmittelbar mit einer Änderung ihres Verhaltens, indem sie den Stoff z.B. zu meiden suchen (Vermeidungsverhalten), der Stoff einen anziehenden Effekt auf sie hat (Attraktion) oder die Organismen physiologisch bedingte Reaktionen (z.B. verstärkte Ventilation („Stressatmung“), schnellere/langsamere Fortbewegung etc.) zeigen. Aus diesem Grund stellen Verhaltensänderungen einen sensitiven Indikator für den Einfluss von Schadstoffen insbesondere im Vergleich zu konventionellen Endpunkten wie der

Mortalität dar (Grue et al., 2002). Laut Little (1990), bieten Verhaltensbeobachtungen eine einzigartige toxikologische Perspektive, die die biochemischen und ökologischen Folgen von Umweltbelastungen miteinander verbindet. Nach Triebskorn et al. (1997) repräsentieren Verhaltensantworten sowohl Kurzzeit- als auch Langzeit-Indikatoren für anthropogene Belastungen mit einer hohen ökologischen Relevanz. Zusätzlich haben Verhaltensveränderungen eine kurze Reaktionszeit (bei kompensatorischen Frühwarn-Reaktionen), sind sensitiv (auf jeden Fall gegenüber Schadstoffen, die sich auf den Nerven- und Muskel-Apparat auswirken) und nicht-invasiv (Gerhardt, 2007).

Mit den Auswirkungen von Schadstoffen auf das Verhalten von Organismen beschäftigt sich das erst in jüngster Zeit begründete Forschungsgebiet der Verhaltensökotoxikologie. Hierbei sollen auch die aus einer Änderung des Verhaltens resultierenden Effekte auf angrenzende biologische Organisationsebenen analysiert werden. Von schadstoffinduzierten Veränderungen im Verhalten sind nicht nur einzelne Individuen betroffen, vielmehr kann dadurch die Lebensfähigkeit von Populationen, die Struktur von Lebensgemeinschaften und die Funktion von Ökosystemen beeinflusst werden (Dell`Omo, 2002). Verhalten spiegelt die Antwort eines Organismus auf interne (physiologische) und externe (Umwelt-, soziale) Faktoren wieder und kann somit als die kumulative Interaktion einer Vielzahl von biotischen und abiotischen Faktoren angesehen werden. Durch spezifische Verhaltensweisen können Organismen miteinander in Beziehung treten. Da Verhaltensmuster angepasst und auch in Typ, Intensität und Erscheinungszeit verändert werden können, sind sie wichtige Mechanismen für Organismen, mit deren Hilfe diese sich an Änderungen in ihrer Umwelt, wie z.B. einer Exposition gegenüber Chemikalien, anpassen können (Evans, 1994).

Der Verhaltensökotoxikologie liegen folgende Annahmen zugrunde:

1. Die meisten Bewegungen und Verhaltensweisen, die ein Tier zeigt, haben einen adaptiven Wert.
2. Signifikante Abweichungen von normalen Reaktionen auf Umweltreize reduzieren die Wahrscheinlichkeit eines Organismus auf Überleben oder Reproduktion.

Man unterscheidet zwischen direkten und indirekten Verhaltenseffekten. Direkte Effekte sind Verhaltensänderungen, die bei Tieren auftreten, die einem bestimmten

Schadstoff oder einer chemischen Mischung ausgesetzt wurden. Aufgrund von Unterschieden im ökologischen Kontext und im Verhaltensrepertoire können die Verhaltensantworten zwischen verschiedenen Arten, aber auch zwischen Chemikalien und mit der Dosis variieren, was Verallgemeinerungen bezüglich dieses Parameters erschwert (Dell`Omo, 2002). Ein direkter Effekt ist beispielsweise eine veränderte Aufnahme (Perzeption) von Umweltreizen. Dazu zählen die Fähigkeit eines Organismus' natürliche chemische Signale in seiner Umwelt zu detektieren und mit einer Änderung des Verhaltens zu reagieren, oder auch Verhaltensveränderungen die aus der Wahrnehmung von chemischen Substanzen resultieren (Vermeidung, Anziehung). Zu direkten Effekten gehören auch Veränderungen in der Lern- und Erinnerungsfähigkeit, bei der Thermoregulation und der Ernährung. Hierbei ist eine Beeinträchtigung von intra-spezifischen Interaktionen, wie z.B. Kommunikation, soziale Organisation und Reproduktion ebenso möglich, wie eine Schädigung von interspezifischen Interaktionen, wie z.B. Räubervermeidung und Konkurrenz (Grue et al., 2002). Beispielsweise zeigten Äschen (*Thymallus thymallus*) bei Exposition gegenüber Methylquecksilber eine verringerte Effizienz bei der Nahrungssuche, wodurch sie gegenüber nichtbeeinträchtigten Artgenossen benachteiligt waren (Fjeld et al., 1998).

Indirekte Effekte dahingegen sind als Verhaltensreaktionen definiert, die auf schadstoffinduzierte Veränderungen in der Umgebung eines Tieres zurückzuführen sind. Hierzu zählen eine Beeinträchtigung der Habitatauswahl und –nutzung, z.B. durch Veränderungen in der Beuteverfügbarkeit und/oder –zusammensetzung, ebenso wie Änderungen von intra- und interspezifischen Interaktionen, wie z.B. Räuber-Beute-Beziehungen oder die Wettbewerbsfähigkeit zwischen Organismen (Grue et al., 2002; Warner et al., 1991).

Beschreibung der Studien

Im ersten Teil der vorliegenden Dissertation wurden die Auswirkungen mehrerer Umweltchemikalien auf Embryonen und Larven von Zebrabärblingen (*Danio rerio*) sowohl auf suborganismischer Ebene (durch die Messung des Biomarkers Acetylcholinesteraseaktivität, Kapitel 4) als auch auf organismischer Ebene (durch die Beobachtung der Embryonal- und Larvalentwicklung ebenso wie die Messung der Bewegungsaktivität, Kapitel 1-4) untersucht. Eine Exposition erfolgte sowohl gegenüber

Einzelchemikalien als auch in Kombination mit Umweltstress (Sauerstoffmangel) (Kapitel 1) bzw. gegenüber binären Mischungen von Chemikalien (Kapitel 2, 3 und 4). Diese Teile der Arbeit wurden im Rahmen des EU-Projektes NoMiracle (*Novel Methods for Integrated Risk Assessment of cumulative stressors in Europe*) durchgeführt, dessen Ziel es ist, Methoden und Modelle zu entwickeln, die eine integrierte Risikobewertung chemischer Stoffe und Stoffgemische im Zusammenspiel mit physikalischen und biologischen Einflussgrößen ermöglichen.

Fische, die Testorganismengruppe in diesem Teil der vorliegenden Arbeit, repräsentieren als Sekundärkonsumenten und teilweise auch als Top-Prädatoren in aquatischen Ökosystemen eine ökologisch äußerst bedeutende Gruppe. Verschiedene Arten dienen zudem als Nahrungsgrundlage (für andere Raubfische, Vögel etc.). Als Vertreter dieser Organismengruppe wurden Zebrabärblinge (*Danio rerio,* Hamilton, 1822) ausgewählt. Diese stammen ursprünglich aus Südostasien, wo sie in makropyhtenreichen Fließgewässern vorkommen (Börries, 2006). Während der letzten Jahre haben sie in der Forschung, besonders als Modellorganismus für Wirbeltiere in Entwicklungsbiologie und Genetik (z.B. Kimmel, 1989; Nüsslein-Volhard, 1994), ebenso wie als Testorganismus in der Ökotoxikologie (z.B. Braunbeck et al., 2005; Nagel, 2002) verstärkte Aufmerksamkeit erhalten. Der Embryo-Test mit *D. rerio* (DarT) wurde von (Nagel, 2002) als Alternativmethode für den akuten Fischtest (OECD, 1992a) mit adulten Fischen vorgeschlagen. Frühe Lebensstadien von Fischen gelten oft als sensitiver im Vergleich zu adulten Fischen (z.B. Hoang et al., 2004). Bei den zahlreichen Studien mit Embryonen und Larven von *D. rerio* (z.B. Bachmann, 2002; Nagel, 2002; Scheil et al., 2009; Strmac, 1999; Versonnen et al., 2004) wurden in der Regel entwicklungsbiologische, biochemische und histologische Parameter untersucht. Verhaltensstudien mit Zebrabärblingen erfolgten bislang hauptsächlich mit adulten Fischen (z.B. Baganz, 2005; Steinberg et al., 1995). Für Larven von Zebrabärblingen existierten bislang lediglich Grundlagen-Daten zum Verhalten (z.B. Bagatto et al., 2001; Budick und O'Malley, 2000), mit Ausnahme einer Studie, die die Auswirkungen eines chemischen Stressors (Aminosäure-Chemostimulantien) auf das Verhalten von Larven des Zebrabärblings untersuchte (Lindsay und Vogt, 2004).

Um die Auswirkungen von Chemikalien mit gleichen und verschiedenen Wirkmechanismen untersuchen zu können, wurden als Testsubstanzen in Kapitel 1-4

sowohl das ubiquitär und natürlich vorkommende Schwermetall Nickel (WHO, 1991), als auch die neurotoxischen Insektizide Diazinon und Chlorpyrifos und ein Abbauprodukt verschiedener Herbizide (3,4-Dichloranilin) ausgewählt.

Zur Quantifizierung der Auswirkungen auf höhere biologische Ebenen wurden Räuber-Beute-Interaktionen zwischen Vertretern zweier trophischer Ebenen, Zuckmücken-Larven (Chironomiden) als Primär-Konsumenten und Detritusfresser am Beispiel der Art *Chironomus riparius*, und Fischen als Sekundärkonsumenten am Beispiel von Zebrabärblingen (*Danio rerio*) untersucht und der Effekt eines neurotoxischen Insektizids auf diese interspezifischen Interaktionen mit Hilfe eines einfachen Testsystems quantifiziert (Kapitel 5). Larven der Zuckmücke *Chironomus riparius* wurden hier als Beuteorganismen ausgewählt, da sie eine wichtige Nahrungsquelle für Fische darstellen und im Bezug auf die Abundanz in Fliessgewässerökosystemen oft eine dominante Rolle einnehmen. Darüber hinaus sind sie als Sedimentbewohner besonders gegenüber an Sedimenten gebundenen Schadstoffen exponiert. Bei Exposition gegenüber chemischen Botenstoffen (Kairomonen) von Fischen, vergruben sich Larven von *Chironomus riparius* signifikant häufiger und tiefer wenn eine zunehmende Räuber-Dichte von Plötzen (*Rutilus rutilus*) simuliert wurde (Hölker und Stief, 2005). Viele bisherige Räuber-Beute-Studien, sowohl mit aquatischen als auch terrestrischen Organismen, waren entweder auf die Beute (z.B. Baker und Ball, 1995; Brown, 2003; Hershey, 1987; Hölker und Stief, 2005; Schulz und Dabrowski, 2001) oder auf den Räuber (z.B. Grippo und Heath, 2003; Hamers und Krogh, 1997; Power, 1990) fokussiert. Daher sollten in der vorliegenden Arbeit beide Interaktionspartner gleichermaßen berücksichtigt werden, da daraus wichtige Schlussfolgerungen über ökologische Auswirkungen gezogen werden können.

Im Bezug auf Mischungstoxizität ist die Untersuchung von Mischungen zweier Chemikalien wichtig zum Verständnis der kombinierten Auswirkungen von Substanzen mit gleichen oder verschiedenen Wirkmechanismen. In der Umwelt sind jedoch meist komplexe Mischungen aus einer Vielzahl von Stoffen vorhanden (Altenburger und Schmitt-Jansen, 2002). In diesem Zusammenhang stellt die *water accommodated fraction* von Rohöl (WAF) vor allem in marinen und Brackwasser-Ökosystemen einen wichtigen Stressfaktor für Organismen dar (Fukuyama et al., 1998). Da Küstenlebensgemeinschaften besonders durch Ölunfälle gefährdet sind, wurde der

marine Amphipode *Corophium volutator* (Schlickkrebs) als Testorganismus ausgewählt, um die Auswirkungen der WAF von Rohöl in verschiedenen Verdünnungen auf die Bewegungsaktivität des marinen Amphipoden zu quantifizieren (Kapitel 6). Die *water accommodated fraction* von Rohöl setzt sich aus einphasigen homogenen Mischungen (den wasserlöslichen Anteilen) von Kohlenwasserstoffen und Dispersionen von feinen Öltröpfchen in Wasser zusammen (Gordon et al., 1973). *C. voluator* ist einer der häufigsten wirbellosen Organismen im ästuarinen Wattenmeer an Küsten des Nordatlantiks und kommt allgemein an amerikanischen und europäischen Küsten vor; er lebt im Sediment als Detritus- und Suspensionsfesser und ist ein wichtiger Beuteorganismus für Fische und Watvögel (Neal und Avant, 2006). *C. volutator* wurde bereits für zahlreiche ökotoxikologische Studien als Testorganismus verwendet, sowohl in akuter, als auch in chronischer Exposition (z.B. Brils et al., 2002; Kirkpatrick et al., 2006; Peters et al., 2002; Scarlett et al., 2007). Da Schadstoffe nicht immer kontinuierlich in die Umwelt eingetragen werden, sondern auch in Pulsen auftreten können (Diamond et al., 2006), z.B. bei einem Ölunfall, wurde im Rahmen dieser Studie auch ein Experiment mit einem Schadstoffpuls durchgeführt, in dem auch eine Erholungsphase der Testorganismen beobachtet wurde.

Zur Quantifizierung von Verhaltensänderungen können Biomonitore verwendet werden (Gruber et al., 1994). Sie bestehen aus drei Komponenten, dem Testorganismus, dem automatischen Detektionssystem und dem Alarmsystem (Osbild et al., 1995). Biomonitore operieren in Echtzeit; lebende Organismen dienen hierbei als Sensoren für Veränderungen der Wasserqualität (Gruber et al., 1994). Der in der vorliegenden Dissertation verwendete *Multispecies Freshwater Biomonitor*® (MFB) (LimCo International, Deutschland) ist ein *Online*-Biomonitor, der zur kontinuierlichen Überwachung von Gewässern eingesetzt werden kann (Gerhardt, 2000; Gerhardt et al., 1994).

Mit dem Ziel die Eignung von Bachflohkrebsen (Gammariden), die Schlüsselorganismen in Fließgewässern darstellen (Welton, 1979), sowie die Eignung des MFB für die kontinuierliche Überwachung von Gewässern zu evaluieren, wurde die letzte Studie dieser Dissertation durchgeführt (Kapitel 7). Im Rahmen der europäischen Wasserrahmenrichtlinie (WFD2000/60/EC, European Commission, 2000) soll die Wasserqualität in Europa verbessert, geschützt und eine weitere Verschlechterung

dieser verhindert werden. Bis zum Jahr 2015 soll in den europäischen Gewässern wieder ein überwiegend guter ökologischer Zustand herrschen (European Commission, 2000). Hierfür sind neben der Verbesserung der Gewässerstrukturgüte auch verschiedene biologische und chemische Erfassungs- und Überwachungs-Methoden, unter anderem auch die kontinuierliche Überwachung der Wasserqualität mit Online-Biomonitoren nötig (Allan et al., 2006). Ein Vergleich und die Validierung verschiedener Techniken zur Überwachung der Gewässerqualität wurde im Rahmen des von der EU-finanzierten Projektes SWIFT-WFD (***S****creening methods for* ***W****ater data* ***I****n****F****orma****T****ion in support of the implementation of the* ***W****ater* ***F****ramework* ***D****irective*) durchgeführt (Roig et al., 2007). Ebenfalls wurden Informationen von chemischen Sensoren und Daten verschiedener biologischer Methoden miteinander in Beziehung gesetzt. Hierzu wurden biologische Frühwarnsysteme (*Biological Early Warning Systems*, BEWS), wie der MFB, eingesetzt, um eine *Online*-Überwachung von Gewässern zu ermöglichen (Roig et al., 2007). Als Testorganismen dienten Gammariden (*Gammarus pulex*). Diese sind in Europa weit verbreitet und besitzen eine Schlüsselrolle in Fließgewässern im Bezug auf die Struktur und Funktion des Ökosystems, indem sie tote organische Substanz zerkleinern und verwerten und so im Nährstoffkreislauf wieder verfügbar machen, außerdem stellen sie wichtige Beuteorganismen für Fische dar (Karaman und Pinkster, 1977; Welton, 1979). Im Rahmen dieser Studie wurde die Gewässerqualität des Rheins mit Hilfe der Bewegungsaktivität von *Gammarus pulex* an einer Gewässerüberwachungsstation bei Huningue (Frankreich) *online* überwacht und mit parallel hierzu aufgenommenen chemischen und weiteren biologischen Parametern in Beziehung gesetzt.

Zusammenfassend wurden im Rahmen meiner Dissertation die Auswirkungen von Einzelstoffen und Mischungen von Umweltstressoren auf Zebrabärblinge (*Danio rerio*) sowohl auf biochemischer/suborganismischer Ebene (Acetylcholinesteraseinhibition) als auch auf der Ebene von Individuen (Verhalten, Embryonal- und Larvalentwicklung) untersucht (Kapitel 1-4). Auf einer höheren biologischen Organisationsebene wurden Interaktionen zwischen Räubern (Zebrabärblingen) und Beuteorganismen (Chironomidenlarven) unter Schadstoffeinfluss untersucht (Kapitel 5). Die Auswirkungen von komplexen Schadstoffmischungen anhand der *water-accommodated fraction* von Rohöl auf das Verhalten des marinen Amphipoden *Corophium volutator*

sind Gegenstand von Kapitel 6 und die kontinuierliche Überwachung der Gewässerqualität mit Hilfe von Verhaltensänderungen des Bachflohkrebses *Gammarus pulex* werden in Kapitel 7 behandelt. Im Abschnitt „Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten“ ist eine Auflistung der Anteile dieser Promotionsarbeit an den jeweiligen Projekten enthalten (Seiten 46-48).

Zielsetzungen

Das Ziel des ersten Teils der vorliegenden Dissertation war es, zu untersuchen, ob und wie sich Einzelsubstanzen, Mischungen von Einzelsubstanzen mit einem abiotischen Stressor (Sauerstoffmangel) und Mischungen zweier Chemikalien auf das Verhalten, die Entwicklung und die Enzymaktivität von Embryonen und Larven des Zebrabärblings auswirken. Hierbei sollte der Parameter Verhalten im Bezug auf die Sensitivität mit entwicklungsbiologischen und biochemischen Größen verglichen und in Beziehung gebracht werden. Im Bezug auf die Bewegungsaktivität wurde bei akuter Exposition eine Erhöhung der Aktivität erwartet, was auf eine Vermeidungsreaktion hindeutet. Durch die Einbeziehung von Mischungen, sowohl von zwei Chemikalien mit ähnlichen bzw. unterschiedlichen Wirkmechanismen als auch von Einzelchemikalien mit Sauerstoffmangel, sollten umweltrelevante Expositionsszenarien getestet werden und die Konzepte der Konzentrationsadditivität und der unabhängigen Wirkung überprüft werden. Bei zusätzlichem Umweltstress wurde eine Erhöhung der Toxizität erwartet. Die Enzymaktivität der Acetylcholinesterase sollte sich mit zunehmendem Alter der Zebrabärblinge erhöhen; bei Schadstoffexposition wurde hierdurch eine verstärkte Hemmung erwartet. Zudem wurde angenommen, dass eine Hemmung des Enzyms Acetlycholinesterase zu einer Beeinträchtigung des Verhaltens bei juvenilen Zebrabärblingen führt.

Im zweiten Teil der Arbeit sollte mit der Untersuchung der Auswirkungen eines Umweltschadstoffs auf Räuber-Beute-Beziehungen zwischen Zebrabärblingen und Zuckmückenlarven eine Verbindung zu Vorgängen auf höheren biologischen Organisationsebenen geschaffen werden. Die Zielsetzungen hierbei waren zum einen die Entwicklung eines einfachen und leicht durchzuführenden Testsystems, mit dem solch komplexe Interaktionen untersucht werden können und zum anderen die

Dokumentation des Einflusses eines neurotoxischen Insektizides (Chlorpyrifos) auf die Räuber-Beute-Beziehungen. Es wurde postuliert, dass sich exponierte Chironomiden weniger vergraben als Kontrolltiere und daher anfälliger für Prädation durch Fische sind. Eine Verstärkung des Eingrabverhaltens sowohl von exponierten als auch von Kontroll-Tieren wurde bei Prädation durch Fische erwartet. Bei Exposition von Räuber- und Beuteorganismen sollten sich die verringerte Fähigkeit des Räubers die Beute zu erkennen und die der Beute sich zu vergraben aufheben und somit zu keinen deutlichen Unterschieden in der Fressrate im Vergleich zur Kontrolle führen.
Detaillierte Arbeitshypothesen finden jeweils sich in der Einleitung der einzelnen Kapitel (Kapitel 1-5).

Ziel des dritten Teils der Arbeit war die Untersuchung der Auswirkungen von Chemikalien in umweltrelevanten Szenarien mit zwei wichtigen Invertebraten-Spezies *(Corophium volutator* und *Gammarus pulex)*. Hierbei sollten in der ersten Studie die Effekte einer umweltrelevanten komplexen Mischung von Chemikalien auf das Verhalten von *C. volutator*, ebenso wie das Potential der Regeneration dieser Organismen untersucht werden. In der zweiten Studie dieses Teils sollte zum einen die Eignung von *G. pulex* als Organismus für die kontinuierliche Gewässerüberwachung überprüft werden. Zudem wurde ein Vergleich der Verhaltens- und Mortalitätsdaten von *G. pulex* aus dem biologischen Monitoring mit Daten aus dem chemischen und weiterem biologischen Monitoring: der Überwachung der Fluoreszenz von einzelligen Grünalgen (Fluotox, www.arnatronic.com) und der Veränderung im Schließverhalten von Muscheln (Mosselmonitor®, www.mosselmonitor.nl) angestrebt. Hierbei sollte ermittelt werden, ob und, wenn ja, wie schnell *G. pulex* auf die Präsenz von Schadstoffen im Flusswasser mit einer Veränderung seines Verhaltens reagiert und ob es empfehlenswert ist, diesen Parameter für die kontinuierliche Gewässerüberwachung einzusetzen.

3. Material und Methoden

3.1 Testorganismen

Die in Kapitel 1-4 beschriebenen Experimente wurden mit Embryonen und Larven des Zebrabärblings (*Danio rerio*) durchgeführt. Die Versuche hierzu erfolgten in Labors der Abteilung Physiologische Ökologie der Tiere, Universität Tübingen mit Eiern des Wildtypstamms WIK, ZFIN ID: ZDB-GENO-010531-2). Zu diesem Zweck wurden die Embryonen und Larven in Glaspetrischalen im Klimaschrank bei 26±1°C und einem 12:12h Licht-Dunkel-Rhythmus entweder (1) in Kunstwasser bis zum Alter von 5 Tagen aufgezogen, um sie anschließend akut gegenüber verschiedenen Umweltschadstoffen einzeln und in Mischungen zu exponieren, oder (2) von Befruchtung an bis zu einem Alter von 11 Tagen gegenüber Einzelstoffen oder binären Mischungen exponiert. In diesem Zeitraum wurden regelmäßig verschiedene entwicklungsrelevante Parameter, wie die Schlupfrate, morphologische Veränderungen und Mortalität, beobachtet und protokolliert. Ebenso wurden im Alter von 5, 8 und 11 Tagen Larven für Verhaltensuntersuchungen (siehe 3.2) entnommen.

Die Expositionen für die in Kapitel 4 beschriebenen biochemischen Messungen fanden unter den gleichen Bedingungen statt, hier wurden Embryonen und Larven im Alter von 2, 5 und 8 Tagen nach Befruchtung entnommen und auf Veränderungen in der Aktivität des Enzyms Acetylcholinesterase hin untersucht.

Die in Kapitel 5 beschriebenen Experimente wurden mit Larven von *C. riparius* im 4. Larvenstadium (L4) und mit adulten Zebrabärblingen des Wildtyp-Stammes WIK (ZFIN ID: ZDB-GENO-010531-2 bzw. Tue. G14) durchgeführt. Die Haltung und Exposition erfolgte in einer Klimakammer bei 25±1°C und einem 12:12h Licht-Dunkel-Rhythmus. Die Expositionsdauer war jeweils 2 h, anschließend wurde das Räuber-Beute-Verhalten in Kontrollwasser untersucht.

Für die Tests in Kapitel 6 wurden Schlickkrebse (*C. volutator*) im Freiland im Avon Aestuar nahe Aveton Gifford, South Devon UK, gesammelt und anschließend in Laboratorien der Universität Plymouth (UK) nach der Größe aufgetrennt in 5 L Hälterungsbecken mit 25±1 ‰ Salzwasser und gesiebtem Freiland-Sediment (Korngrösse <300 µm) gegeben. Die Akklimatisationszeit betrug 1 Woche bei Standardbedingungen von 15±1°C und einem 12:12 h Licht-Dunkel-Rhythmus. Die Exposition gegenüber der WAF erfolgte akut über eine Dauer von 2 h.

Gammarus pulex in Kapitel 7 wurde einige Tage vor Beginn der Exposition aus dem Freiland (Kander, Deutschland) gesammelt und im Anschluss mehrere Tage in Bachwasser an die Bedingungen der Monitoringstation in Hunigue (Frankreich) akklimatisiert.

3.2 Entwicklungsbiologische und Verhaltens-Untersuchungen mit *Danio rerio*

Akute Tests

Für die in Kapitel 1 und 2 beschriebenen akuten Verhaltenstests wurden je Schadstoffkonzentration 12 Larven im Alter von fünf Tagen einzeln in MFB-Messkammern exponiert und die Bewegungsaktivität nach einer Akklimatisationszeit von 10 min über eine Dauer von 2 h vom *Multispecies Freshwater Biomonitor* aufgezeichnet (siehe auch Abschnitt 2.3 und 2.4 in Kapitel 1). Je Chemikalie wurden acht Schadstoffkonzentrationen und zwei Negativkontrollen getestet. Der gleiche Versuchsaufbau wurde auch für die Untersuchung der Auswirkungen unterschiedlicher Sauerstoffkonzentrationen und von Mischungen aus zwei Substanzen bzw. aus einer Substanz mit unterschiedlichen Sauerstoffkonzentrationen verwendet. Getestet wurden Nickelchlorid, Chlorpyrifos und Nickelchlorid plus Chlorpyrifos, ebenso wie Kombinationen aus Nickelchlorid mit verschiedenen Sauerstoffkonzentrationen. Die unterschiedlichen Sauerstoffkonzentrationen wurden mittels Belüftung durch Stickstoff, der den Sauerstoff verdrängt, hergestellt. Um den Sauerstoffgehalt über die Messzeit konstant zu halten, wurden die Testgefässe in ein umgebendes Glasaquarium gestellt, aus dem durch Stickstoffbelüftung der Sauerstoff teilweise entfernt wurde. Dieses Aquarium wurde während der Messzeit von zwei Stunden dicht abgeschlossen um so den verringerten Sauerstoffgehalt über die Messzeit konstant zu halten (siehe Abb. 1 in Kapitel 1).

Subchronische Tests

Die subchronischen Tests in den Kapiteln 1-4 wurden nach dem Protokoll „*Fish, Short-Term Toxicity Test on Embryo and Sac-Fry Stages*“ (Veterinary Medicines Directorate, 1996) durchgeführt. Die Exposition erfolgte von Befruchtung an bis zum Alter von 11 d. Die Eier der Zebrabärblinge wurden mit Hilfe von Laichboxen, in denen ein für die Eier durchlässiges Sieb, ebenso wie grüne Laichwatte und ein Stein platziert waren, gewonnen. Die Weibchen laichten am Morgen, nach Anschalten des Lichtes (Trigger für

Laichverhalten) über den Laichboxen ab, die Eier fielen durch das Sieb und konnten anschließend (nach ca. 1 h) abgesiebt werden. Die gewonnenen Eier wurden zufällig auf Petrischalen mit den jeweiligen Schadstoffkonzentrationen (pro Substanz jeweils 5 Konzentrationen) bzw. der Negativkontrolle verteilt. Nach wenigen Stunden erfolgte eine Trennung der befruchteten von den unbefruchteten Eiern. Je Konzentration wurden drei Replikate getestet. Untersucht wurden die Auswirkungen von Nickelchlorid, Chlorpyrifos, Diazinon und 3,4-Dichloranilin, ebenso wie von binären Mischungen aus Nickelchlorid plus Chlorpyrifos, Diazinon plus Chlorpyrifos und Diazinon plus 3,4-Dichloranilin. Die Embryonal- bzw. Larvalentwicklung wurde täglich in einem 24-stündigen Intervall beobachtet und Abweichungen von der normalen Entwicklung, wie z.B. Ödeme und Deformationen, notiert. Des Weiteren wurden die Schlupfrate und die Anzahl toter Embryonen bzw. Larven bestimmt. Ergänzt wurde dies durch jeweils zweistündige Messungen der Bewegungsaktivität im MFB in regelmäßigen Abständen (im Alter von 5, 8 und 11 Tagen), die wie im Abschnitt „akute Tests“ beschrieben durchgeführt wurden.

3.3 Biochemische Untersuchungen mit *Danio rerio*

Für die Analyse der Acetylcholinesteraseaktivität (Kapitel 4) wurden jeweils 8 Replikate pro Schadstoffkonzentration bzw. Kontrolle á 20 Embryonen im Alter von 2 Tagen nach Befruchtung bzw. á 10 Larven im Alter von 5 bzw. 8 Tagen nach Befruchtung für die weitere Untersuchung in flüssigem Stickstoff schockgefroren. Alle Proben wurden anschließend wie in Küster (2005) beschrieben analysiert. Die Embryonen bzw. Larven wurden in einer jeweils adäquaten Menge an Phosphatpuffer homogenisiert und anschließend zentrifugiert. Der Gesamtproteingehalt des Überstandes wurde nach Lowry et al. (1951) quantifiziert. Die Aktivität der Acetylcholinesterase wurde nach einem Protokoll von Ellman et al. (1961), das von Küster (2005) für Zebrabärblingsembryonen modifiziert wurde, photometrisch durch den Umsatz von S-Phenylthioacetat mit DTNB (5,5-Dithiobis-(2-Nitro-Benzoesäure)) als chromogenes Reagens quantifiziert. Die prozentuale Hemmung der Acetylcholinesterase wurde anhand des prozentualen Anteils der Enzymaktivität exponierter Tiere an der Aktivität der Kontrolltiere dargestellt.

3.4 Untersuchung von Räuber-Beute-Beziehungen zwischen *Danio rerio* und *Chironomus riparius*

Zur Untersuchung des Räuber-Beute-Verhaltens (Kapitel 5) wurden die Larven von *C. riparius* und adulte Zebrabärblinge akut, jeweils 2 h, gegenüber Chlorpyrifos in zwei Konzentrationsstufen (1 und 6 µg/L) exponiert. Hierbei wurden eine Kontrolle und drei Ansätze mit dem Schadstoff getestet: (1) nur Chironomiden kontaminiert (Cc), (2) nur Fische kontaminiert (Dc) und (3) beide Interaktionspartner (Chironomiden und Fische) exponiert (Bc). Die zweistündige Exposition der Chironomiden erfolgte in Glasschalen mit der jeweiligen Schadstofflösung und Sediment, anschließend wurden die Tiere in die Testaquarien (10 L, mit ca. 2 cm Sediment und Wasser befüllt) gegeben. Dieser Zeitraum wurde in Vorversuchen ermittelt und war nötig um den Chironomiden ein natürliches Eingraben zu ermöglichen. Die Fische wurden direkt nach dem Einsetzen der Chironomiden in die Testaquarien für 2 h in 3 L Aquarien gegenüber Chlorpyrifos exponiert. Im Anschluss wurden sie in die jeweiligen Testaquarien eingesetzt. Vor und nach Einsetzen der Fische erfolgte eine Zählung der Chironomiden an der Oberfläche und der teilweise bzw. vollständig eingegrabenen Individuen. Nach einer 2stündigen Nahrungssuchzeit, wurden die Fische wieder aus den Aquarien entnommen und mit Benzocain betäubt. Nach Trockentupfen wurde ihre Länge bestimmt und die Tiere zur weiteren Analyse in Eppendorf-Gefäßen in flüssigem Stickstoff schockgefroren (1 Fisch/Gefäß). Der Anteil von Chironomiden die überhaupt nicht, teilweise oder ganz eingegraben waren, wurde erneut bestimmt. Nachdem die noch verbliebenen Chironomiden gezählt worden waren, wurden sie wie die Zebrabärblinge schockgefroren (5 Tiere/Gefäß).

3.5 Verhaltensuntersuchungen mit *Corophium volutator*

Für die in Kapitel 6 beschriebenen Verhaltensuntersuchungen wurden juvenile *C. volutator* jeweils einzeln in Messkammern in 100 ml Bechergläsern gegenüber verschiedenen Verdünnungen der *water accommodated fraction* (WAF) von verwittertem Rohöl ausgesetzt, ebenso wie gegenüber mit 100 % WAF kontaminiertem Sediment. Anschließend wurde die Bewegungsaktivität akut über 2 h gemessen. In einem Experiment mit Kurzzeitexposition und einer anschließenden Erholungsphase wurde der WAF-Anteil von 50 % über 25 % sukzessive erniedrigt und schließlich

vollkommen mit 25±1 ‰ Salzwasser (Kontrollmedium) ersetzt. Die Bewegungsaktivität der Amphipoden wurde hier insgesamt über 20 h aufgezeichnet. Zusätzlich wurden visuelle Beobachtungen notiert und die Mortalität bestimmt.

3.6 Gewässerüberwachung mit *Gammarus pulex*

Zur kontinuierlichen Überwachung der Gewässerqualität des Rheins (Kapitel 7) wurde jeweils ein Individuum von *Gammarus pulex* in eine Messkammer des MFB eingesetzt, die in einem von Rheinwasser durchströmten Durchflussaquarium platziert wurde. Die Kammer enthielt ein Stück Laub aus dem Herkunftsgewässer der Tiere als Schutz und Nahrung. Die Messung der Bewegungsaktivität erfolgte kontinuierlich über einen Zeitraum von 6 Wochen. In bestimmten Zeitabständen wurde das Überleben der Tiere überprüft und tote Organismen ersetzt. Die Auswertung der Daten erfolgte durch die Analyse der Bewegungs- und Ventilationsaktivität über die Zeit. Chemische und biologische Auffälligkeiten aus einer kontinuierlichen Überwachung von physikalisch-chemischen Parametern (Temperatur, pH, Sauerstoffgehalt, Leitfähigkeit), des Gehalts an Spurenmetallen, wie z.B. Kupfer, Cadmium und Blei und des Gehalts an organischem Kohlenstoff (*total organic carbon*, TOC), sowie Veränderungen in der Fluoreszenz von einzelligen Grünalgen (Fluotox) und Veränderungen im Verhalten von Muscheln beim Schließen und Öffnen der Schalen (Mosselmonitor), wurden mit den entsprechenden Verhaltensdaten verglichen und auf Übereinstimmungen überprüft.

4. Ergebnisse und Diskussion

4.1 Toxizität von Einzelsubstanzen und binären Mischungen auf *Danio rerio* Embryonen und Larven in Kombination mit Sauerstoffmangel

Kapitel 1: *Kienle C, Köhler H-R, Filser J, Gerhardt A (2008): Effects of nickel chloride and oxygen depletion on behaviour and vitality of zebrafish Danio rerio (Hamilton, 1822) (Pisces, Cypriniformes) embryos and larvae. Environmental Pollution, 153(3): 612-620.*

Um die Auswirkungen des Schwermetalls Nickel alleine und in Kombination mit Sauerstoffmangel als zusätzlichem Umweltstress auf frühe Lebensstadien des Zebrabärblings zu untersuchen, wurden akute und subchronische Tests durchgeführt. Diese Studie zeigte eine signifikante Verringerung der Bewegungsaktivität von *D. rerio* Larven bei akuter Exposition gegenüber Nickelchlorid (7,5 - 15 mg Ni/L). Sauerstoffmangel (≤2,45 ± 0,16 mg O_2/L) führte ebenfalls zu einer signifikant verminderten Bewegungsaktivität. Im subchronischen Test wiesen die gegenüber ≥10 mg Ni/L exponierten Tiere im Alter von 96 h eine im Vergleich zur Kontrolle verringerte Schlupfrate auf. Im Alter von 5 Tagen war in diesem Konzentrationsbereich die Bewegungsaktivität ebenfalls reduziert. Bei 11 Tage alten Larven, die gegenüber Nickelchlorid exponiert waren, trat verstärkt Mortalität auf. Die LC_{20}, d.h. die Konzentration bei der eine Mortalität von 20 % auftrat, lag hier bei 9,5 mg Ni/L.
Die beobachtete LOEC von 7,5 mg/L liegt im Bereich umweltrelevanter Konzentrationen (0,05-2 mg Ni/L in natürlichen Gewässern in der Nähe von Industrieanlagen und 183 mg/L in der Nähe einer Nickel-Raffinerie (Chau und Kulikovsky-Cordeiro, 1995; Kasprzak, 1987). In der Natur könnte eine Verringerung der Bewegungsaktivität von Fischen zu einer Erhöhung der Drift und/oder des Prädationsrisikos führen, daher stellt die Bewegungsaktivität einen ökologisch relevanten Parameter für die Gesundheit und das Überleben der Art dar.
In Mischungen von hohen Nickel- mit geringen Sauerstoffkonzentrationen lösten die kombinierten Stressoren vermutlich eine Vermeidungs-/Fluchtreaktion aus, was durch die erhöhte Bewegungsaktivität im Vergleich zu den einzelnen Stressoren widergespiegelt wird. Die Verringerung des pH-Wertes um 0,4 bei geringen Sauerstoffkonzentrationen sollte die Toxizität von Nickel ebenso wie die Zebrabärblingslarven nicht stark beeinflussen. In Studien mit Dickkopfelritzen

(*Pimephales promelas*) (Hoang et al., 2004) führte ein Anstieg des pH-Wertes von 7.97 auf 8.54 nur zu einer leichten Erhöhung des 96h LC_{50} von 1.75 auf 1.80 mg Ni/L (bei einer Wasserhärte von 100 mg/L (als $CaCO_3$)).

Die verringerte Schlupfrate lässt sich vermutlich auf eine Hemmung des Schlupfenzyms Chorionase zurückführen (Hagenmaier, 1974) und wurde auch in anderen Studien beobachtet z.B. bei Zebrabärblingen (45 µg/L (Geometrisches Mittel von NOEC und LOEC, GM NOEC-LOEC), 6 mg Ni/L (LOEC) im Alter von 96 h) (Dave und Xiu, 1991; Grabner, 2005) oder Karpfen (*Cyprinus carpio*) (6 mg Ni/L) (Blaylock und Frank, 1979).

Ein Grund für die relativ geringe Toxizität von Nickel für *D. rerio* Larven in der vorliegenden Studie könnte die geringe Bioverfügbarkeit von Nickel bei der recht hohen Wasserhärte (13°dH; 231,4 mg/L as $CaCO_3$) und dem hohen pH (~ 8,0) des verwendeten Kunstwassers sein. Wie auch für viele andere Metalle nachgewiesen nimmt die Toxizität von Nickel für aquatische Organismen mit zunehmender Wasserhärte ab (Hoang et al., 2004; Pyle et al., 2002), was durch verminderte Bioverfügbarkeit erklärt werden kann. Als Wirkungsmechanismus für die Toxizität von Nickel wird in mehreren Veröffentlichungen eher ein respiratorischer als ein ionenregulatorischer Wirkmechanismus angegeben (Brix et al., 2004; Pane et al., 2003).

Da die Bewegungsaktivität bereits bei akuter Exposition beeinträchtigt war, wird empfohlen, diesen Parameter als Ergänzung zu sonst üblichen Endpunkten für Toxizität zu verwenden. Bisher wurden ökotoxikologische Studien, die auf Verhaltensparametern basierten, hauptsächlich mit adulten Fischen durchgeführt. Die vorliegende Studie hat gezeigt, dass solche Tests auch für Fischlarven geeignet sind. Das Verhalten erwies sich im Bezug auf die Expositionszeit und die Effektkonzentration als sensitiver als der konventionelle Parameter Mortalität. Zudem sind auch aus ethischen Gründen ein verminderter Einsatz von adulten Fischen und eine Erfassung sublethaler Parameter wünschenswert.

Kapitel 2: *Kienle C, Köhler, H.-R., Gerhardt A. (in press): Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (Danio rerio) embryos and larvae. Ecotoxicology and Environmental Safety, doi:10.1016/j.ecoenv.2009.04.014.*

Um die Mischungstoxizität von Umweltchemikalien mit unterschiedlichen Wirkungsmechanismen zu untersuchen, wurden in dieser Studie die Auswirkungen des neurotoxischen Insektizids Chlorpyrifos und des allgemein toxischen Metalls Nickel auf Embryonen und Larven des Zebrabärblings untersucht. Chlorpyrifos bewirkte bei akuter Exposition einen tendenziellen Anstieg der Bewegungsaktivität bei ≥0,25 mg/L, wohingegen die Aktivität ab ≥7,5 mg Ni/L signifikant vermindert war. Wurden die Zebrabärblinge subchronisch gegenüber Chlorpyrifos (CHP) exponiert, traten Verhaltenseffekte bei viel geringeren Konzentrationen (≥0,01 mg/L) und bedeutend früher (bereits nach 5 d Exposition) als ein erhöhter Anteil an morphologischen Veränderungen und Mortalität auf. Die LC_{50} von Chlorpyrifos lag bei 10 d alten Zebrabärblingen bei 0,43 mg/L, die LC_{20} von Nickelchlorid bei 9,5 mg Ni/L. Binäre Mischungen von CHP und $NiCl_2$ bewirkten im Bezug auf die Bewegungsaktivität eine antagonistische Abweichung vom Konzept der unabhängigen Wirkung, wie durch Analyse mit dem MixTox-Modell (Jonker et al., 2005) ermittelt werden konnte.

In dieser Studie wurden erstmals die Auswirkungen der Kombination eines Schwermetalls und eines Pestizids auf Fische untersucht. Die beobachtete Erhöhung der Bewegungsaktivität deutet vermutlich nicht auf eine Vermeidungsreaktion hin, sondern ist stattdessen eher mit den Auswirkungen von Chlorpyrifos auf das Nervensystem zu erklären, wodurch auch Muskelkrämpfe ausgelöst werden (Kamrin, 1997), wie nach akuter Exposition beobachtet wurde. Insgesamt erwies sich Chlorpyrifos als deutlich toxischer als Nickel (Effekte von Chlorpyrifos bei jeweils ≥0,25 mg/L und ≥0,01 mg/L in akuten und subchronischen Tests bzw. von Ni bei ≥7,5-10 mg/L). Aufgrund der unterschiedlichen Wirkmechanismen (CHP: AChE Hemmstoff und $NiCl_2$: Beeinträchtigung der Enzymfunktionen und von respiratorischen Mechanismen) wirken beide Substanzen vermutlich unabhängig voneinander. Zu den Auswirkungen von Mischungen aus Metallen und Pestiziden gegenüber Fischen existiert bislang keine publizierte Literatur. Eine Studie mit *Tigriopus brevicornis* (Copepoda) konnte Synergismus für verschiedene Kombinationen von Metallen und Pestiziden (Kupfer bzw. Cadmium in Kombination mit Malathion bzw. Dichlorvos) nachweisen (Forget et al.,

1999). Insgesamt zeigte der Parameter Bewegungsaktivität bei Exposition gegenüber dem Acetylcholinesterasehemmstoff Chlorpyrifos in dieser Studie die höchste Empfindlichkeit im Vergleich zu entwicklungsbiologischen Parametern oder Mortalität. Die Auswirkungen von Nickel und Chlorpyrifos auf die untersuchten Endpunkte Bewegungsaktivität, Deformationen und Mortalität waren im subchronischen Test abhängig von der Expositionszeit. Bei akuter Exposition bewirkten die beiden Parameter unterschiedliche Verhaltensantworten. Unsere Ergebnisse zeigen, dass es von der untersuchten Substanz und deren jeweiligem Wirkmechanismus abhängt, welcher Endpunkt zuerst und in welchen Konzentrationsbereichen und Mischungsverhältnissen reagiert.

Im Bezug auf die Umweltrelevanz kann das akute Risiko von Chlorpyrifos für Zebrabärblinge vermutlich als gering angesehen werden. Langfristige Auswirkungen sind jedoch hochrelevant, da in Studien eine subchronische Exposition von *D. rerio* gegenüber Chloryprifos im Embryonal- und Larvalstadium das Verhalten bis ins Adultstadium beeinflussen konnte (Levin et al., 2003, 2004). Bei Berücksichtigung von sensitiveren Arten, wie z.B. der Regenbogenforelle und gemessenen Umweltkonzentrationen von 0,3 µg/L (Gilliom et al., 2006) kann eine Gefährdung einheimischer Fische nicht ausgeschlossen werden.

Kapitel 3: *Scheil V*, Kienle C*, Osterauer R, Gerhardt A, Köhler H-R (2009): Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (Danio rerio) embryos and larvae. Ecotoxicology, 18(3): 355-363.*

*beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.

Die Auswirkungen der polar-narkotisch wirkenden Substanz 3,4-Dichloranilin (3,4-DCA), einem Abbauprodukt verschiedener Herbizide (u.a. Propanil und Diuron) und des Acetylcholinesterasehemmstoffs Diazinon, einem neurotoxischen Insektizid, auf frühe Lebensstadien von Zebrabärblingen (*Danio rerio*) waren Gegenstand dieser Studie. Zur Beurteilung der Auswirkungen auf verschiedene biologische Organisationsebenen (von der molekularen Ebene bis hin zum Gesamtorganismus), wurden die molekulare Stressantwort in Bezug auf Hsp70, die Embryonal- und Larvalentwicklung und die Bewegungsaktivität als integrative Biomarker untersucht (Gegenstand der Dissertation sind die subchronischen Tests über 11 Tage mit Verhaltensuntersuchungen zu Diazinon, 3,4-DCA und binären Mischungen. Die Stressproteinanalysen (Hsp70) und die Embryotests wurden von Dr. V. Scheil bzw. R. Osterauer durchgeführt).

3,4-Dichloranilin führte im subchronischen Test ab einer Konzentration von 0,25 mg/L zu einer erhöhten Deformationsrate bei der Individualentwicklung. Die Bewegungsaktivität und die Mortalität waren ab 0,5 mg/L beeinträchtigt. Auswirkungen von Diazinon auf diese Parameter konnten ab 2 mg/L beobachtet werden, mit Ausnahme der Deformationsrate, die bei 11 Tage alten Zebrabärblingen ab 1 mg/L signifikant erhöht war. In äquitoxischen Mischungen wirkten beide Substanzen im Bezug auf die Deformationsrate und Mortalität im Alter von 11 Tagen additiv. Ein erhöhter Hsp70-Gehalt trat bei Zebrabärblingen, die gegenüber 0,25 mg 3,4-DCA/L exponiert waren, ebenso wie bei jenen, die gegenüber 0,05 mg Diazinon/L exponiert waren, auf. Auch hier konnte in Mischungen Additivität nachgewiesen werden, antagonistische oder synergistische Effekte, die eine gegenseitige Beeinflussung der beiden Substanzen indizieren würden, traten nicht auf. Insgesamt führten beide Substanzen, ebenso wie die binären Mischungen zu schwerwiegenden Schädigungen bei Embryonen und Larven von *D. rerio*.

Die niedrigsten Effektkonzentrationen (LOECs) lagen bei Diazinon mindestens 33fach und bei 3,4-DCA mindestens 167fach über den gemessenen Umweltkonzentrationen von jeweils 1,5 µg/L (Bailey et al., 2000; Planas et al., 2006), Effekte von Mischungen lagen

ebenfalls über dem umweltrelevanten Konzentrationsbereich. Bei Berücksichtigung von sensitiveren Arten wie z.B. adulten Regenbogenforellen, die 4,5-6 fach geringere 96 h LC_{50} Werte aufwiesen als adulte Zebrabärblinge (Becker et al., 1990; Hodson, 1985; Keizer et al., 1993; Meier et al., 1979), zeitweise hohen Konzentrationen in räumlichen *Hotspots* und chronischer Exposition im Freiland kann eine Umweltrelevanz jedoch nicht ausgeschlossen werden.

Die vorliegende Studie zeigte mit Hilfe eines mehrstufigen Ansatzes, dass verschiedene Endpunkte je nach Chemikalie mit unterschiedlicher Sensitivität reagieren können. Der Hsp70-Gehalt und Deformationen waren im Bezug auf 3,4-DCA die sensitivsten Parameter, bei Diazinon reagierte der Hsp70-Gehalt am empfindlichsten. Daher sollte zum besseren Verständnis der Auswirkungen von Chemikalien und ihrer Mischungen eine Batterie verschiedener Testmethoden angewendet werden. Aufgrund von Unsicherheiten in der Vorhersage, welcher Endpunkt von einer bestimmten Substanz beeinflusst werden könnte, erscheint es sinnvoll, eine angemessene Anzahl von Endpunkten zu verwenden. Diese sollten bei einem solchen Ansatz idealerweise unterschiedlicher Art sein und verschiedene biologische Organisationsebenen umfassen.

Kapitel 4: *Kienle C, Küster E, Gerhardt A., Köhler H-R (submitted): Linking behaviour to acetylcholinesterase inhibition in embryos and larvae of zebrafish (Danio rerio) exposed to pesticides. Aquatic Toxicology.*

Um Änderungen im Verhalten in Beziehung zu suborganismischen Veränderungen zu setzen, wurden die Auswirkungen zweier Insektizide mit dem gleichen Wirkmechanismus auf Verhalten, Enzymaktivität, Deformationen und Mortalität von Embryonen und Larven des Zebrabärblings in subchronischen Tests über 11 Tage untersucht. Die neurotoxischen Insektizide Diazinon und Chlorpyrifos (CHP) hemmen beide das Enzym Acetylcholinesterase und wurden in Oberflächengewässern häufig in Mischungen gefunden (Gilliom et al., 2006).

Die Aktivität der Acetylcholinesterase (AChE) nahm mit dem Alter der Zebrabärblinge signifikant zu. Die deutlichsten Auswirkungen beider Pestizide auf die Enzymaktivität traten im Alter von 120 und 196 h auf. Die Acetylcholinesteraseaktivität wurde hier bereits ab 10 µg CHP/L signifikant gehemmt. Bei der gleichen Konzentration konnten auch Auswirkungen auf die Bewegungsaktivität gemessen werden. Effekte auf diese beiden Parameter traten somit bei deutlich geringeren Konzentrationen als morphologische Veränderungen und Mortalität (jeweils 0,25 und 0,5 mg/L) auf. Diazinon war im Bezug auf Verhaltensänderungen und die Enzymhemmung deutlich weniger toxisch, obwohl beide Substanzen, aufgrund ihrer spezifischen Wirkungsweise als Acetylcholinesterase-Hemmstoffe, das Verhalten voraussichtlich beeinträchtigen. Bei binären Mischungen trat bei allen beobachteten Parametern Konzentrationsadditivität auf.

Die niedrigste Effektkonzentration bezüglich Enzymaktivität und Bewegungsaktivität lagen mit 10 µg CHP/L nur wenig über in der Umwelt gemessenen Konzentrationen von 0,3 µg CHP/L (Gilliom et al., 2006). Bei einer, z.B. in REACH vorgeschriebenen, Anwendung eines Sicherheitsfaktors von 100 (beim Vorliegen eines chronischen NOECs) oder 1000 (beim Vorliegen von akuten Tests) zur Abschätzung eines Risikos für aquatische Organismen könnte Chlorpyrifos daher ein deutliches Risiko für Fische in der Umwelt darstellen. Das Verhältnis einer PEC (Predicted Environmental Concentration) von 0,3 µg/L zur PNEC (Predicted No Effect Concentration) (10 µg/L/100) läge hier bei 3. Ein Risiko wird bei PEC/PNEC-Verhältnissen >1 angenommen. Daher sollte Chlorpyrifos weiter beobachtet werden. Das Risiko für Fische durch Exposition gegenüber Diazinon ist vermutlich geringer, da die Effektkonzentrationen in der

vorliegenden Studie relativ hoch lagen.

Die Wirkung von Chlorpyrifos und Diazinon als Acetylcholinesterasehemmstoffe auf das Nervensystem (Kamrin, 1997) lässt auf einen engen Zusammenhang zwischen der Enzymaktivität und dem Verhalten schließen, was durch die gleichen Effektkonzentrationen für Chlorpyrifos bestätigt wurde. In Studien mit Silberlachsen (*Oncorhynchus kisutch*) konnte bei Exposition gegenüber Chlorpyrifos (0,6 - 2,5 µg/L) ebenfalls ein Zusammenhang zwischen der Hemmung der Acetylcholinesterase und Verhaltensbeeinträchtigungen gefunden werden (Sandahl et al., 2005). Ein Grund für die geringe Toxizität von Diazinon könnten Unterschiede im log K_{ow} sein (Literaturwerte für Diazinon: 3,02 (Suntio et al., 1988) bzw. 3,81 (Ladaa et al., 1998) und Chlorpyrifos: 4,99 (Kamrin, 1997) bzw. 5,11 (Ladaa et al., 1998), was in einer verstärkten Aufnahme von Chlorpyrifos ebenso wie einer langsameren Elimination und einer verstärkten Bioakkumulation resultieren könnte.

Die vorliegende Studie zeigt somit, dass die Insektizide Chlorpyrifos und Diazinon, bezogen auf die Effektkonzentrationen, trotz ihres gleichen Wirkmechanismus' unterschiedliche Auswirkungen auf Embryonen und Larven von Zebrabärblingen haben können, sowohl auf organismischer als auch auf suborganismischer Ebene. Mischungen beider Substanzen zeigten, wie erwartet, Konzentationsadditivität für die Parameter Acetlycholinesterasehemmung, Bewegungsaktivität, Anteil morphologischer Deformationen und Mortalität. Im Bezug auf die Risikoabschätzung für die untersuchten Substanzen bedeutet das auch, dass die Wirkungen von Konzentrationen, bei denen keine Effekte der einzelnen Stoffe festgestellt wurden, sich in Mischungen zu toxischen Effekten addieren können.

Die Ergebnisse dieser Studie zeigen, dass das Verhalten von Zebrabärblingen gut mit dem Parameter Acetylcholinesteraseaktivität korreliert werden kann. Die Evaluation des Endpunktes ‚Acetylcholinesteraseaktivität' in mehreren Studien mit Embryonen und Larven des Zebrabärblings (Küster, 2005; Küster und Altenburger, 2006, 2007), sowie die vorliegende Studie machen deutlich, dass die Acetylcholinesteraseaktivität als sinnvoller und verlässlicher wirkmechanismus-basierter *biomarker of exposure* von Embryonen und Larven des Zebrabärblings gegenüber neurotoxischen Pestiziden dienen kann. Die zusätzliche Integration von Verhaltensveränderungen als *biomarker of effect* sollte angestrebt werden, um neben der Exposition auch deren Auswirkungen auf Organismenebene quantifizieren zu können.

4.2 Auswirkungen akuter Schadstoffexposition auf höhere biologische Organisationsebenen (Räuber-Beute-Beziehungen)

Kapitel 5 *Kienle C*, Langer ME*, Gerhardt A, Köhler H-R (unpublished manuscript): Impairment of trophic interactions between zebrafish (Danio rerio) and midge larvae (Chironomus riparius) by chlorpyrifos.*

*beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.

Umweltschadstoffe wirken sich nicht nur auf den Organismus selbst aus, sondern können auch seine Interaktionen mit anderen Organismen beeinflussen. In der vorliegenden Studie wurde eine natürlich vorkommende Räuber-Beute-Interaktion zwischen Vertretern zweier trophischer Ebenen, Zuckmücken-Larven (Chironomiden), als Primär-Konsumenten und Detritusfresser am Beispiel der Art *Chironomus riparius* und Fischen als Sekundärkonsumenten am Beispiel von Zebrabärblingen (*Danio rerio*) untersucht. Die Testorganismen wurden akut, jeweils 2 h, gegenüber Chlorpyrifos in zwei Konzentrationsstufen (1 und 6 µg/L) exponiert. Das hierzu verwendete Versuchsdesign umfasste vier Ansätze: (1) Exposition der Räuber (*D. rerio*), (2) Exposition der Beuteorganismen (*C. riparius*), (3) Exposition beider Interaktionspartner und (4) Kontrolle. Es konnten Unterschiede in der Fressrate der Zebrabärblinge von exponierten Chironomiden nach akuter, zweistündiger Exposition gegenüber 6 µg CHP/L nachgewiesen werden. Ebenso zeigten sich Unterschiede im Eingrabverhalten der Chironomiden; hier gruben sich exponierte Chironomiden signifikant seltener ein als Kontrolltiere. Bei Exposition von Räubern und Beuteorganismen konnten keine signifikanten Unterschiede in der Fressrate der Zebrabärblinge beobachtet werden. Eine geringere Konzentration von 1 µg CHP/L rief ebenso in keinem der Ansätze Unterschiede in der Fressrate der Zebrabärblinge und im Eingrabverhalten der Chironomiden hervor.

Die Untersuchungen von Räuber-Beute-Interaktionen waren bisher meist auf einen der beiden Interaktionspartner fokussiert, entweder auf die Beute (z.B. Baker und Ball, 1995; Hölker und Stief, 2005; Schulz und Dabrowski, 2001) oder auf den Räuber (z.B. Fjeld et al., 1998; Grippo und Heath, 2003; Power, 1990). Studien die, wie von Lima (2002) angeregt, beide Interaktionspartner gleichermaßen berücksichtigten sind rar (z.B. Bridges, 1999; Hamers und Krogh, 1997). Dabei ist es, wenn Räuber und Beute im

gleichen Habitat leben, wahrscheinlich, dass beide Interaktionspartner bzw. deren Interaktion durch ein Verschmutzungsereignis beeinträchtigt werden.

Schadstoffe können die in unserer Studie untersuchten Räuber-Beute-Beziehungen zwischen Fischen und Chironomiden verändern, indem sie bei Fischen die Fähigkeit zur Nahrungssuche und bei Chironomiden die Fähigkeit zur Räubervermeidung beeinträchtigen. Unsere Studie zeigt, dass es essentiell ist, beide Ebenen, sowohl die der Räuber als auch die der Beute in der Untersuchung von Räuber-Beute-Interaktionen zu beachten. Wir schlagen daher vor, eine Erfassung dieser Interaktionen in der Chemikalientestung zu verwenden, z.B. in *higher tier* Studien oder wenn ein umfassenderes Verständnis zu den Auswirkungen von Chemikalien auf verschiedenen trophischen Ebenen erzielt werden sollen. Aufgrund des einfachen Versuchsaufbaus in dieser Studie kann dieser Ansatz gut in der Praxis, auch mit unterschiedlichen Fischarten und Beuteorganismen, angewandt werden.

4.3 Biomonitoring in verschiedenen umweltrelevanten Szenarien mit zwei Crustaceen-Arten (*Corophium volutator* und *Gammarus pulex*).

Kapitel 6 *Kienle C, Gerhardt A (2008): Behaviour of Corophium volutator (Crustacea, Amphipoda) exposed to the water accommodated fraction (WAF) of oil in water and sediment. Environmental Toxicology and Chemistry 27(3): 599-604.*

Da Lebensgemeinschaften in der Gezeitenzone besonders durch Ölunfälle gefährdet sind (Fukuyama et al., 1998), wurden in dieser Studie die kurzzeitigen Auswirkungen der *water accommodated fraction* (WAF) von verwittertem Forties Rohöl auf das Verhalten des marinen Amphipoden *Corophium volutator* mit Hilfe des Multispecies Freshwater Biomonitors® (MFB) untersucht. Die WAF ist der Anteil des Öls, der für aquatische Organismen das größte Risiko darstellt. Bei Exposition gegenüber 25 bzw. 50% WAF zeigten die Amphipoden Hyperaktivität mit einem anschließenden Anstieg der Ventilation wie im Stepwise Stress Model postuliert (Gerhardt, 1999; Gerhardt et al., 2005). Dahingegen führte Exposition gegenüber 100 % WAF zu einem narkotischen Effekt (Hypoaktivität). Bei Exposition gegenüber 100 % WAF im Sediment zeigte *C. volutator* eine erhöhte Tendenz zur Hyperaktivität. In einem Pulsexperiment trat überwiegend Hyperaktivität bei und nach einer 130minütigen Exposition gegenüber 50 % WAF auf.

Insgesamt waren die Auswirkungen der WAF auf die Bewegungsaktivität von *C. volutator* im Wasser deutlicher als bei Exposition gegenüber Sediment. Hierbei wird die höhere Sensitivität der Wasser-Exposition jedoch teilweise durch die geringere Umweltrelevanz relativiert, da *C. volutator* den größten Teil seiner Lebenszeit im Sediment lebt. Womöglich kann *C. volutator* im Sediment auch vor Ölverschmutzungs-Ereignissen Schutz suchen und so akut toxische Kurzzeiteffekte minimieren. In früheren Studien vermieden Individuen von *C. volutator* bei Exposition gegenüber mit Forties Rohöl gespiktem Sediment tendenziell das Eingraben und tauchten auch häufig wieder aus dem Sediment auf (Scarlett et al., 2007), was die Ergebnisse der vorliegenden Studien unterstützt.

C. volutator schien sich von einer zweistündigen Exposition gegenüber WAF nach ungefähr 18 h erholt zu haben. Da die Wiedererholung von Organismen nach einem Ölverschmutzungspuls eine wichtige Rolle spielt, sollten hierzu weitere und längere

Studien durchgeführt werden. Bisher waren diesbezüglich keine Literaturdaten verfügbar. In einem Pulsexperiment mit dem Süßwasser-Amphipoden *Hyalella azteca* hatte die Wiedererholungszeit zwischen Pulsen von Kupfersulfat ($CuSO_4$) oder Natrium-Pentachlorphenol (NaPCP) eine signifikante Auswirkung auf die Mortalität bei einer zweiten Exposition (Zhao und Newman, 2006). Bei einer genügend langen Zeit zwischen den Expositionen konnten sich die Amphipoden wieder nahezu auf den ursprünglichen Zustand erholen. Nach Diamond et al. (2006) hängen die Effekte von Pulsexpositionen von deren Häufigkeit, Stärke und Dauer ebenso wie von der Erholungszeit zwischen den Pulsen ab. Kriterien auf der Basis chronischer Tests für die Wasserqualität und Abwassergrenzwerte könnten daher nicht ausreichen, um solchen Effekten vorzubeugen. Im Bezug auf die Überwachung von Küsten könnte das Verhalten von *C. volutator* einen geeigneten Parameter darstellen.

Kapitel 7 *Gerhardt A, Kienle C, Allan IJ, Greenwood R, Guigues N, Fouillac A-M, Mills GA, Gonzalez C (2007): Biomonitoring with Gammarus pulex at the Meuse (NL), Aller (GER) and Rhine (F) rivers with the online Multispecies Freshwater Biomonitor®. Journal of Environmental Monitoring 9(9): 979-985.*

Biologische Frühwarnsysteme sind im Rahmen des chemischen und biologischen Monitorings in der gegenwärtigen europäischen Gesetzgebung, der Wasserrahmenrichtlinie (WFD2000/60/EC) zur Überwachung der Gewässergüte geeignet (Allan et al., 2006). In der vorliegenden Studie wurde ein *In situ* Biomonitoring an den Flüssen Maas (NL), Aller (GER) und Rhein (F) im Rahmen des von der europäischen Union geförderten Projektes SWIFT-WFD durchgeführt. Als Testorganismus wurde *Gammarus pulex* eingesetzt und das Verhalten und Überleben über mehrere Wochen mit dem Multispecies Freshwater Biomonitor (MFB) aufgezeichnet (Gegenstand der Dissertation sind die Messungen am Rhein; die Experimente an der Maas und an der Aller wurden von Dr. A. Gerhardt mit verschiedenen Mitarbeitern der jeweiligen Monitoringstationen durchgeführt).
G. pulex überlebte in den MFB-Messkammern an der Monitoring-Station an der Aller problemlos (100 %). Die Messung der Bewegungsaktivität zeigte keine Unregelmäßigkeiten in der Expositionssituation an, auch die chemische Analytik bestätigte dies. An der Maas reagierte *G. pulex* auf Puls-Exposition gegenüber einer Mischung von Spurenmetallen oder mehreren organischen Xenobiotika mit einer um bis zu 20 % verringerten Bewegungsaktivität (beim ersten Puls) und erhöhter Mortalität (beim 2. bzw. 3. Puls). An der Monitoring-Station am Rhein, zeigte sich, dass die Testorganismen chemische Unregelmäßigkeiten im Gewässer wahrnehmen konnten und mit bis zu 20 % verringerter Lokomotion darauf reagierten. Hier konnten gemessene chemisch-analytische Auffälligkeiten wie z.B. ein Anstieg der Kupferkonzentration im Rheinwasser oder die Präsenz von Öl und/oder Pestiziden (angezeigt durch einen Anstieg der Fluoreszenz im Algenmonitor, bzw. durch einen Ölmonitor) mit Veränderungen des Verhaltens in Beziehung gebracht werden. Die Mortalität von drei der insgesamt acht Testorganismen nach zwei Wochen könnte ebenso auf die Exposition gegenüber Kupfer und/oder organischen Verbindungen (angezeigt durch einen Anstieg des *Total Organic Carbon*, TOC) zurückzuführen sein. Die beobachtete Mortalität gegen Ende des Monitoring-Zeitraumes ging vermutlich auf

organische Stoffe und eine Abnahme des Sauerstoffgehaltes zurück.

Gammarus pulex erwies sich als geeigneter Testorganismus für die Gewässerüberwachung mit dem MFB, da er als Zerkleinerer und Detritusfresser auch Auswirkungen durch partikelgebundene Schadstoffe zeigen kann. Die Testorganismen konnten unter den Monitoringbedingungen gut überleben und reagierten auf Änderungen in Schadstoffkonzentrationen mit Verhaltensänderungen. Insgesamt hat sich die Verwendung des MFB mit einer einheimischen und ökologisch relevanten Art wie *Gammarus pulex* als Testorganismus als verlässliches Biomonitoring-System für die *Online*-Überwachung der Qualität von Oberflächengewässern erwiesen.

5. Schlussfolgerungen

Organismen sind in ihrer Umwelt einer Vielzahl von Stressoren ausgesetzt. Hierbei können abiotische Parameter die Wirkung von Schadstoffen modifizieren, wie in Kapitel 1 für Larven des Zebrabärblings gezeigt werden konnte. Daher dürfen diese Stressfaktoren bei der Abschätzung der ökotoxikologischen Risiken von Umweltchemikalien nicht außer Acht gelassen werden. Die Untersuchung von Schadstoffmischungen mit gleichen oder unterschiedlichen Wirkmechanismen (Kapitel 2-4) zeigte in Untersuchungen mit Embryonen und Larven von Zebrabärblingen in der Mehrheit der Fälle eine additive Wirkung der kombinierten Stressoren auf (Ausnahme: akute Toxizität bei Mischungen aus Nickel und Chlorpyrifos), die in der Regel auch für alle untersuchten Parameter (Enzymaktivität, Verhalten, Entwicklungsstörungen, Mortalität) konsistent vorhanden war. Aufgrund der hohen Umweltrelevanz von Mischungseffekten und der beobachteten additiven Wirkungen ist es insbesondere wichtig, Effekte von Chemikalienmischungen baldmöglichst in die Risikoabschätzung von Umweltchemikalien mit einzubeziehen. Bei den Auswirkungen von Umweltstressoren auf Organismen sollte auch die Beeinflussung von interspezifischen Interaktionen, wie z.B. Räuber-Beute-Beziehungen beachtet werden. In der vorliegenden Arbeit konnten Auswirkungen auf Interaktionen zwischen Fischen und Chironomiden in umweltrelevanten Konzentrationsbereichen detektiert werden. In Bezug auf komplexe Mischungen stellt die *water accommodated fraction* von Rohöl ein großes Problem für aquatische Organismen in Küstenlebensgemeinschaften dar. Die Wirkung dieser Chemikalienmischung konnte in Kapitel 6 durch Änderungen im Verhalten des marinen Amphipoden *Corophium volutator* angezeigt werden. Es liegt somit nahe, dass quantitative Verhaltensstudien für ein Monitoring in Küstenbereichen geeignet sein können. Hier besteht jedoch noch weiterer Forschungsbedarf. Eine Eignung von Verhaltensveränderungen für die kontinuierliche Überwachung der Gewässerqualität an Monitoringstationen konnte im letzten Kapitel der vorliegenden Arbeit aufgezeigt werden (Kapitel 7). Der einheimische Bachflohkrebs *Gammarus pulex* stellt hierfür einen geeigneten und relevanten Testorganismus dar, da er auf komplexe Schadstoffmischungen im Oberflächenwasser sensitiv reagiert.

Verhaltensparameter haben sich in der vorliegenden Arbeit als integrative, relevante und sensitive Parameter bei den verschiedenen Fragestellungen bewährt. Auch bei der

Betrachtung interspezifischer Interaktionen können sie für die Bewertung der Auswirkung von Umweltstressoren auf aquatische Organismen dienen. Der Vorteil dieser Parameter ist auch eine kurze Reaktionszeit, wodurch die Auswirkungen von Schadstoffen zeitnah detektiert werden können. Im Idealfall sollten Verhaltensmessungen, welche durchaus in der kontinuierlichen Überwachung von Gewässerqualität eingesetzt werden sollten, jedoch mit weiteren Parametern, wie chemischen Messungen der Substanzkonzentration, biochemischen Biomarkern (z.B. Enzymaktivität, Hsp70-Gehalt), histologischen Veränderungen, Veränderungen der Embryonal- und Larvalentwicklung und letztlich auch mit auftretenden Mortalitäten in Beziehung gesetzt werden, um eine maximale Aussagekraft der Ergebnisse zu ermöglichen und den bestehenden Unterschieden in der Sensitivität der Parameter gegenüber verschiedenen Schadstoffen gerecht zu werden.

6. Literatur

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

<u>Kapitel 1</u> Kienle C, Köhler H-R, Filser J, Gerhardt A (2008): Effects of nickel chloride and oxygen depletion on behaviour and vitality of zebrafish *Danio rerio* (Hamilton, 1822) (Pisces, Cypriniformes) embryos and larvae. Environmental Pollution 153(3): 612-620.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die fachliche Betreuung erfolgte durch Dr. Almut Gerhardt (LimCo International, Ibbenbüren), Prof. Dr. Juliane Filser (Universität Bremen) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

<u>Kapitel 2</u> Kienle C, Köhler, H-R, Gerhardt A (in press): Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology and Environmental Safety, doi:10.1016/j.ecoenv.2009.04.014.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die analytischen Messungen der Substanzen wurden von Eva Pfefferle (Steinbeis-Transfer-Zentrum, Reutlingen), Dr. Peter Kühn und André Velescu (Universität Tübingen) durchgeführt. Die fachliche Betreuung erfolgte durch Dr. Almut Gerhardt (LimCo International, Ibbenbüren) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

<u>Kapitel 3</u> Scheil V*, Kienle C*, Osterauer R, Gerhardt A, Köhler H-R (2009): Effects of 3,4-dichloroaniline and diazinon on different biological organisation levels of zebrafish (*Danio rerio*) embryos and larvae. Ecotoxicology 18(3): 355-363.

*beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung der subchronischen Tests und der Verhaltensuntersuchungen zu 3,4-Dichloranilin, Diazinon sowie der Mischungen beider Substanzen. Die Embryotests zu Diazinon wurden von R. Osterauer durchgeführt. Die gesamten Stressproteinanalysen ebenso wie die Embryotests mit 3,4-Dichloranilin und Mischungen aus Diazinon und 3,4-Dichloranilin wurden von Dr. V. Scheil durchgeführt. Die fachliche Betreuung erfolgte durch Prof. Dr.

H.-R. Köhler (Universität Tübingen) und Dr. A. Gerhardt (LimCo International, Ibbenbüren).

<u>Kapitel 4</u> Kienle C, Küster E, Gerhardt A, Köhler H-R (submitted): Linking behaviour to acetylcholinesterase inhibition in embryos and larvae of zebrafish (*Danio rerio*) exposed to pesticides.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die Bestimmung der Acetylcholinesteraseaktivität wurde unter Mithilfe von Silke Aulhorn und Andrea Beyerle (Helmholtz-Zentrum für Umweltforschung –UFZ, Leipzig) durchgeführt. Die analytischen Messungen der Substanzen wurden von Eva Pfefferle (Steinbeis-Transfer-Zentrum, Reutlingen) durchgeführt. Die fachliche Betreuung erfolgte durch Prof. Dr. H.-R. Köhler (Universität Tübingen), Dr. Almut Gerhardt (LimCo International, Ibbenbüren) und PD Dr. Eberhard Küster (Helmholtz-Zentrum für Umweltforschung –UFZ, Leipzig).

<u>Kapitel 5</u> Kienle C*, Langer ME*, Gerhardt A, Köhler H-R (unpublished manuscript): Impairment of trophic interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) by chlorpyrifos.

*beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.

Die gesamte Versuchsplanung, Durchführung und Auswertung wurde gemeinsam mit M. Langer durchgeführt. Die fachliche Betreuung erfolgte durch Prof. Dr. H.-R. Köhler (Universität Tübingen) und Dr. Almut Gerhardt (LimCo International, Ibbenbüren).

<u>Kapitel 6</u> Kienle C, Gerhardt A (2008): Behaviour of *Corophium volutator* (Crustacea, Amphipoda) exposed to the water accommodated fraction (WAF) of oil in water and sediment. Environmental Toxicology and Chemistry 27(3): 599-604.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung. Die Versuchsdurchführung erfolgte unter Mithilfe von Dr. Alan Scarlett und Prof. Dr. Tamara Galloway (University of Exeter, England). Die fachliche Betreuung erfolgte durch Dr. Almut Gerhardt (LimCo International, Ibbenbüren).

<u>Kapitel 7</u> Gerhardt A, Kienle C, Allan IJ, Greenwood R, Guigues N, Fouillac A-M, Mills GA, Gonzalez C (2007): Biomonitoring with *Gammarus pulex* at the Meuse (NL), Aller (GER) and Rhine (F) rivers with the online Multispecies Freshwater Biomonitor®. Journal of Environmental Monitoring 9(9), 979–985.

Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung der Halbfreilandversuche mit *Gammarus pulex* am Rhein. Die Versuchsdurchführung erfolgte unter Mithilfe von Miriam Langer (Universität Tübingen) und Fabien Toulet (Aprona, Huningue Monitoring Station). Die Versuche an der Maas wurden von Dr. Almut Gerhardt (LimCo International, Ibbenbüren), Dr. Ian J. Allan (University of Portsmouth, UK) und Nel Frijns (*Institute for Inland Water Management and Wastewater Treatment*, RIZA, NL) in Zusammenarbeit mit dem RIZA Monitoring Team durchgeführt. Prof. Richard Greenwood und Dr. Graham A. Mills (University of Portsmouth, UK) betreuten Dr. Ian J. Allan fachlich. Die Versuche an der Aller erfolgten durch Dr. Almut, Dr. Nathalie Guigues, Dr. Anne-Marie Fouillac und Andreas Austen (*Bureau de Recherche Géologique et Minière*, BRGM, France) in Zusammenarbeit mit dem BRGM Monitoring Team. Dr. Catherine Gonzalez leitete das Projekt SWIFT-WFD. Die fachliche Betreuung erfolgte durch Dr. Almut Gerhardt.

<u>Kapitel 1</u>: Effects of nickel chloride and oxygen depletion on behaviour and vitality of zebrafish *Danio rerio* (Hamilton, 1822) (Pisces, Cypriniformes) embryos and larvae

Cornelia Kienle[a,c], Heinz-R. Köhler[a], Juliane Filser[b], Almut Gerhardt[c]

[a]Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b]Department of General and Theoretical Ecology, University of Bremen, Leobener Strasse, D-28359 Bremen, Germany

[c]LimCo International, An der Aa 5, D-49477 Ibbenbüren, Germany

Abstract

We examined acute (2 h exposure of 5-day-old larvae) and subchronic (exposure from fertilization up to an age of 11 days) effects of $NiCl_2 \cdot 6H_2O$ on embryos and larvae of zebrafish (*Danio rerio*), both alone and in combination with oxygen depletion. The following endpoints were recorded: acute exposure: locomotory activity and survival; subchronic exposure: hatching rate, deformations, locomotory activity (at 5, 8 and 11 days) and mortality. In acute exposures nickel chloride (7.5-15 mg Ni/L) caused decreasing locomotory activity. Oxygen depletion (≤2.45 ± 0.16 mg O_2/L) also resulted in significantly reduced locomotory activity. In the subchronic test, exposure to ≥10 mg Ni/L resulted in delayed hatching at an age of 96 h, in decreased locomotory activity at an age of 5 days, and increased mortality at an age of 11 days (LC_{20} = 9.5 mg Ni/L). The observed LOEC for locomotory activity (7.5 mg Ni/L) is in the range of environmentally relevant concentrations. Since locomotory activity was already affected by acute exposure, this parameter is recommended to supplement commonly recorded endpoints of toxicity.

"Capsule": Increasing concentrations of nickel chloride and decreasing concentrations of oxygen lead to reduced vitality and locomotory activity in *Danio rerio* embryos and larvae.

Keywords: behaviour; $NiCl_2$; O_2; Multispecies Freshwater Biomonitor®

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1. Introduction

Zebrafish (*Danio rerio,* Hamilton, 1822) which originally live in stream habitats rich in macrophytes in South East Asia (Börries, 2006) have received special attention in research during the last years, especially as model vertebrates in developmental biology and genetics (e.g. Kimmel, 1989; Nüsslein-Volhard, 1994). The embryo test with *D. rerio* (DarT) was proposed as an alternative method for the acute fish test with adult fish (Nagel, 2002) and numerous studies with *D. rerio* embryos and larvae have been conducted so far (Bachmann, 2002; Nagel, 2002; Strmac, 1999; Versonnen et al., 2004). Some studies investigated the effects of pollutants on the behaviour of zebrafish using adolescent or adult fish (Baganz et al., 1997; Levin et al., 2003; Steinberg et al., 1994; Vogl et al., 1999) while others described baseline data on the behaviour of larval zebrafish (Bagatto et al., 2001; Budick and O'Malley, 2000; Orger et al., 2000), but only a single study so far considered the effect of a chemical stressor (aminoacid chemostimulants) on zebrafish larval behaviour (Lindsay and Vogt, 2004). To further reduce the use of adult fish in ecotoxicological tests, however, it might be reasonable to establish behavioural tests with fish larvae.

Behavioural ecotoxicology deals with the effects of pollutants on the behaviour of organisms, and their link to adjacent levels of biological organisation (e.g. biochemical, physiological or general metabolic processes within the animal as well as population maintenance) (Dell`Omo, 2002). Behaviour integrates the animals' responses to internal (physiological) and external (environmental, social) factors and relates one organism to another (Evans, 1994).

Behavioural tests represent a sensitive method to detect effects of contaminants (Dell`Omo, 2002) compared to conventional endpoints as mortality (e.g. Levin et al., 2003). Moreover, alterations in behaviour are measurable already after a short time (e.g. avoidance, attraction). Lindsay and Vogt (2004) were able to detect effects of amino acid chemostimulants on the behaviour of four-day-old *D. rerio* larvae within only few minutes of exposure.

To measure behavioural alterations automated online biomonitors can be used. They use living organisms as sensors for alterations in water quality and work in real-time (Gruber et al., 1994; Osbild et al., 1995). In our study, the Multispecies Freshwater Biomonitor® (MFB) (LimCo International, Germany) has been used to record the

locomotory activity of *D. rerio* larvae.

Next to biotic factors, abiotic factors determine the constitution and the efficiency of an organism's physiological and behavioural performance in an ecosystem. Abiotic stressors like oxygen depletion can occur during summer in the hypolimnion of eutrophic lakes and in streams dominated by organic matter degradation (Schwörbel, 1992). Fish from mountain streams usually react most sensitive to oxygen deficiency: whereas salmonids need at least 6 mg O_2/L and show stress in respiration at 40 – 50 % O_2 saturation, the more insensitive carps are capable of living at oxygen contents down to 1 mg/L, resp. 13 % saturation (at 26°C) (Schönborn, 2000).

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) is a water-soluble nickel compound, not biologically degradable, very toxic for aquatic organisms and may cause long-term harmful effects (Merck, 2004). Nickel (Ni) is a ubiquitous, naturally occurring trace metal (0.0086 % of the earth crust; Duke, 1980), with increased concentrations in waterbodies e.g. in the area of nickel-processing industries (WHO, 1991). Unpolluted Canadian rivers and lakes exhibit background concentrations of 0.1 – 10 µg Ni/L but natural waters near industrial sites have been shown to contain between 50 and 2000 µg Ni/L, with a maximum of 183 000 µg Ni/L near a nickel refinery in Sudbury, Ontario (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987).

The aim of the present study was to examine the effects of nickel chloride on locomotory behaviour, survival and vitality of early life stages of zebrafish (*D. rerio*) in hard water. The innovative approach in our study was based on (1) the evaluation of behaviour as sensitive test parameter for short- and long-term tests, (2) the potential of replacing adult fish by young larvae considering ethical reasons as well as sensitivity aspects and (3) increased ecological realism by adding oxygen depletion as an interfering natural stressor.

The following hypotheses were tested for juvenile zebrafish:

1. Exposure to $NiCl_2$ results in a higher locomotory activity (avoidance reaction).
2. Sensitivity to Ni is exposure time-dependent.
3. Additional environmental stress (oxygen depletion) increases $NiCl_2$ toxicity.

2. Materials and Methods

2.1 Test animals and acquisition of eggs

Adult zebrafish (*D. rerio*, strain WIK, MPI for Developmental Biology, Tübingen) of both sexes were kept in the laboratory in 150 – 230 L aquaria with aerated and filtered water (50/50 % mixture of tap and distilled water with a conductivity of approx. 400 µS/cm), with a minimum of 1 L water per fish on the average. Culture conditions were 26 ± 1°C at a 12 h:12 h light:dark cycle without dimming. The adult fish were fed ad libitum twice per day with dry flake food (Nutrafin Max, Hagen, Germany) and frozen crustaceans or midge larvae (MM Aquaristik, Germany), respectively.

The eggs used in the tests were collected using spawn traps which had been placed on the bottom of each aquarium the evening before spawning was required. In the morning (1 h after triggering the spawning via switching on the light) the spawn traps were removed from the aquaria, the eggs were sieved and cleaned under flowing tap water and transferred to Petri dishes. Embryos and larvae were kept in glass Petri dishes in reconstituted water (OECD-Guideline 203; ISO-Standard 6341-1982), which had been aerated for 12 hours before use with an aquarium pump. The Petri dishes with the embryos and larvae were kept in a climate chamber at 26 ± 1°C and a 12 h:12 h light:dark cycle up to an age of 5 days. Two to four hours after fertilization the fertilized eggs were separated from unfertilized eggs and distributed over several Petri dishes with test water. An appropriate amount of water (~1/3 of the volume) was exchanged daily. After 24 h the eggs were put into new Petri dishes with fresh reconstituted water. Every day the condition of the larvae was checked under a stereomicroscope, and malformed or inactive embryos and larvae were removed.

2.2 Test substance

Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) (Roth, Germany) was dissolved in reconstituted water in order to prepare a stock solution of 1000 mg Ni/L at pH 7.5. From this stock solution the test solutions were prepared directly before use. Eight different nominal concentrations (0.25, 1, 2.5, 5, 7.5, 10, 12.5 and 15 mg Ni/L) and two negative controls with pure reconstituted water were examined for the acute test. The subchronic test comprised five nominal concentrations (0.5, 1, 5, 10 and 15 mg Ni/L) and one negative control.

2.3 The Multispecies Freshwater Biomonitor® (MFB)

The Multispecies Freshwater Biomonitor (LimCo International, Germany) is an online biomonitor which continuously and quantitatively records the behaviour pattern of animals (Gerhardt et al., 1994). The MFB consists of flow-through sensor chambers, a measuring unit and a personal computer with specific software for data evaluation (Gerhardt, 2001). The measuring principle in the sensor chamber is based on quadropole impedance conversion (Gerhardt et al., 1994). The behavioural signal of the animal is analysed by a Fast Fourier Transformation, resulting in a histogram of different signal frequencies, hence being able to distinguish different types of behaviours, such as locomotion and ventilation (Gerhardt et al., 1994). The chambers, sealed with a lid (mesh size: 0.25 mm) at both ends, used for the fish larvae were 4 cm in length with a diameter of 1 cm, allowing for free movement of the fish (size of fish larvae: ~ 3.8 mm in length, ~ 0.5 - 1 mm in diameter). Previous tests with chambers of different lengths revealed that the above mentioned size was suitable for short-term exposure of 2 h.

2.4 Acute behavioural tests with nickel chloride

Five-day-old larvae have been chosen based on the results of pilot studies (data not shown) which showed that larvae first display constant swimming activity with an intermediate overall activity and low variation in locomotory activity at this age and thus seemed to be most suitable to allow for the detection of increased as well as decreased activity due to environmental stress.

The chambers were placed into polyethylene-vessels (208×208×64 mm^3, 2 L) filled with 2 L of the respective nickel solution, which were arranged in duplicate in a surrounding black basin (to eliminate disturbance from movement along the vessels) with temperature adjusted water (to 26 ± 1°C). Only healthy larvae were used and transferred carefully into the chambers; the remaining air bubbles in the chambers were removed with a Pasteur pipette. Subsequently the chambers were placed horizontally on the bottom of the test vessel (Fig. 1a). After an acclimation time of 10 min the measurement was started. The behaviour of 11 – 12 larvae per treatment was recorded continuously for 2 h in intervals of 10 min and for a duration of 4 min each. Several abiotic parameters (temperature, pH, conductivity, oxygen concentration and

saturation) were determined at the beginning and the end of each measurement period. The test vessels were illuminated from above during the measurements (58 W neon light, distance to chambers: 145 cm). No food was provided during the experiments.

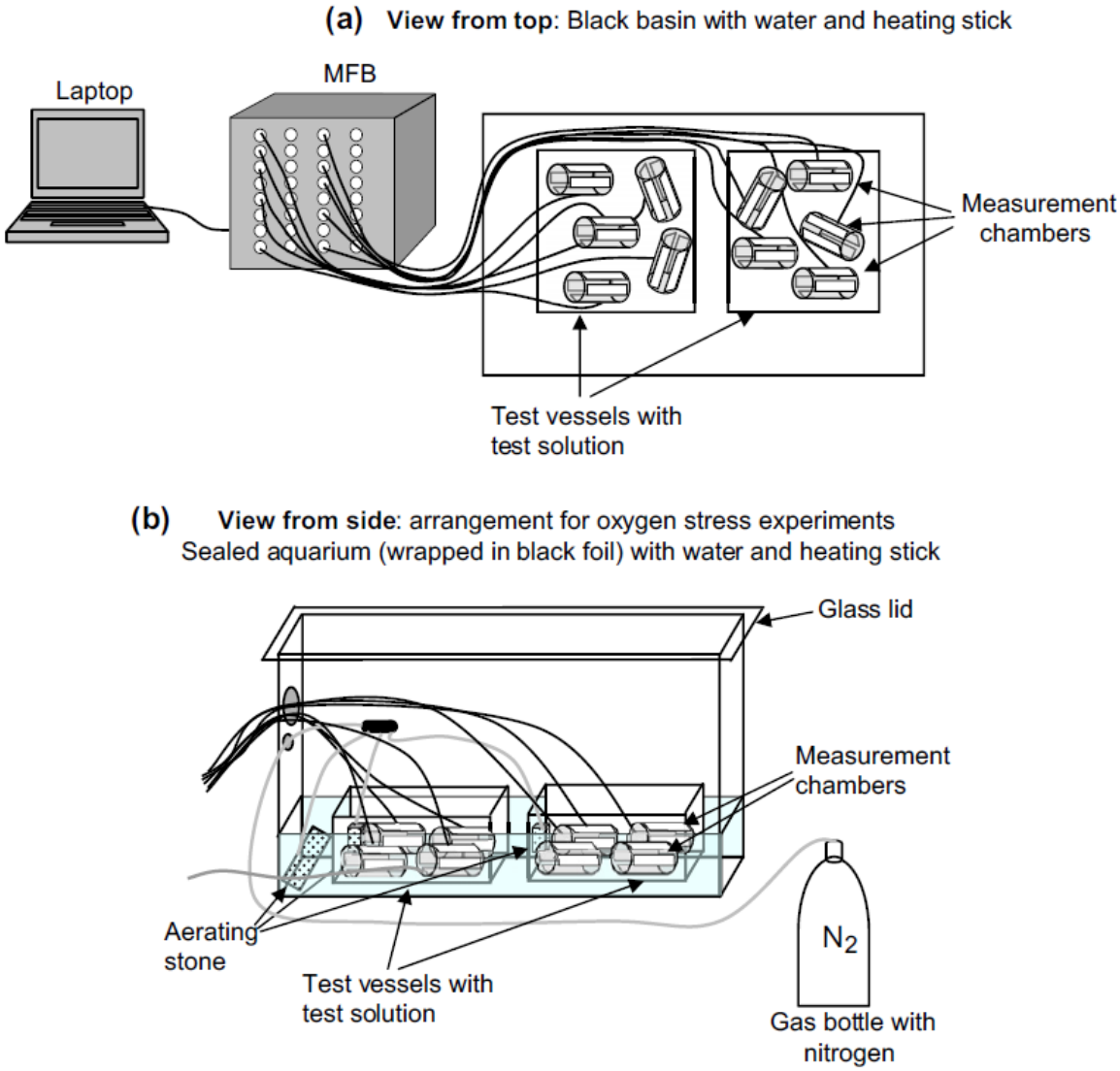

Fig. 1. Experimental setup for behavioural measurements with and without oxygen stress (explanation in the text).

2.5 Subchronic test with nickel chloride

The test was conducted according to the VMD Guidance Note "Ecotoxicity testing of medicines intended for use in fish farming" (Veterinary Medicines Directorate, 1996). The organisms were exposed to Ni from the time of fertilization (≤1 h) up to an age of 11 days in plastic Petri dishes with 30 fertilized eggs each and three replicates per nickel concentration. Plastic Petri dishes were used to avoid possible Ni–glass interactions. After 96 h of embryonic development, the hatching rate and mortality was recorded.

Furthermore, mortality and unusual swimming behaviour at the surface were recorded daily up to the 11th day after fertilization. For behavioural measurements in the MFB, four larvae from each replicate were randomly removed for analysis at regular intervals (5, 8 and 11 days after fertilization). The behaviour measurements of the animals were performed in the same Ni concentration as used for the subchronic exposure. No food was provided during the experiments.

2.6 Test with different oxygen levels

The experiments were performed in a completely air-tight construction (Fig. 1b), oxygen was removed by pumping gaseous nitrogen in the test solutions, the surrounding waterbath and the overlaying atmosphere via an aeration stone for an appropriate time (~5 to 30 min), depending on the oxygen concentration which was aimed to be reached. To keep the oxygen level constant, the test vessels were arranged in a surrounding glass aquarium (60×30×30 cm) with appropriate holes for the cables of the measuring chambers and for the aeration tube. As soon as the appropriate oxygen level was reached, the larvae were placed into the chambers as described in Section 2.4. Subsequently the top was covered by a glass plate and the waterbath and the surrounding atmosphere aerated once again to reach the appropriate oxygen concentration. The air-tightness of the construction was guaranteed through sealing with tape. As confirmed by repeated oxygen measurements, this construction kept the oxygen level in the water nearly constant over a period of 2 h. Six different oxygen concentrations between 0.81 and 7.94 mg O_2/L were tested (for detailed data see "Results" section). Each oxygen concentration was combined with different concentrations of Ni (Table 1). The behaviour of 9 – 12 replicate *Danio* specimens was recorded for each treatment.

Table 1: Combinations of nickel and oxygen concentrations

	O_2 [mg/L]						
Nickel [mg/L]	0.81	2.45 ± 0.16	3.23 ± 0.25	4.19 ± 0.28	4.75 ± 0.60	5.33 ± 0.40	7.94 ± 0.24
0	+	+	+	+	+	+	+
0,25				+			+
0,5				+			
1		+		+			+
2,5				+			+
5			+	+			+
7,5		+		+		+	+
10			+	+		+	+
12,5				+			+
15		+	+	+		+	+

2.7 Data analysis

For each larva, means of locomotory activities (percentage time spent on locomotion) were calculated separately for the first and the second hours, to take into account possible early warning reactions and the decrease of activity over time. For statistical evaluation the data on "percentage time spent on locomotion" were arcsine transformed from proportional values. Nonparametric methods were chosen because the data were only partially normally distributed (one-sample-Kolmogorov-Smirnov-Test, SPSS 10.0.1, USA). Linear regression analysis (JMP 4.0, SAS systems, USA) was performed in order to detect treatment differences in abiotic parameters. The data of all tests were analysed for significance using Friedman's ANOVA (Statistica 5.0, StatSoft, USA), followed by a Wilcoxon two group test (JMP 4.0, SAS systems, USA) to examine differences between control and exposure treatments. The response surface for mixture data of $NiCl_2$ and oxygen depletion was calculated with Statistica 5.0 (StatSoft, USA) and mixture responses were calculated with the MixTox Model (Jonker et al., 2005). The LC_{20} after 11 days was estimated with Table Curve™ 2D 5.1 (SYSTAT Software Inc., USA).

3. Results

3.1 Abiotic parameters

In the experiments with Ni alone, the abiotic parameters matched optimal conditions for the larvae, such as 25.3 ± 0.8°C, 7.94 ± 0.24 mg O_2/L (99.6 ± 2.6 %), pH: 7.99 ± 0.14 and conductivity: 640 ± 17 µS/cm (mean ± SD of the control treatments, *n* = 6).

The oxygen concentrations in the tests with oxygen depletion were: 0.81 mg O_2/L (~10 %, single value), 2.45 ± 0.16 mg O_2/L (31.1 ± 2.4 %), 3.23 ± 0.25 mg O_2/L (41.7 ± 3.5 %), 4.19 ± 0.28 mg O_2/L (53.6 ± 3.9 %), 4.75 ± 0.60 mg O_2/L (60.5 ± 7.0 %) and 5.33 ± 0.40 mg O_2/L (68.3 ± 4.7 %) respectively. The pH increased significantly with decreasing oxygen concentration to 8.39 ± 0.33 ($p < 0.018$, $pH = 8.409 - 0.005[O_2]$, $r^2 = 0.414$, $n = 13$). With increasing nickel concentrations, electric conductivity increased significantly to 719 ± 19 µS/cm ($p < 0.001$, $conductivity = 648.658 + 4.721[Ni]$, $r^2 = 0.815$, $n = 13$), but in a tolerable range for the embryos and larvae.

3.2 Locomotory acitivity of D. rerio larvae

D. rerio larvae showed nearly constant locomotory movements in the control treatments (Fig. 2).

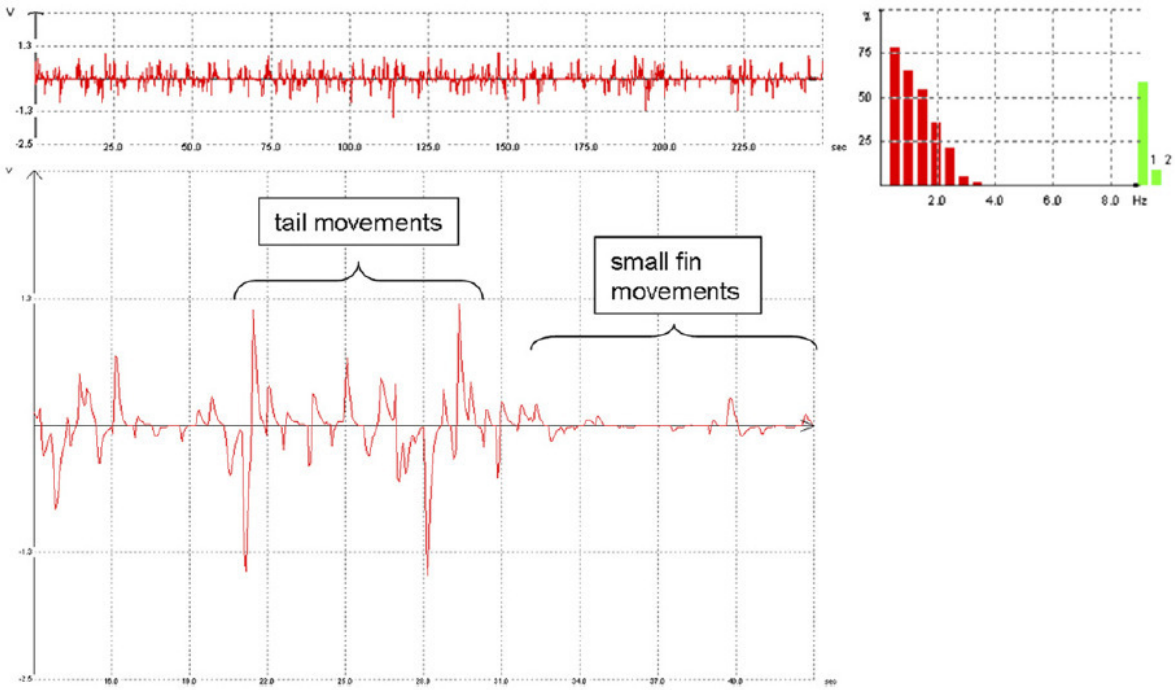

Fig. 2. Example of the spontaneous locomotory movement pattern (left: original signal: amplitude [V] vs. time [s], right: FFT-histogram [activity in % of the time (250 s)] vs. frequency [Hz] of a 5-day-old *Danio rerio* larva under control conditions.

Occasionally, short pauses in locomotion were recorded. The movement pattern was characterized of alternating high peaks (high amplitude, corresponding to tail movements) and weaker movements with lower amplitude (corresponding to small fin movements). The comparison of the data recorded for the respective first hour of measurement with those recorded for the second hour revealed the effect of Ni and O_2 depletion to be more pronounced during the second hour of movement recording. Therefore, in the following, we exclusively refer to data recorded during the second hour of the measurement.

3.3 Acute test with nickel chloride

The locomotory activity decreased significantly with increasing nickel concentration ($p < 0.001$, activity = 0.702 – 0.0168[Ni], $r^2 = 0.188$, $n = 117$). The LOEC with a significant difference vs. the control was 7.5 mg/L ($p < 0.001$, Friedman's ANOVA; $p < 0.005$, Wilcoxon test) (Fig. 3).

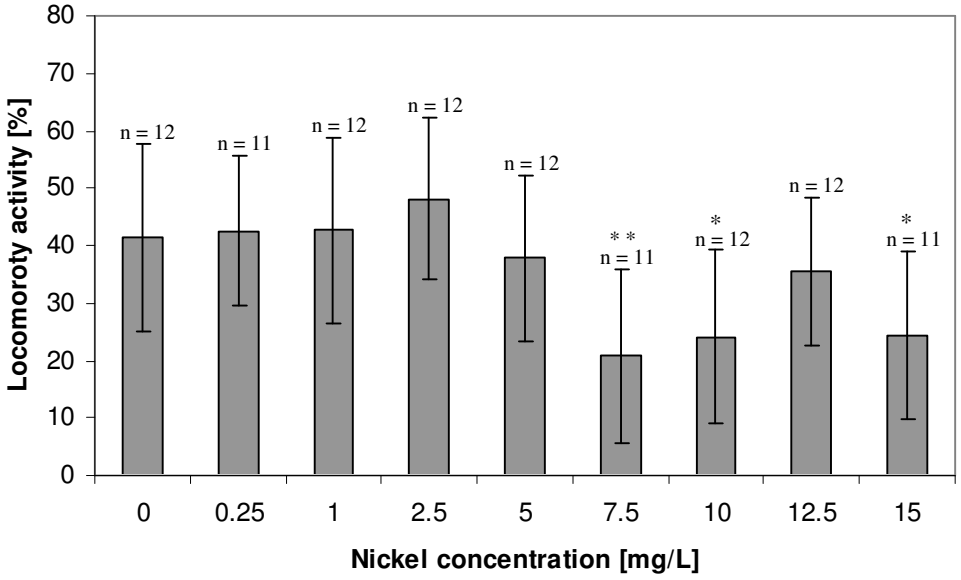

Fig. 3. Acute exposure: Concentration-response relationship of locomotory activity [%] (0.5 - 2 Hz frequency band) of five-days-old *D. rerio* larvae against the Ni concentration [mg/L] (mean ± SD). Significant differences to the control (0 mg Ni/L): ** $p < 0.01$, * $p < 0.05$ (Wilcoxon test).

3.4 Subchronic test with nickel chloride

In the subchronic test with $NiCl_2$, larvae exhibited different symptoms of Ni toxicity with increasing exposure time.

A significant delay of hatching was observed at concentration levels of 10 mg/L and above at an age of 96 h ($p < 0.019$, Friedman's ANOVA, $p < 0.046$, Wilcoxon test) (Fig. 4). In treatments with 15 mg Ni/L on the average 23.3 % of the larvae had not hatched as compared to 1.1 % in the control treatment. No increased mortality could be observed at this age.

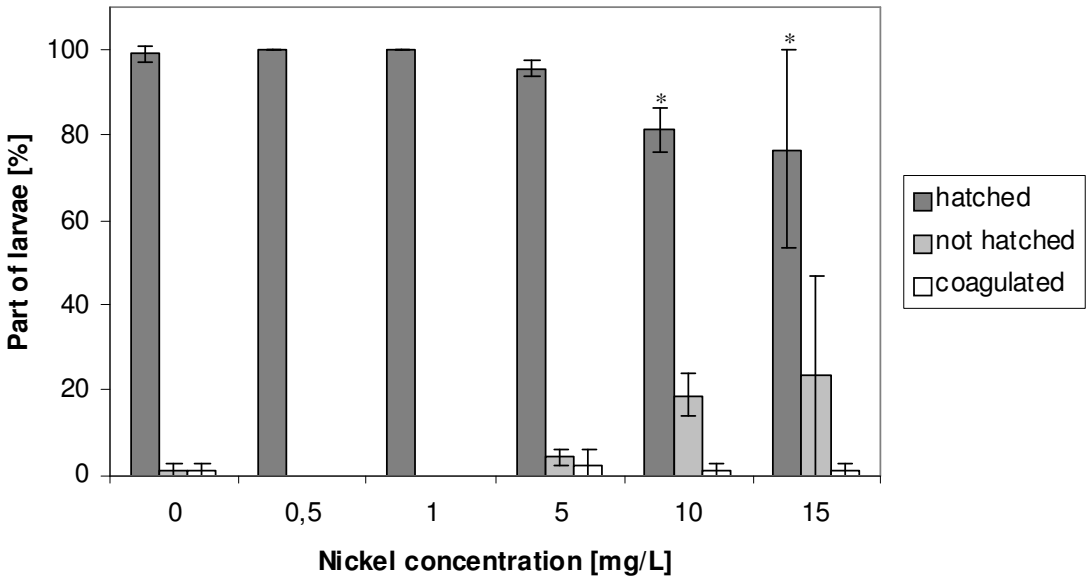

Fig. 4. Hatching rate and mortality of 96-h old *D. rerio* larvae. Percentage of hatched or coagulated (dead) larvae versus the nickel concentration [mg/L] (means ± SD, 30 larvae per replicate for each concentration, three replicates each). Significant differences to the control: * $p < 0.05$ (Wilcoxon test).

Locomotory activity decreased with exposure time. After 11 days of exposure, the activity of the larvae was generally much lower than in the first days. The most obvious differences in the activity of the larvae between the treatments, were recorded at the age of 5 days ($p < 0.001$, Friedman's ANOVA) (Fig. 5), e.g. decreased activity vs. the control was found at 10 mg Ni/L ($p < 0.028$) and at 15 mg Ni/L ($p < 0.042$ first hour, $p < 0.067$, second hour, Wilcoxon test).

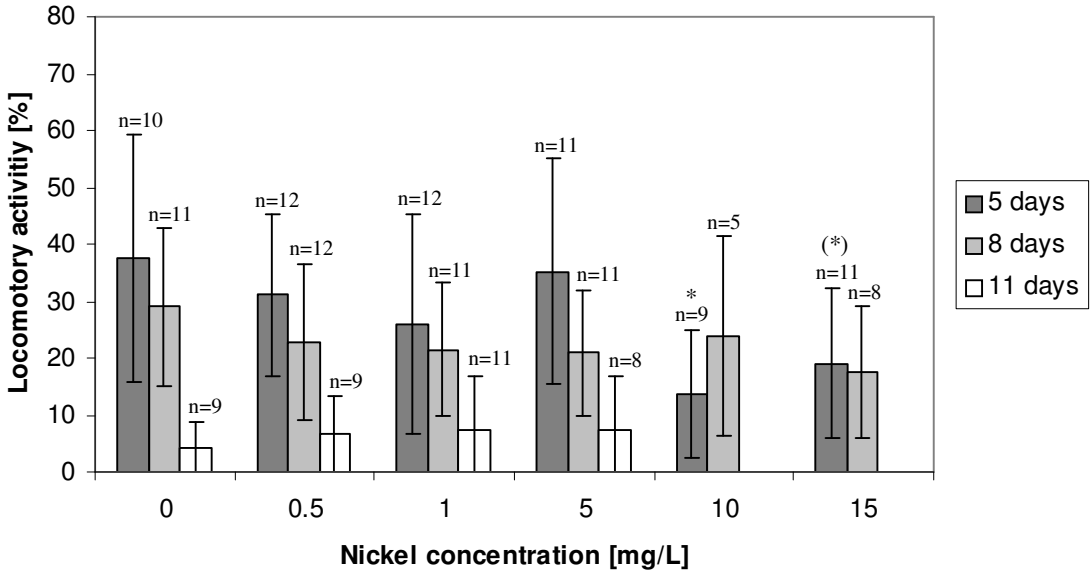

Fig. 5. Subchronic exposure: concentration–response relationship of locomotory activity [%] (0.5 - 2 Hz frequency band) of 5-day-old *D. rerio* larvae against the concentration [mg/L] (mean ± SD). Significant differences vs. control: * $p < 0.05$, (*) $p < 0{,}067$ (Wilcoxon test).

Significant differences in the number of larvae which stayed constantly at the water surface (in the following ´surface swimming`) were observed ($p < 0.0103$, Friedman's ANOVA) in treatments with 10 mg Ni/L at an age of 8 days and more ($p < 0.034$, Wilcoxon test) and with 15 mg Ni/L at an age of 7 days and more ($p < 0.037$, Wilcoxon test) (Fig. 6).

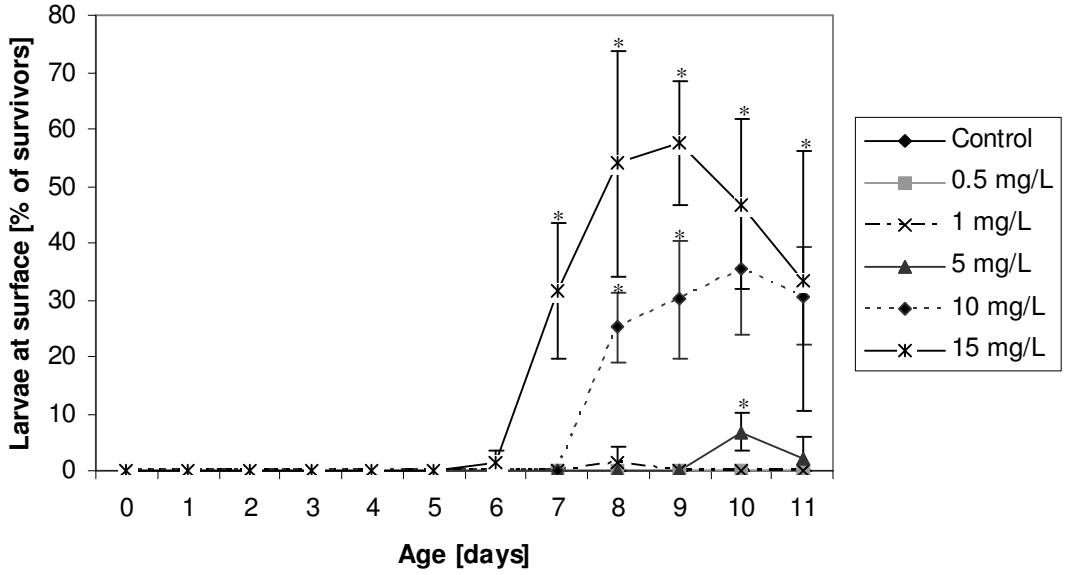

Fig. 6. Proportion of *D. rerio* larvae [% of survivors] which stayed constantly at the water surface at different nickel concentrations (mean ± SD); number of larvae for each replicate 30 (days 0–5); 26 (days 6–8); 22 (days 9–11), three replicates each. Significant differences to control treatment: * $p < 0.05$ (Wilcoxon test).

These larvae went back to the surface after having been pushed under water with a pipette tip and were not able to remain in the water column or at the bottom of the Petri dish. This effect prevented behavioural data recording for 10–15 mg Ni/L-exposed larvae at day 11 in our setup with chambers, filled completely with water, without any air-space.

Mortality increased significantly at an age of 11 days after fertilization at concentrations of 10–15 mg Ni/L ($p < 0.016$, Friedman's ANOVA, $p < 0.046$ and $p < 0.043$, Wilcoxon test) up to 39.4 ± 5.3 %. The LC_{20} at 11 days was 9.52 mg Ni/L.

3.5 Acute test with O_2-deficiency

A lower and less frequent locomotory activity of larvae in oxygen-deficient water (2.45 mg O_2/L) compared to control larvae in water with 7.94 mg O_2/L was recorded ($p < 0.004$, Wilcoxon test). The locomotory activity between treatments with 2.45 and both 4.19 and 7.94 mg O_2/L differed significantly ($p < 0.001$, Wilcoxon test). After 2 h of measurement, mortality occurred in treatments with 0.81 (~100 %), 2.45 (42 %) and 3.23 mg O_2/L (25 %). Accordingly no sublethal effect threshold for oxygen depletion on the behaviour of zebrafish larvae could be detected.

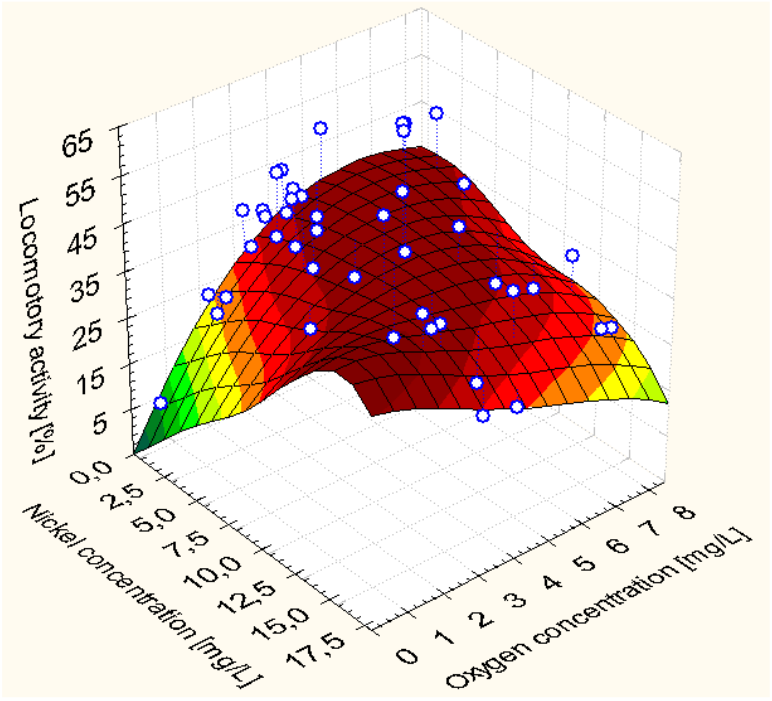

Fig. 7. Locomotory activity of *D. rerio* larvae 5 days after fertilization in acute tests with $NiCl_2$ at different oxygen concentrations (n = 9-12).

In combination of oxygen deficiency and nickel treatment, locomotory activity decreased with increasing nickel concentrations in tests with high oxygen saturation levels (4.19 mg O_2/L and higher) (Fig. 7).

At lower oxygen concentrations (< 4.19 mg O_2/L), nickel had rather a stimulating than an inhibitory effect on locomotory activity. Mixture toxicity modelling (MixTox model;

Jonker et al., 2005) indicated a significant antagonistic action of O_2 deficiency and Ni treatment ($p < 0.003$).

4. Discussion

In the present study fish larvae were examined for the first time in the MFB which proved to be well suitable for such young larvae. Small fin and tail movements could be distinguished, which resembled the signals of swimming movements and movements with the small fins of the three spined-stickleback, also recorded in the MFB (Craig and Laming, 2004). The electrical field of the MFB did neither disturb behaviour of adult three-spined stickleback (Craig and Laming, 2004) nor crustaceans (Kirkpatrick et al., 2005).

In our study acute behavioural investigations were most sensitive with decreasing effects on the locomotory activity at 7.5 mg Ni/L and above whereas significant decreasing effects on hatching rate, locomotory activity and mortality in the subchronic test occurred first at 10 mg Ni/L and above (see Table 2). According to these results the first hypothesis (Exposure to $NiCl_2$ results in a higher locomotory activity (avoidance reaction)) has to be rejected for exposure to Ni alone, but could be accepted for combined exposure to Ni and reduced oxygen levels. The second hypothesis (Sensitivity to Ni is exposure time-dependent) could be accepted. According to Triebskorn et al. (1997) behavioural answers should be integrated as short-time and long-time indicators of contaminations with high ecological relevance.

The observed effect concentrations for Ni are in the range of measures near industrial sites (50 - 2000 µg Ni/L in natural waters near industrial sites and 183 000 µg/L near a nickel refinery; Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1997). In nature decreased locomotory activity in fish might lead to increased downstream drift and/or predation risk, hence representing an ecologically relevant parameter for the species' health and survival. A similar decreasing effect of metals on the activity of rainbow trout and brook trout (*Salvelinus fontinalis*) exposed to aluminium as well as walleyes (*Stizostedion vitreum vitreum*) exposed to mercury was reported in Atchison et al. (1987).

Table 2
Comparison of effect concentrations in different studies concerning nickel toxicity to fish.

Species	Age	Acute studies: Parameter	Nickel	pH	Water hardness [mg/L] (as CaCO3)	Source
Cyprinus carpio (carp)	E+L	Hatching rate	6 mg/L	7.4	128	Blaylock and Frank (1979)
Oncorhynchus mykiss (rainbow trout)	A	Attraction	6 µg/L	7-7.5	28.4	Giattina et al. (1982)
O. mykiss	A	Avoidance reaction	> 19 µg/L	7-7.5	28.4	Giattina et al. (1982)
Danio rerio (zebrafish)	E+L	Hatching rate	45 µg/L	7.5-7.7	100	Dave and Xiu (1991)
D. rerio	E+L	Hatching rate	10 mg/L	8.0	231.4	Present study
D. rerio	L	Diminished locomotory activity	7.5 mg/L	8	231.4	Present study
		Subchronic and chronic studies				
O. mykiss	E+L	Growth	35 µg/L	7.0	53	Nebeker et al. (1985)
O. mykiss	E+L	Embryo survival, swim-up, hatching, fingerling survival, growth	> 466 µg/L	7.9	89	Brix et al. (2004)
D. rerio	E+L	Mortality (after 14 days)	90 µg/L	7.5-7.7	100	Dave and Xiu (1991)
D. rerio	E+L	Mortality (after 11 days)	10 mg/L	8.0	231,4	Present study

A, adult, L, larvae, E, embryos. Displayed are the lowest effect thresholds.

The delay of hatching at ≥10 mg Ni/L might be caused by an interaction of nickel with the hatching enzyme chorionase, a metal-protease (Hagenmaier, 1974). This effect is supported by other data, e.g. studies for zebrafish (45 µg/L (Geometric Mean of NOEC and LOEC, GM NOEC–LOEC), 6 mg Ni/L (LOEC) at an age of 96 h) (Dave and Xiu, 1991; Grabner, 2005) and studies for carp (*Cyprinus carpio*) (6 mg Ni/L) (Blaylock and Frank, 1979).

The additionally observed ´surface swimming` at ≥10 mg Ni/L could possibly be explained by a delay of hatching in these concentrations. Here the yolk sac seemed to be resorbed to a minor degree than in the control larvae of the same age. In histological sections small lipid droplets, presumably non-resorbed degradation products of the yolk, were visible below the swim bladder (R. Triebskorn, Tübingen, personal communication) in the respective nickel treatments, which might have provided buoyancy and therefore were responsible for the swimming behaviour at the surface.

Increased mortality of *D. rerio* due to exposure with Ni has been observed in another study as well. Dave and Xiu (1991) observed increased mortality at 360 µg Ni/L (GM NOEC-LOEC) and above when exposing *D. rerio* larvae (from 2 to 4 h after fertilization up to an age of 16 days without feeding) to nickel sulfate hexahydrate ($NiSO_4 \cdot 6H_2O$) (at a water hardness of 100 mg/L (as $CaCO_3$), pH 7.5 – 7.7). The fact that the mortality inducing concentrations in this study are clearly below those of the present study can be explained by the prolonged exposure time (11 vs. 16 days). So the starvation stress could have been considerably higher. Additionally it is also possible that the *D. rerio* strain used by Dave and Xiu (1991) was more sensitive.

In acute exposures oxygen concentrations of 2.45 ± 0.16 mg O_2/L and below both alone and in combination with low nickel concentrations resulted in significantly decreased activity compared to the control. Oxygen stress resulted in increased mortality in treatments with 3.23 ± 0.25 mg O_2/L and lower (both alone and in combination with Ni). At high nickel and low oxygen concentrations, the combined stressors possibly elicited an avoidance/escape response, reflected by higher locomotory activity compared to the single stressors. Decreased oxygen concentrations were associated with an increase in pH of up to 0.4 units. This should not strongly influence the toxicity of Ni as well as the zebrafish larvae. In studies of Hoang et al. (2004) an increase in pH from 7.97 to 8.54 increased the 96 h LC_{50} for fathead minnows (*Pimephales promelas*) only slightly, from 1.75 to 1.80 mg Ni/L, at a water hardness of 100 mg/L (as $CaCO_3$).

Naturally, zebrafish live in streams with high plant density at the riparian zone (Börries, 2006), so in these waterbodies low oxygen concentrations may locally occur. Various studies have shown that embryos and larvae of zebrafish can cope with low oxygen concentrations in certain age stages (Braunbeck et al., 2005; Padilla and Roth, 2001). These studies indicate that an age of 5 days seems suitable for the investigation of effects of oxygen depletion on *D. rerio* larvae. In earlier stages the tolerance to low oxygen levels is still very high (Braunbeck et al., 2005; Padilla and Roth, 2001). The oxygen consumption of 5-day-old larvae is higher than at the age of 7 and 8 days (Grillitsch et al., 2005).

No data were available on combined effects of pollutants and oxygen depletion. As a significant antagonistic action of O_2 deficiency and Ni treatment was detected in our study, the third hypothesis (Additional environmental stress (oxygen depletion)

increases $NiCl_2$ toxicity) could be accepted.

One reason for the relatively low toxicity of nickel to *D. rerio* larvae in the present study could be the low bioavailability of nickel at the relatively high water hardness (13 °dH; 231.4 mg/L as $CaCO_3$) and the high pH of the used reconstituted water (~ 8.0). According to Ji and Cooper (1996) at a $NiCl_2$ concentration of 10^{-1} and 10^{-2} M and a pH of 8.0 – 8.4 almost 100 % of Ni is available as $Ni(OH)_4^{2-}$. The shift in pH in our study as well as the tested concentration range of 0.5 – 15 mg Ni/L (corresponding to 8.52×10^{-3} M – 2.56×10^{-1} M $NiCl_2$) therefore should not have affected this availability. As for many other metals, toxicity of nickel on aquatic organisms decreases with increasing water hardness (Hoang et al., 2004; Pyle et al., 2002). Differences in bioavailability and therefore in toxicity for fish were emphasised in several studies (Hoang et al., 2004; Pyle et al., 2002). For larval fathead minnows the LC_{50} increased from 0.40 mg Ni/L when exposed in soft water (water hardness 20 mg/L as $CaCO_3$) to 1.57 mg Ni/L in hard water (water hardness 52 mg/L as $CaCO_3$) (Pyle et al., 2002). As mechanism for the toxicity of nickel to fish, several papers mentioned a respiratory rather than an ionoregulatory mechanism (Brix et al., 2004; Pane et al., 2003). Ionoregulatory toxicants like cadmium and copper disturb the Na or Ca balance at the gill what leads to several physiological dysfunctions that eventually cause mortality (Brix et al., 2004). Respiratory toxicity on the contrary resulted in accumulation of metals at the gills and related with this diminished oxygen consumption (Pane et al., 2003).

Adult rainbow trout reacted most sensitive to exposure to nickel by an avoidance reaction (Giattina et al., 1982). Reasons for the strong differences to the LOEC in the present study (7.5 mg/L) may be differences in water parameters, test and exposure systems, or a higher sensitivity of adult rainbow trout. Exposure of carp (*C. carpio*) to 6 mg Ni/L resulted in more dramatic effects on hatching rate (51.7 % compared to 92.3 % in the control) (Blaylock and Frank, 1979) than at the highest nickel concentration (15 mg/L) in the present study (77 % compared to 98.9 % in the control at the age of 96 h), probably due to the higher water hardness in this study (see Table 2). In general, adult rainbow trout seem to react more sensitive (attraction) to exposure with nickel than embryos and larvae of zebrafish, rainbow trout and carp. It has to be kept in mind that the results are only limited comparable because of the different test systems.

5. Conclusions

Nickel and low oxygen concentrations lead to diminished locomotory activity of 5-day-old zebrafish larvae in acute and subchronic exposures. In subchronic Ni exposures hatching rate and locomotory activity of the larvae were found to be equally sensitive but occurred at different age stages. Combined exposures to high Ni and low oxygen concentrations seemed to elicit an escape response of the *D. rerio* larvae.

Ecotoxicological studies based on behavioural parameters, which have yet been mainly conducted with adult fish, are also appropriate to fish larvae, since (1) behaviour was shown to be more sensitive in respect to exposure time and concentration than conventional parameters like mortality, and (2) a reduced use of adult fish is required for ethical reasons.

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Kapitel 2: Behavioural and developmental toxicity of chlorpyrifos and nickel chloride to zebrafish (*Danio rerio*) embryos and larvae

Cornelia Kienle[a], Heinz-R. Köhler[a], Almut Gerhardt[b]

[a]Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b]LimCo International, Oststrasse 24, D-49477 Ibbenbüren, Germany

Abstract

In order to assess the combined toxicity of environmental chemicals with different modes of action in acute (2h) and subchronic (11d) exposures, embryos and larvae of *Danio rerio* were exposed to a heavy metal salt, nickel chloride ($NiCl_2$), the insecticide chlorpyrifos (CHP) and their binary mixtures. Chlorpyrifos is an acetylcholine esterase inhibitor, which is likely to affect behaviour of the organism. $NiCl_2$ targets the active sites of enzymes and is regarded as an unspecific toxicant for aquatic organisms. Several endpoints, such as locomotor activity, morphological abnormalities, and mortality of *D. rerio* embryos and larvae were studied. During acute exposures to ≥0.25 mg/L of chlorpyrifos, locomotor activity tended to increase. However, this activity decreased significantly at ≥7.5 mg Ni/L. Subchronic exposures to CHP resulted in behavioural changes at much lower concentrations (≥0.01 mg/L) and considerably earlier than the observed increase in morphological abnormalities and mortality (LC_{50} (10d): 0.43 mg/L). Combined CHP and $NiCl_2$ mixtures led to an antagonistic deviation from the concept of independent action, in the case of locomotor activity. Compared to developmental or survival parameters, behaviour was the most sensitive endpoint for CHP exposure in this study: therefore we recommend this parameter to complement already established endpoints.

Keywords: locomotion, Multispecies Freshwater Biomonitor®, vitality, MixTox model

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1. Introduction

In the environment, organisms are usually exposed not just to a single pollutant but rather to a mixture of these chemicals. Different concepts exist to describe the combined toxic effects of these compounds. For example, the concept of concentration addition is based on the assumption that components of a mixture have a common molecular target site and therefore show a "similar" mode of action. This implies that the toxicity remains constant when a compound is replaced, completely or partially, by an equally effective amount of another chemical. This concept can also be applied when pollutants exhibit different modes of action, but still lead to a common toxicological endpoint, e.g. mortality or inhibition of reproduction (Faust et al., 1996). Another concept used to describe chemical mixture toxicity is that of independent action. This is based on the assumption that substances acting in combination attack different target sites of an organism. Therefore, they should show a 'dissimilar' mode of action (Faust et al., 1996). Consequently 'synergism', i.e. stronger effects than those expected from concentration addition, and 'antagonism', i.e. effects weaker than those predicted by the independent action model, may occur (Escher and Hermens, 2002).

An animal's behaviour integrates responses to internal (physiological) and external (environmental, social) factors and relates one organism to another (Evans, 1994). In this context, behavioural tests represent a sensitive method to detect effects of contaminants (Dell`Omo, 2002) as compared to conventional endpoints, such as mortality (e.g. Levin et al., 2003). Behavioural changes can be measured a short time after toxic chemical exposure (e.g Lindsay and Vogt, 2004). Developmental parameters are regarded to be sensitive as well (e.g. Nagel, 2002). Nagel (2002), proposed the embryo test with *Danio rerio* (DarT) as replacement for the acute fish test with adult fish. In a prolonged embryo test, which lasted up to 96 h (Scheil et al., 2009) only a few effects of chlorpyrifos and $NiCl_2$ on developmental parameters were found, such as a decreased hatching rate due to $NiCl_2$ exposure. In this study, we extended the DarT test for up to 11 d to investigate whether prolonged exposure would reveal developmental and behavioural effects of test substances on *D. rerio*, or whether the results of the embryo test were representative for prolonged exposure as well.

For the mixed chemical experiments in the present study two dissimilarly acting compounds, the insecticide chlorpyrifos (CHP) and the heavy metal nickel, were chosen.

Mixtures of pesticides and metals may occur in surface waters (e.g. near vineyards) as well as in agricultural areas near metal-processing industries. As nickel is also a naturally occurring trace metal (Duke, 180) and highly soluble in water (Merck, 2004), co-occurrence of nickel and pesticides in surface waters is highly probable.

Nickel(II) chloride ($NiCl_2$) is of high environmental importance, because it is not biologically degradable and has been shown to exert long-term harmful effects to aquatic biota (Merck, 2004). Ni can act in an unspecific way on the active sites of enzymes and, furthermore, it can behave as an oxidative stressor and carcinogen. Environmental concentrations of this heavy metal range from 0.001 – 0.01 mg Ni/L (unpolluted Canadian rivers and lakes) up to 0.5 and 2 mg Ni/L (natural waters near industrial sites) with a maximum of 183 mg Ni/L near a nickel refinery in Sudbury, Ontario, Canada (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987).

The insecticide chlorpyrifos (CHP) is a broad-spectrum organophosphate compound (Kamrin, 1997), which forms the active ingredient in Dursban™ and Lorsban™ insecticides, which are among the most widely used insect control products (Dow AgroSciences, 2008). They act on pests primarily as a contact poison, with some additional effectiveness as a stomach poison, and they are regarded as highly toxic to freshwater fish. CHP acts on the nervous system as an inhibitor of the enzyme acetylcholinesterase and accumulates in the tissues of aquatic organisms (Kamrin, 1997). The highest measured environmental concentrations of CHP were about 0.3 µg/L in surface waters in the United States (Gilliom et al., 2006).

To date, no studies concerning the effects of pesticide and metal mixtures on the behaviour and development of fish are available. Therefore, the aim of the present study is to quantify these effects by exposing early life stages of zebrafish (*D. rerio*) to the organophosphate insecticide chlorpyrifos and the heavy metal $NiCl_2$ in acute and subchronic tests. The following hypotheses were tested for embryos and larvae of zebrafish:

1. Acute exposure to $NiCl_2$ and/or CHP results in a higher locomotor activity (LA), indicating a possible avoidance reaction of the test organisms.
2. The toxicity expected by mixtures of CHP and $NiCl_2$ deviates from the concept of independent action.
3. Sensitivity to CHP, $NiCl_2$, and their mixture is exposure time-dependent for developmental and behavioural parameters.

2. Materials and Methods

2.1 Maintenance of test animals

Adult zebrafish (*D. rerio*, WIK strain, MPI for Developmental Biology, Tübingen) of both sexes were kept in 150 – 230 L aquaria with aerated and filtered water (50/50 % mixture of tap and distilled water to achieve a conductivity of approximately 400 µS/cm) at a density of ≤1/L. A temperature of 26 ± 1°C and a pH of ~8 were maintained, with a 12:12 h light:dark cycle without dimming. Dry flake food (Nutrafin Max, Hagen, Germany) and frozen crustaceans (*Moina* sp., Bosmidae) or midge larvae (MM Aquaristik, Germany) were given as food twice per day *ad libitum*.

2.2 Acquisition of eggs

The eggs for the tests were gathered with spawn traps placed on the bottom of each aquarium the evening before spawning. The spawn traps were removed from the aquaria in the morning (1 h after triggering the spawning via switching on the light). The eggs were transferred to Petri dishes containing reconstituted water (OECD, 1992, Guideline 203). Two to four hours after fertilization the fertilized eggs were separated and distributed over several Petri dishes containing test water (30 eggs per Petri dish). To prevent contamination with proliferating Protozoa, the eggs were transferred into new Petri dishes with fresh reconstituted water once after 24 h. The eggs were kept at a temperature of 26±1 °C with a 12:12 h light:dark cycle. Approximately half of the test water was exchanged every second day. The condition of the larvae was checked daily under a stereomicroscope for morphological abnormalities, mortality as well as behavioural anomalies.

Any studies involving experimental animals were conducted in accordance with national and institutional guidelines for the protection of animal welfare.

2.3 Acute exposure experiments with nickel chloride, chlorpyrifos and binary mixtures

For the acute exposure experiments (2 h exposure at an age of 5 d post fertilization, dpf), embryos and larvae were raised in glass Petri dishes with reconstituted water as described previously up to an age of 5 dpf. Malformed or inactive embryos and larvae were removed prior to the experiments. At 5 dpf, the larvae were exposed to the respective test chemical concentrations acutely for 2 hours while measuring locomotor activity (procedure described in 2.5). Eight nominal concentrations for each substance

were examined with two negative controls each. For Ni alone, concentrations of 0.25, 1, 2.5, 5, 7.5, 10, 12.5, and 15 mg Ni/L were tested and for CHP alone amounts of 0.0001, 0.001, 0.01, 0.1, 0.25, 0.5, 0.75 and 1 mg of CHP/L were used. The mixed chemical concentrations were chosen following the Box-Behnken design (Box and Behnken, 1960), aiming at a rational distribution of the data points over the response surface. Nine ratios of $NiCl_2$ to CHP, plus two negative controls, were tested: 0.5 + 0.1, 2.5 + 0.25, 5 + 0.5, 7.5 + 0.25, 7.5 + 1, 10 + 0.5, 12.5 + 0.75, 15 + 0.25 and 15 + 1 (mg Ni/L + mg CHP/L, respectively) (Fig 1A).

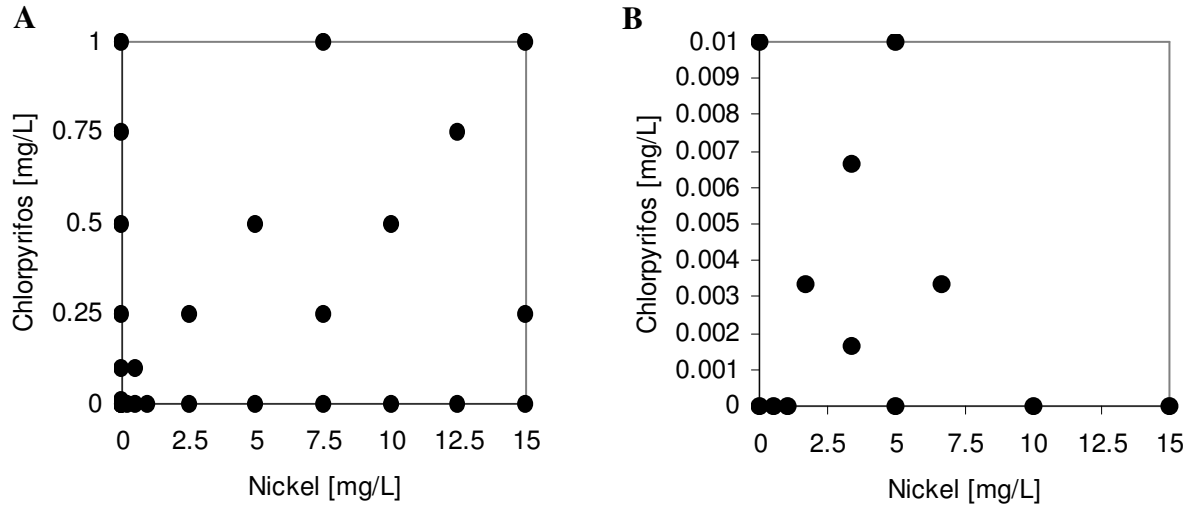

Fig. 1. Test design for acute (A) and subchronic (B) mixture experiments with nickel [mg/L] and chlorpyrifos [mg/L]. For exact concentrations see text. Concentrations were chosen to be as evenly distributed in the Ni×CHP matrix as possible.

2.4 Subchronic test with nickel chloride and/or chlorpyrifos

The subchronic test (exposure from ≤1 hpf up to 11 dpf) was conducted according to the VMD Guidance Note "Ecotoxicity testing of medicines intended for use in fish farming" (VMD, 1996) using a semi-static test design, with partly water exchange every second day. The exposure of the organisms to CHP, $NiCl_2$, and their mixture started at the time of fertilization (≤1 h) and was terminated at an age of 11 d. Experiments were performed in glass Petri dishes (for CHP exposures and CHP/$NiCl_2$ mixtures) and in plastic Petri dishes (for $NiCl_2$ exposures) with 30 fertilized eggs in each dish, and three replicates per concentration. Glass or plastic Petri dishes were used to avoid possible interactions of the chemicals with the vessel. During the exposure time selected developmental endpoints were recorded daily, from one up to 11 dpf, including the rate

of deformations and mortality. Four larvae from each replicate were randomly removed at regular intervals (5, 8, and 11 d post fertilization) for measurements of the locomotor activity (see 2.7). During behavioural measurements, the larvae remained exposed to the same solutions as for the respective subchronic exposures. No food was provided during the experiments, as zebrafish can live from their yolk sack up to 12 d after fertilization (Rombough, 2002). As we could observe no increased mortality in the control treatments, we assumed that the animals were well and not starving.

Five nominal concentrations for each single substance were tested with one negative control each (for Ni alone: 0.5, 1, 5, 10, and 15 mg Ni/L and for CHP alone: 0.01, 0.1, 0.25, 0.5, and 1 mg CHP/L). The calculation of mixed concentrations was based on the LOECs (= 1 toxic unit, 1 TU) for the most sensitive parameter obtained in the single substance tests (LOECs for locomotor activity: 0.01 mg CHP/L, 10 mg Ni/L). In the mixed chemical experiment, combinations of the two substances were equal to either 0.5, 1, or 1.5 TU in a two-ray design with 1/3 of the TU of chemical 1 and 2/3 of the TU of chemical 2 combined and vice versa (see Fig. 1B). Five Ni/CHP combinations with one negative control were examined: 3.333 + 0.0017, 1.667 + 0.003, 6.667 + 0.003, 3.333 + 0.0067, 5 + 0.01 (mg Ni/L + mg CHP/L, respectively) (Fig 1B). Approximately half of the respective test solutions were changed every second day.

Optimal conditions for the larvae were provided in control treatments (25.3 ± 0.8 °C, 7.94 ± 0.24 mg O2/L, pH 7.99 ± 0.14, 640 ± 17 µS/cm; means ± SD, n = 6). An increase in electric conductivity up to 719 ± 19 µS/cm (mean ± SD, n = 13) with increase in Ni salt concentration was detected, however, still within a tolerable range for the zebrafish embryos and larvae (Grabner, pers. comm., 2005).

2.5 Measurement of locomotor activity

Measurement of locomotor activity in acute and subchronic exposure experiments was performed with the Multispecies Freshwater Biomonitor® (MFB) (LimCo International, Germany), an online biomonitor for continuous and quantitative recording of the behaviour pattern of animals (Gerhardt et al., 1994) as described in Kienle et al. (2008). The behavioural signal of the animal was analysed by a fast Fourier transformation, resulting in a histogram of different signal frequencies (Gerhardt et al., 1994).

In summary, the test chambers were placed into glass aquaria (20x20x15 mm^3, 5 L) or polyethylene vessels (208x208x64 mm^3, 2.77 L) filled with 1.5 L (chlorpyrifos, Ni/CHP-

Mixtures) or 2 L (nickel) of the respective solution. To eliminate disturbance from movement along the vessels, they were arranged in duplicate in a surrounding black basin with temperature-adjusted water (26 ± 1°C) and illuminated from above during the measurements (58 W neon light at 145 cm distance to the chambers). The larvae were transferred carefully into the chambers (one larva per chamber), the lid was closed and the remaining air bubbles in the chambers were removed with a Pasteur pipette. Subsequently, the chambers were placed horizontally on the bottom of the test vessel. The measurements were started after an acclimation time of 10 min and the behaviour of 11 - 12 larvae per treatment was continuously recorded for 2 h in intervals of 10 minutes. Each measurement was performed for 4 min. No food was provided to the larvae during the experiments.

2.6 Test substances

Chlorpyrifos (Sigma-Aldrich, Germany) was dissolved in reconstituted water (OECD, 1992, Guideline 203), which was constantly stirred for at least 4 hours in order to prepare a stock solution of 1 mg/L at a water temperature of 45 °C and a pH of 8.0. Subsequently, the solution was kept at 35 °C overnight until use with constant stirring. Nickel(II) chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) (Roth, Germany) was dissolved in reconstituted water in order to prepare a stock solution of 1 g Ni/L at pH 7.5. All test solutions were prepared directly before use.

2.7 Analytical conformation of test substance concentrations

The chlorpyrifos concentration was determined using gas chromatography–mass spectrometry (GC-MS) (HP5890 series II, Hewlett-Packard, Waldbronn, Germany) using a chlorpyrifos standard (Dr. Ehrenstorfer, Augsburg, Germany) at a concentration of 1 mg/L in reconstituted water. The substance was extracted from the aqueous, acidified solution with dichloromethane by shaking in a separating funnel for 3 minutes. Once the two phases had separated thoroughly, the solvent phase was dried using Na_2SO_4, and was then filled in a 50 mL rotovap bulb. Subsequently, the solvent was ablated in the rotating evaporator to a volume of 1 mL. The sample volume, containing the chlorpyrifos insecticide, was filled in GC-MS sampling vials and the concentration of insecticide was determined by GC/MS. The stock solution and highest test concentration of 1 mg/L was measured using an injection volume of 1 µL.

Analytical confirmation of nickel concentrations was performed by flame atomic absorption spectroscopy (F-AAS, Perkin-Elmer M1100, Waltham, MA, USA) at two characteristic wavelengths (232 and 341.5 nm) using a Tritisol nickel standard (1000 ± 2 mg nickel(II) chloride in water, Merck Darmstadt, Germany). The analysis was performed with an air/acetylene mixture at a flow rate of 2.5 L/min (C_2H_2) and 8 L/min (oxidant) and a gap width (monochromator) of 0.7 H. For the calibration curve, nickel concentrations of 0.5, 1, 2, and 5 mg/L were diluted from the Tritisol standard in MilliQ® (18.2 mΩ/cm) (Millipore Corporation, Billerica, MA, USA). The stock solution for the tests (1000 mg Ni/L) and test dilutions of 0.5, 1, 5, 10, and 15 mg/L were measured for nickel concentrations using F-AAS.

2.6 Data analysis

Means of the percentage of time spent on locomotion were calculated for each larva separately for the first and the second hour in order to take into account early warning reactions and the decrease of locomotive activity over time. The data on the 'percentage of time spent on locomotion' were arcsin transformed, from proportional values, for statistical evaluation. As the data were only partially normally distributed (one sample-Kolmogorov-Smirnov-Test, SPSS 10.0.1, USA), non-parametric methods of statistical analysis were chosen. The data from all tests were analysed for significance by means of Friedman's ANOVA (Statistica 5.0, StatSoft, USA) with a subsequent Wilcoxon two-group test (JMP 4.0, SAS systems, USA) to detect differences between control and substance exposure treatments. A linear regression analysis was performed for acute and subchronic nickel and chlorpyrifos measurements, using the equation $y = ax + b$, with the locomotor activity (LA) as y and the toxicant concentration [CHP] or [Ni] as x (JMP 4.0, SAS systems, USA). In the regression equation a is the slope of the line and b the intercept. The MixTox Model (Jonker et al., 2005) was applied to calculate the type of responses to mixtures. Significance levels were defined as follows: $p < 0.001$ highly significant: ***, $p < 0.01$ strong significance: **, $p < 0.05$ significant: * and $0.05 < p > 0.1$ tendency to be significant: (*). The LC_{50} after 10 d for CHP and the LC_{20} after 11 d for Ni were calculated using Table Curve™ 2D 5.1 (SYSTAT Software Inc., USA) Software.

3. Results

3.1 Measured concentrations

The retrieval rate of chlorpyrifos was 51.6 % (nominal concentration 1 mg/L). Nickel retrieval rates were in the range of 101.6-104.7 % of the nominal concentrations (see Table 1).

Table 1: Nominal and measured nickel concentrations [mg/L] and retrieval rate of nominal concentrations; mean ± SD of six replicate measurements (mean 232 and 341.5 nm)

Ni [mg/L] (nominal)	Ni [mg/L] (measured) (mean 232 and 341.5 nm)	Retrieval rate of nominal concentrations
Control	0.00 ± 0.00	-
0.5	0.52 ± 0.06	104.2 ± 12.7
1	1.02 ± 0.06	102.1 ± 5.9
5	5.08 ± 0.18	101.6 ± 3.6
10	10.47 ± 0.92	104.7 ± 9.2
15	15.42 ± 0.47	102.8 ± 3.1
1000	944.98 ± 16.42	94.5 ± 1.6

3.2 Behavioural toxicity in acute and subchronic exposures

Movement pattern: In control treatments, *D. rerio* larvae showed continuous locomotor movements without pauses, as suggested by the regular and constant peaks in the movement pattern over time (Fig. 2A). When exposed to CHP, the larvae showed a typical aberrant behaviour at CHP concentrations of 0.25 mg/L and higher. This abnormality consisted of paused jerky movements, as shown by the movement pattern in figure 2B. Muscular cramps could also be observed. No such effect was visible during $NiCl_2$ exposure.

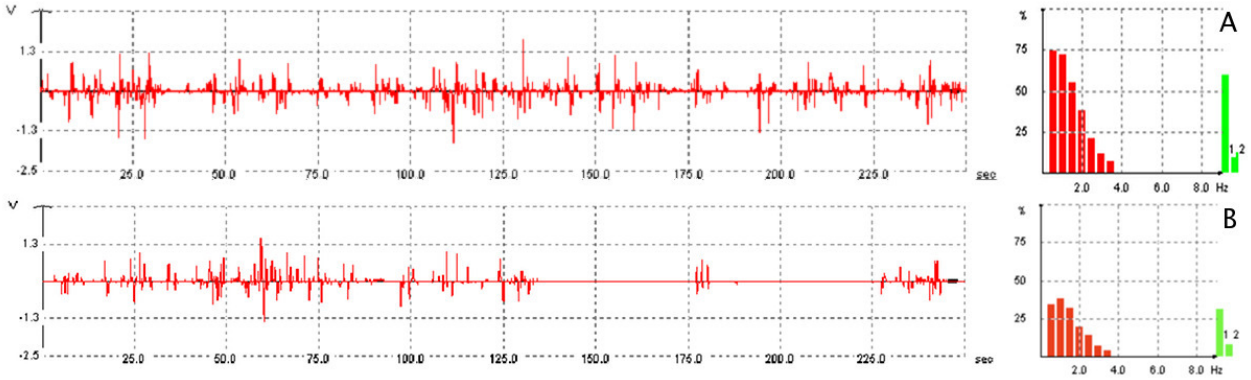

Fig. 2. Examples of spontaneous locomotor movement patterns **A:** movement pattern (amplitude [V] vs. time [sec]) (left) and fast Fourier transformation (FFT) histogram [activity in % of the time (250 s)] vs. frequency [Hz] (right) of a 5-d-old *Danio rerio* larva in control treatment showing continuous locomotor movements over time. **B:** Movement pattern (left) and FFT histogram (right) of a 5-d-old *D. rerio* larva acutely exposed to 1 mg CHP/L, showing decreased locomotor activity with pauses.

Acute exposure to chlorpyrifos resulted in a slight concentration-dependent increase in locomotor activity. This was defined as the percentage of time the animal spent on locomotion (linear regression analysis: $p = 0.083$; $LA = 0.581 + 0{,}068[CHP]$, $r^2 = 0.194$, $n = 130$) (Fig. 3B). A significant decrease in locomotor activity with increasing nickel concentration was detected ($p < 0.001$, $LA = 0.702 - 0.0168[Ni]$, $r^2 = 0.188$, $n = 117$) (Fig. 3A). This resulted in a calculated LOEC of 7.5 mg Ni/L (significant difference vs. the control: $p < 0.001$, Friedman's ANOVA; $p = 0.005$, Wilcoxon test).

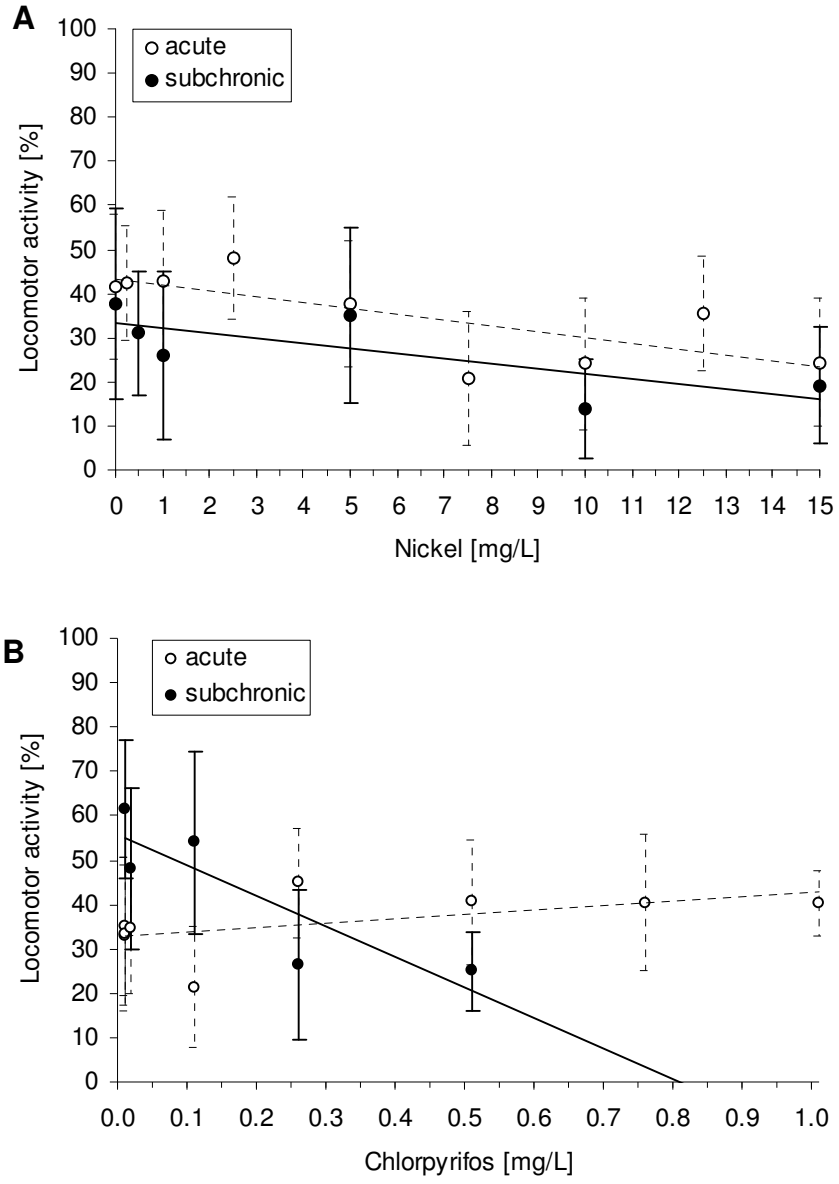

Fig. 3. Locomotor activity (percent of total time spent in locomotion) of 5-d-old *D. rerio* larvae acutely (2 h exposure at 5 dpf) and subchronically (from ≤1 hpf up to 11 dpf) exposed to different nickel (**A**) and chlorpyrifos (**B**) concentrations [mg/L] in single exposures. Data of the second hour of measurement are displayed respectively.

When testing nickel in combination with low CHP concentrations (0.25 mg CHP/L) in acute exposures, the locomotor activity-decreasing effect of Ni dominated over CHP. Whenever Ni was combined with high CHP concentrations (1 mg/L), the activity-increasing effect of CHP dominated (Fig. 4). The calculation of the data with the MixTox model (Jonker et al., 2005) did not reveal any significant results for independent action. No mortality was observed in the acute exposure experiments.

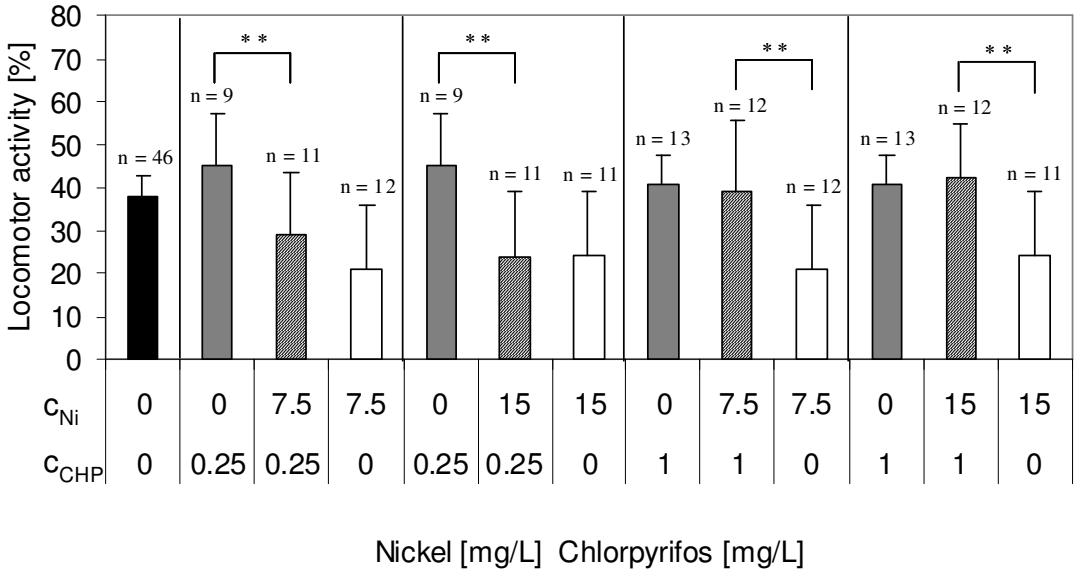

Fig. 4. Comparison of the locomotor activity (percent of total time spent in locomotion) of *D. rerio* acutely exposed to different nickel and chlorpyrifos concentrations [mg/L] single and in binary mixtures. Significant differences between treatments: **p<0.01. Data of the second hour of measurement are displayed respectively.

In subchronic exposures, locomotor activity decreased significantly at an age of 5 d (linear regression analysis: Ni: $p = 0.008$, $LA = 0.589 - 0.0130[Ni]$, $r^2 = 0.106$, $n = 65$, CHP: $p < 0.001$; $LA = 0.849 - 0.778[CHP]$, $r^2 = 0.358$, $n = 64$) (Fig. 3A and B), resulting in significant differences to the control treatments at concentrations of ≥10 mg Ni/L and ≥0.01 mg CHP/L (Ni: $p < 0.001$, Friedman ANOVA; $p = 0.028$, Wilcoxon test; CHP: $p < 0.001$, Friedman ANOVA; $p = 0.013$, Wilcoxon test). Data analysis with the MixTox model revealed a significant antagonistic deviation from independent action ($p = 0.006$). The response surface for mixture exposures is displayed in Fig. 5.

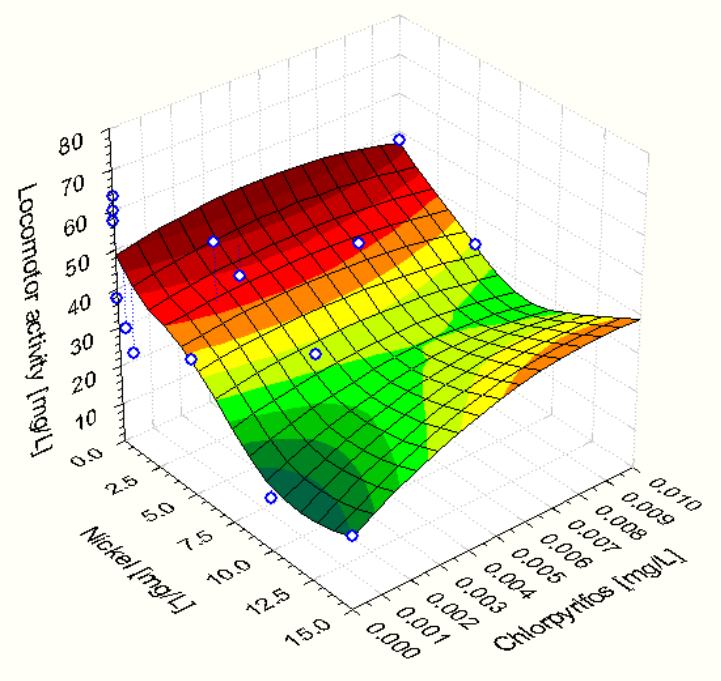

Fig. 5. Locomotor activity (percent of total time spent in locomotion) of 5-d-old *D. rerio* larvae exposed to different nickel and chlorpyrifos concentrations [mg/L] alone and in binary mixtures in subchronic exposure (from ≤1 hpf up to 11 dpf). Data of the second hour of measurement are displayed as surface plot with isobolic lines calculated on the basis of means.

3.3 Developmental toxicity in subchronic exposures

3.3.1 Deformations

D. rerio larvae exposed to CHP showed a significant increase in the percentage of individuals with morphological deformations from an age of 4 and 5 d onwards at both 0.25 and 0.5 mg CHP/L (Fig. 6). In these treatments the larvae suffered from an unnatural bending of the spine (100 %, 0.5 mg CHP/L, day 7, n = 24) in concentrations of 0.25 and 0.5 mg CHP/L. Additionally, heart oedema (23.3 %, 0.5 mg CHP/L, day 7, n = 24) and other deformations of the spine occurred, apart from bending (5.4 %, 0.5 mg CHP/L, day 7, n = 24) (Fig. 7A-D). In contrast, $NiCl_2$ did not induce morphological deformations. Also, in the subchronic test with binary mixtures of CHP and $NiCl_2$, which comprised only CHP concentrations of 0.01 mg/L and lower, no spine deformations were observed.

3.3.2 Mortality

Up to an age of 12 d, no increased mortality occurred in the control treatments. Mortality increased significantly at 8 dpf and 0.5 mg CHP/L ($p = 0.034$, Friedman`s ANOVA; $p = 0.046$, Wilcoxon test) up to 100 % mortality at 10 dpf, with a calculated $LC_{50}$ of 0.43 mg CHP/L. In the case of $NiCl_2$, mortality increased significantly at 11 dpf and 10-15 mg Ni/L ($p = 0.016$, Friedman`s ANOVA; $p = 0.046$ and $p = 0.043$, Wilcoxon test) up to

39.4 ± 5.3 %, resulting in a calculated LC_{20} of 9.5 mg Ni/L. In the subchronic test with binary mixtures of CHP and $NiCl_2$, no increased mortality was observed due to the lower concentration range tested.

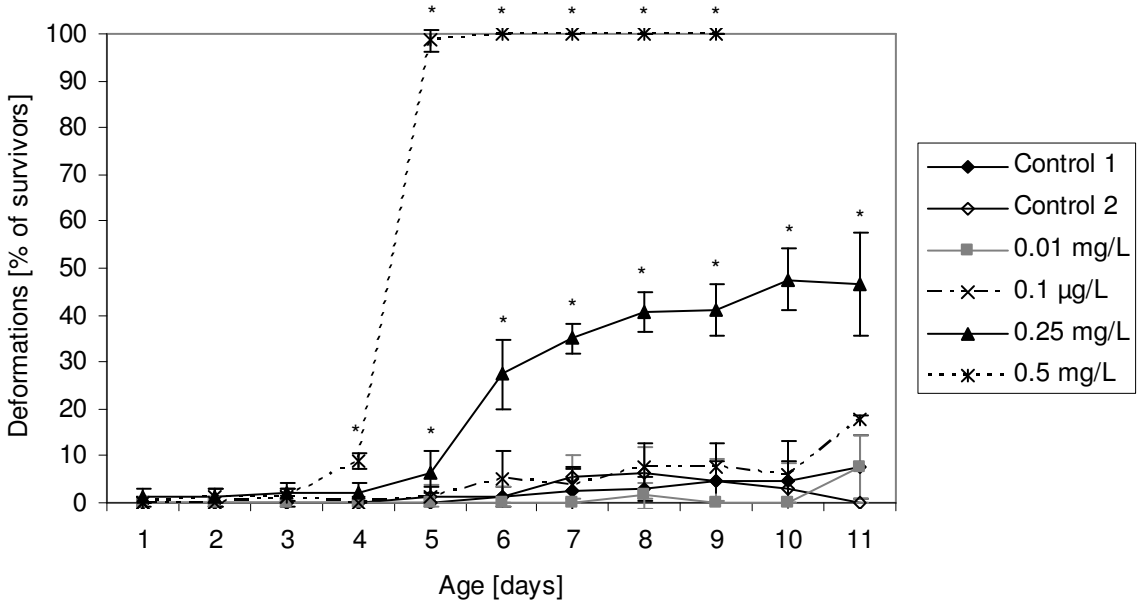

Fig 6. Deformations (% of survivors) of *D. rerio* larvae exposed to different chlorpyrifos concentrations [mg/L] (mean ± SD; number of larvae per replicate: 30 (days 0 - 5); 26 (days 6 - 8); 22 (days 9 - 11), 3 replicates each experiment). Significant differences to control treatment: *p<0.05. Only the deformations of surviving larvae are displayed.

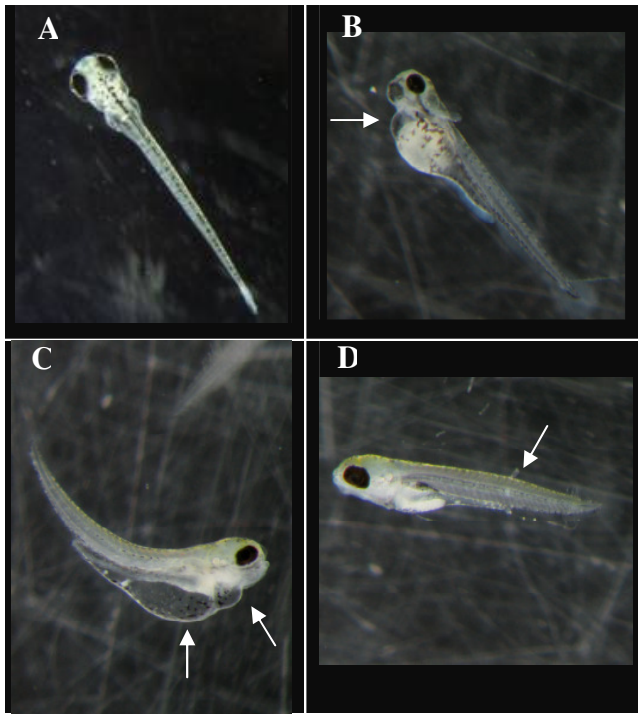

Fig. 7. Selected morphological malformations: (**A**) 5-day-old control larva of *Danio rerio*, (**B**) 5-day-old larva exposed to chlorpyrifos (CHP) with heart edema, (**C**) 6-day-old larva exposed to CHP with heart edema and abnormal bending of the spine, (**D**) 5-day-old larva exposed to CHP with deformations of the spine.

4. Discussion

This study represents the first approach to investigate behavioural and developmental effects of mixtures of pesticides and metals on fish. We aimed at quantifying this aspect in acute as well as subchronic exposures with early life stages of zebrafish (*D. rerio*) using the organophosphate insecticide chlorpyrifos and the heavy metal nickel chloride.

Measured concentrations

The detection rates for nickel were about 100 % of the nominal concentrations (101.6-104.7 %). This corresponds well with the good water solubility of nickel(II) chloride of 2540 g/L (Merck, 2004). The lower detection rate of chlorpyrifos (51.6 %) may be explained by the partial solubility of the added pesticide, even though a water solubility of 2 mg/L at 25 °C was expected (Kamrin, 1997) and no suspended particles could be observed. Adhesion of the pesticide to the surface of the glass bottles could also have played a role.

Mixture toxicity of CHP and $NiCl_2$ regarding acute and subchronic behavioural effects

In acute exposures, locomotor activity decreased significantly at 7.5 mg Ni/L but tended to increase with increase in chlorpyrifos concentrations as well as in binary mixtures containing $NiCl_2$ (Fig. 3). However, the observed increase in locomotor activity with increase in CHP concentrations and in binary mixtures also containing Ni suggests the lack of an avoidance reaction, but an increase in activity due to the effects of CHP on the nervous system, causing muscular cramps (Kamrin, 1997). This was observed after an acute exposure time of 2 hours. A first escape response to CHP, indicated by a higher degree of locomotor activity compared to the control, would only be possible when the larvae were able to detect the substance by means of their chemosensory systems before the effects on the nervous system occurred. This could not be verified, as no literature data were available concerning this matter. Due to the reasons stated above, our hypothesis, stating that 'Acute exposure to $NiCl_2$ and/or CHP results in a higher locomotor activity indicating a possible avoidance reaction of the test organisms' could not be proven for the investigated substances and mixtures.

Generally, CHP was shown to be much more toxic than Ni in our tests (effects at ≥0.25 mg/L and ≥0.01 mg/L in acute and subchronic tests, respectively, with Ni at ≥7.5-10 mg/L). Because of the different modes of action of CHP (AChE inhibitor) and $NiCl_2$

(enzyme function, respiratory mechanism), it is likely that both substances act completely independent of each other. For acute exposures, the two substances elicited different results regarding locomotor activity (an increase was observed with CHP and a significant decrease with Ni exposure). This made it quite difficult to judge mixture toxicity. In subchronic exposures, a significant antagonistic deviation from the independent action concept could be detected.

Examples of the effects of binary mixtures on aquatic organisms can be found in the literature. Atrazine and organophosphate insecticides, both having different modes of action, showed a synergistic effect on *Chironomus tentans* (Pape-Lindstrom and Lydy, 1997) whereas mixtures of Ni and chromium (1:1-ratio with LC_{50} concentration) (96 h-LC_{50} 16.46 mg/L (nickel) and 13.58 mg/L (chromium)) elicited an additive effect in guppies (*Poecilia reticulata*) (Khangarot and Ray, 1990). Binary pesticide-metal mixtures (copper-malathion, cadmium-malathion, cadmium-dichlorvos) showed synergistic effects on the marine microcrustacean *Tigriopus brevicornis* (Copepoda) (Forget et al., 1999). However, no studies concerning the effects of metal and pesticide mixtures have been found in the literature so far for zebrafish.

Altogether, our hypothesis 'The toxicity expected by mixtures of CHP and $NiCl_2$ deviates from the concept of independent action.' could not easily be proven for mixtures of CHP and $NiCl_2$ in acute exposures, as both substances elicited different behavioural responses; however an antagonistic deviation from independent action could be proven for subchronic exposures regarding locomotor activity.

Time dependency of CHP, Ni, and mixture toxicity in subchronic exposures

The most pronounced behavioural effects occurred at an age of 5 d for single substance, as well as for mixture exposures. Compared to acute exposures, the subchronic test was much more sensitive to CHP concerning locomotor activity (0.25 mg/L (acute) *vs.* 0.01 mg/L (subchronic)), whereas the response levels for Ni exposure were quite similar in acute and subchronic exposures (7.5 mg/L (acute) *vs.* 10 mg/L (subchronic)). Owing to the fact that subchronic exposures with nickel were carried out in plastic Petri dishes and subchronic exposures with chlorpyrifos and mixtures of chlorpyrifos and nickel chloride in glass vessels, different adsorption behaviour of nickel to the surface of the glass vessels could have played a role in mixture toxicity. However, as the concentration of nickel in the mixtures was much higher (1 TU = 10 mg/L) compared to chlorpyrifos (1

TU = 0.01 mg/L), the probable difference in adsorption seems to be of minor importance.

Thirty-day-old Japanese medaka (*Oryzias latipes*) exposed to chlorpyrifos displayed different behavioural and morphological symptoms like loss of equilibrium. These included hypoactivity, underreactivity to startle response, haemorrhage in the caudal area, and deformities (scoliosis and/or lordosis, forward pointing of the pectoral fins) (Rice et al., 1997). These symptoms were consistent with the three general modes of action response syndromes (hyperactivity, hypoactivity, and physical deformity) Drummond and Russom (1990) mentioned for categorizing a range of investigated neurotoxic chemicals, with each syndrome or sign of stress indicating a different mode of action. At higher CHP concentrations, a shorter time until initial onset of morphological effects and mortality was observed. The symptoms of CHP toxicity observed in *D. rerio* embryos and larvae, in the present study, were qualitatively similar to the results in those studies with respect to behavioural responses (hypoactivity, Fig. 2 and 3) as well as to deformation types, such as lordosis, a sustained, abnormal spinal curvature with a convex form of the dorsal surface (Rice et al., 1997) (Fig. 7C).

A significant increase in deformations could only be observed for subchronic exposure to chlorpyrifos (Fig. 6), whereas no deformations were observed in exposures with $NiCl_2$ and binary mixtures, most likely due to the low CHP concentrations used (max. 0.01 mg/L) in the mixture experiments. In the subchronic test, mortality increased with exposure time in higher concentration levels (0.5 mg CHP/L, 10 mg Ni/L). However, in mixture experiments no increased mortality was observed also due to the reason stated above. In a prolonged embryo test with zebrafish, Scheil et al. (2009) did not observe any increase in mortality within the exposure period of 96 h at concentrations of up to 1 mg CHP/L as well as in mixtures of Ni and CHP. Exposure to 100 ng CHP/L from 1-5 dpf was found to lead to elevated mortality rates in zebrafish from 20-38 weeks of age (Levin et al., 2003). In 30-day-old Japanese medaka (*Oryzias latipes*), the 48-h LC_{50} for chlorpyrifos was 0.25 mg/L (Rice et al., 1997). From our results and those in the cited literature, it becomes clear that at these low concentrations of CHP no mortality should be expected.

As shown above, the toxic effects of CHP on the monitored parameters: locomotor activity, deformations, and mortality increased with exposure time for development and

survival. No significant differences in behaviour were recorded after prolonged exposures to Ni and CHP at 8 and 11 dpf. Our hypothesis that 'Sensitivity to CHP, Ni and binary mixtures of these chemicals is exposure time-dependent for developmental and behavioural parameters' was therefore proved true.

Comparison of test systems related to their sensitivity towards different endpoints

The reaction of an organism to pollutants occurs on a biochemical as well as on an individual level. On a biochemical level, e.g. with nerve poisons, a physiological reaction already must have taken place to elicit a behavioural response. Similar effects on behaviour occur within certain groups of action, e.g. muscular cramps with AChE inhibitors; hence, in this case, for example, it is possible to conclude effects on a biochemical or histological level. In the following interpretation, the methods used in this study shall be compared with previously used test routines for their sensitivity to different endpoints.

Parallel to this work, stress protein (hsp 70) and histopathological investigations have been conducted in the presence of nickel chloride and chlorpyrifos using prolonged embryo tests with zebrafish (Scheil et al., 2009). Hatching rate decreased significantly with increase in Ni concentrations, but no effects on deformations and mortality were observed in the prolonged embryo test up to an age of 96 h in concentrations of up to 15 mg Ni/L and 1 mg CHP/L. The hsp70 level tended to be higher than in the control level (1 mg Ni/L), indicating an increase in stress protein production, and significantly lower at ≥10 mg Ni/L, indicating a pathological response. With CHP, an increased hsp70 level occurred only at 0.1 mg/L. Histopathological effects were obvious in the gut at ≥20 mg Ni/L and in liver, gut, pancreas, and skin at ≥0.6 mg CHP/L (Scheil et al., 2009).

These results show that the sensitivity of the test systems can be varied in the presence of different substances. Concerning the metal Ni, the prolonged embryo test revealed effects on the hatching rate earlier, but in the same concentration range than the other test systems. Considering the organophosphate, chlorpyrifos showed no morphological effects in the prolonged embryo test up to concentrations of 1 mg CHP/L, whereas with longer exposure duration such effects occurred already at lower concentration levels. Behavioural effects in the subchronic test occurred at lower CHP concentrations of 0.01 mg/L.

It can be concluded that behaviour was the most sensitive parameter for the

acetylcholinesterase inhibitor chlorpyrifos but was equally sensitive to the other parameters for nickel chloride exposure. This shows that it depends very much on the substance and its mode of action which parameter reacts first. Also the exposure time is important, e.g. if one only looks at the prolonged embryo test for CHP exposure results, no risk for fish might be concluded. However, in the subchronic test nearly all relevant effects occurred after the test duration of 96 h for this test.

Environmental relevance

The effects of CHP and $NiCl_2$ on *D. rerio* larvae were tested within the range of environmentally relevant concentrations (0.0003 mg CHP/L in different surface waters in the USA (Gilliom et al., 2006); 0.001-0.01 mg Ni/L in unpolluted Canadian rivers and lakes, 0.5-2 mg Ni/L in natural waters near industrial sites with a maximum of 183 mg/L (Chau and Kulikovsky-Cordeiro, 1995; Kasprzak, 1987)).

Hence, an acute risk of chlorpyrifos for fish, even if they were much more susceptible to pollutants than the zebrafish, can presumably be excluded. But subchronic effects are more relevant, especially regarding behaviour, as they occur already at lower concentrations. Exposure of zebrafish larvae to CHP can, although occurring only over a short time, influence the behaviour up to the adult stage (Levin et al., 2003), which might also be true for other fish species. In the environment, a decreased activity caused by exposure to nickel and/or chlorpyrifos could, on the one hand, lead to an easier capture of fish larvae by predators. However, their diminished activity could make them less recognizable for predators, as observed with mummichog larvae (*Fundulus heteroclitus*) (Zhou and Weis, 1999). Additionally, food-searching behaviour could also be affected due to the diminished activity with subsequent negative impacts on their growth and fitness. Acute or subchronic exposure (at high concentrations) could provide an easier capture of fish larvae in the wild due to the toxicant-related cramps and jerky movements, as have already been observed with juvenile (21-32 d old) medaka (*Oryzias latipes*) (Carlson et al., 1998). They also would have only a low chance of escape, since the inhibition of acetylcholine esterase also influences perception (Kamrin, 1997).

The highest concentrations we tested for CHP are unlikely in natural surface waters because of the low solubility and the fast adsorption of CHP on sediment particles. However, as shown in our study, subchronic effects can occur at very low concentrations and therefore make the observed effects environmentally relevant. It has to be kept in

mind that more sensitive species than the zebrafish might be at a much greater risk from CHP exposures at lower concentrations than those discussed in this study.

Conclusions

The effects of nickel and chlorpyrifos on critical parameters, including: deformations, mortality and locomotor activity were dependent on exposure time in subchronic exposures. Behaviour was the most sensitive parameter for the acetylcholinesterase inhibitor chlorpyrifos in subchronic exposures. In acute exposures, the two substances elicited different behavioural results. Our results show that it depends on the investigated substance and its respective mode of action which endpoint reacts first and in which concentration range.

Acknowledgements

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

Volker Scheil[a*], Cornelia Kienle [a*], Raphaela Osterauer [a],
Almut Gerhardt[b], Heinz-R. Köhler[a]

[a]Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b]LimCo International, An der Aa 5, D-49477 Ibbenbüren, Germany

* Both authors contributed equally to this paper and share first authorship.

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 (acetylcholinesterase inhibitor *vs.* polar narcosis) 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 elicited deformations at ≥0.25 mg/L during the 11 days subchronic test, whereas locomotor activity and mortality were impaired at ≥0.5 mg/L. Diazinon effects on those parameters were obvious at ≥2 mg/L, except for the deformation rate (11 days: 1 mg/L). In equitoxic mixtures of both substances concentration additivity was observed for deformation rate and mortality (11 days). 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, with deformations and Hsp70 levels as most sensitive parameters concerning 3,4-DCA and Hsp70 as most sensitive parameter concerning diazinon. 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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1. Introduction

In the field, 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 mainly results 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 to 1.5 µg/L were found (EU, 2006, Planas et al., 2006). The toxicity of 3,4-DCA can be described by the polar narcosis mode of action as indicated by

quantitative structure activity analysis so far (Arnold et al., 1990). 96 h 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 acetylcholinesterase which inactivates the neurotransmitter acetylcholine (Pesando et al., 2003). 96 h LC_{50} 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 embryos and larvae of zebrafish *Danio rerio* in a multi-biological-level approach.

The following hypotheses were tested for juvenile zebrafish:

1. Endpoints at lower levels of biological organisation (molecules), measured as general proteotoxicitiy (Hsp70 level), should exhibit higher sensitivity to 3,4-dichloroaniline, diazinon and mixtures of them than those on higher levels of biological organisation (organisms) with the parameters hatching rate, locomotor activity, deformations and mortality.
2. The acetylcholinesterase inhibitor diazinon should lead to more severe effects than the unspecific toxicant 3,4-dichloroaniline.
3. 3,4-Dichloroaniline and diazinon should act independently in equitoxic mixtures for all investigated endpoints.

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 deionised 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 (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. For the prolonged embryo test, nominal concentrations of 0.5, 0.7, 1, 1.5 and 2 mg 3,4-DCA/L were tested. For the Hsp70 analysis nominal concentrations of 0.05, 0.1, 0.15, 0.2 and 0.25 mg 3,4-DCA/L were tested. The subchronic test comprised six nominal concentrations (0.005, 0.01, 0.1, 0.25, 0.5 and 1 mg 3,4-DCA/L).

Diazinon (Pestanal, analytical standard, Sigma-Aldrich, Seelze, Germany) was dissolved in reconstituted water 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 prolonged embryo test comprised nominal concentrations of 0.1, 0.5, 1, 2 and 3 mg/L diazinon and, for the biochemical investigations, nominal concentrations of 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. 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.

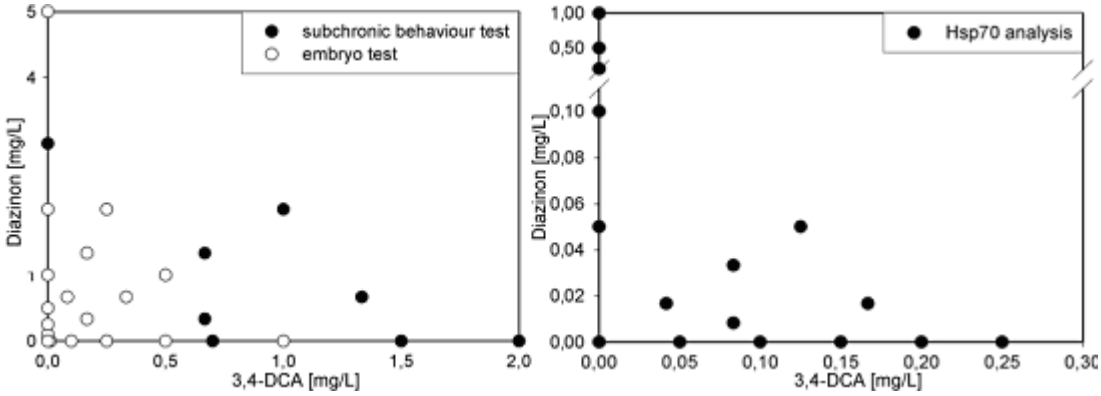

Fig. 1. Test design for the mixture experiments with 3,4-dichloroaniline [mg/L] and diazinon [mg/L]

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 (ten embryos per Petri dish, four replicates per concentration) containing the respective test solutions. The tests were performed in climate chambers at a 12:12 h 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 for 96 h.

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 Section 2.3) onwards up to an age of 11 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 a, b). For behaviour measurements in the subchronic test, the measurement chambers were placed into glass aquaria (15×20×20 cm) filled with 1.5 L 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 W 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 (replicates) 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 (three dishes per concentration) were exposed in the way described for the prolonged embryo test. The tests lasted for 168 h. Ten times eight embryos (ten replicates) 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 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. The methodological variability of this method was proved to be ±2.7 % (Köhler et al. 2005).

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. For 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).

3. Results

3,4-Dichloroaniline

During the prolonged embryo test a significant increase of heart and yolk sac oedemas was found in fish exposed to 1 mg 3,4-DCA/L and higher concentrations (Fig. 2) at 96 h post fertilisation when compared to controls. Mortality was not significantly increased in any test concentration during the 96h embryo test.

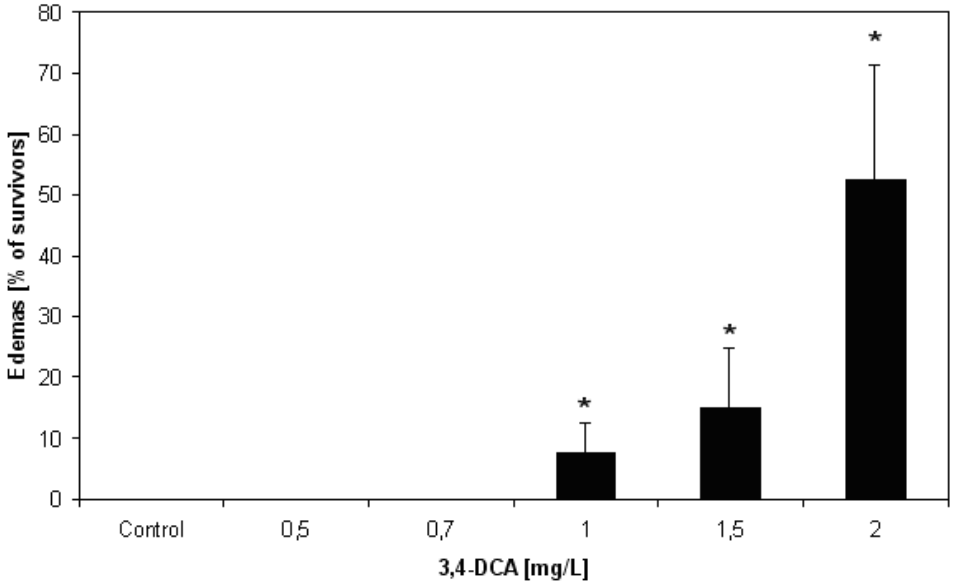

Fig. 2. Percentages of larvae exposed to 3,4 dichloraniline which showed oedemas 96h post fertilisation. * Significantly different to the control, $P < 0.05$. $n = 10$, bars represent means ± SD

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 edema (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.

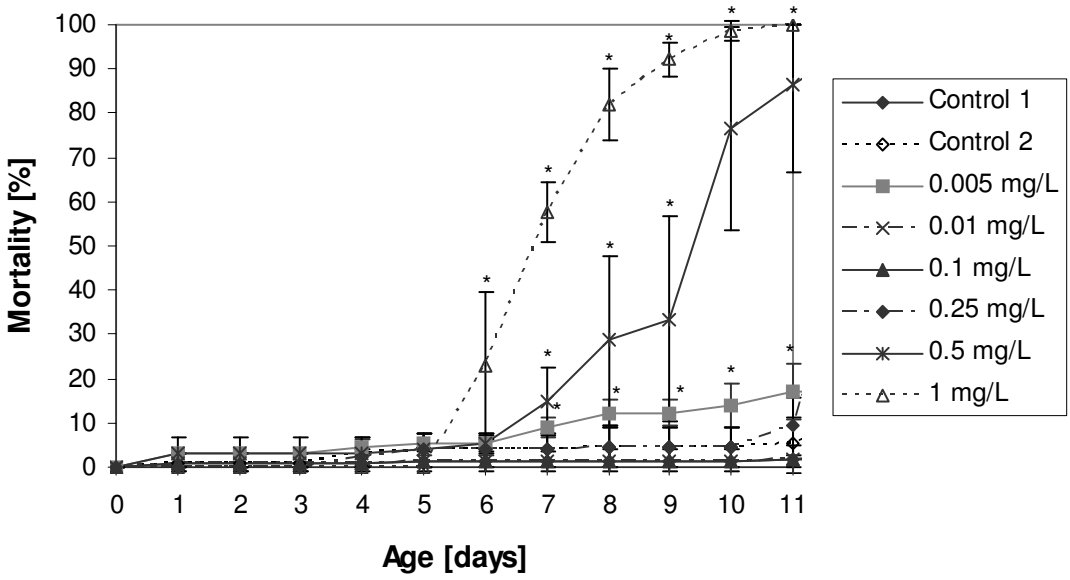

Fig. 3. Cumulative mortality [%] of *D. rerio* larvae exposed to different 3,4-dichloroaniline concentrations [mg/L] (means ± SD; number of larvae per replicate: 30 (days 0- 5); 26 (days 6-8); 22 (days 9-11), three replicates for each experiment). * Significantly different to control treatment at $P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

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. 3), resulting in an LC_{50} of 0.388 mg 3,4-DCA/L 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. 4).

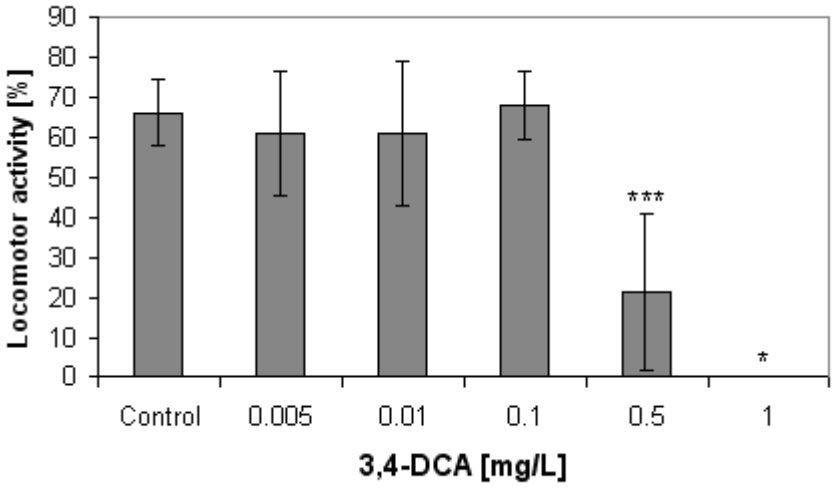

Fig. 4: Locomotor activity (percent of total time spent in locomotion) of 5- and 8-days-old *D. rerio* larvae exposed to different 3,4-dichloroaniline concentrations [mg/L]

A significant increase of the Hsp70 level was observed if the embryos and larvae were exposed to 250 µg 3,4-DCA/L for 168 h. Hsp70 levels of all investigated groups are displayed in Fig. 5.

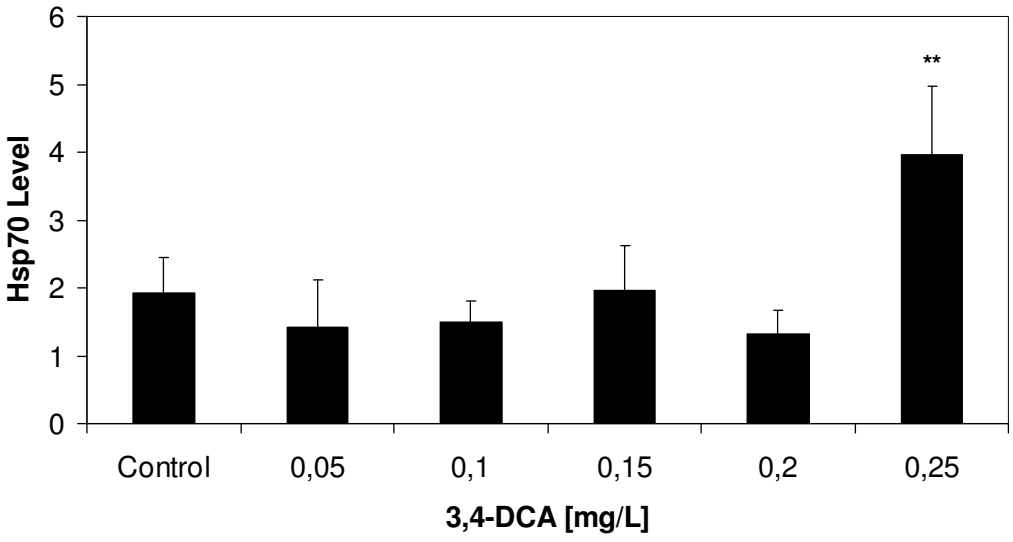

Fig. 5 Hsp70 levels of zebrafish larvae exposed to 3,4-dichloroaniline (n = 10, means ± SD). ** Significantly different to the control at $P < 0.01$

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 an age of 96 h. At 2 mg/L a significant increased deformation rate, mostly edema and spine deformations, was observed. Even after 1 day of exposure, 5 mg/L induced deformities, edema and an abnormal bending of the spine in 100 % of the larvae. In the subchronic test 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. 6).

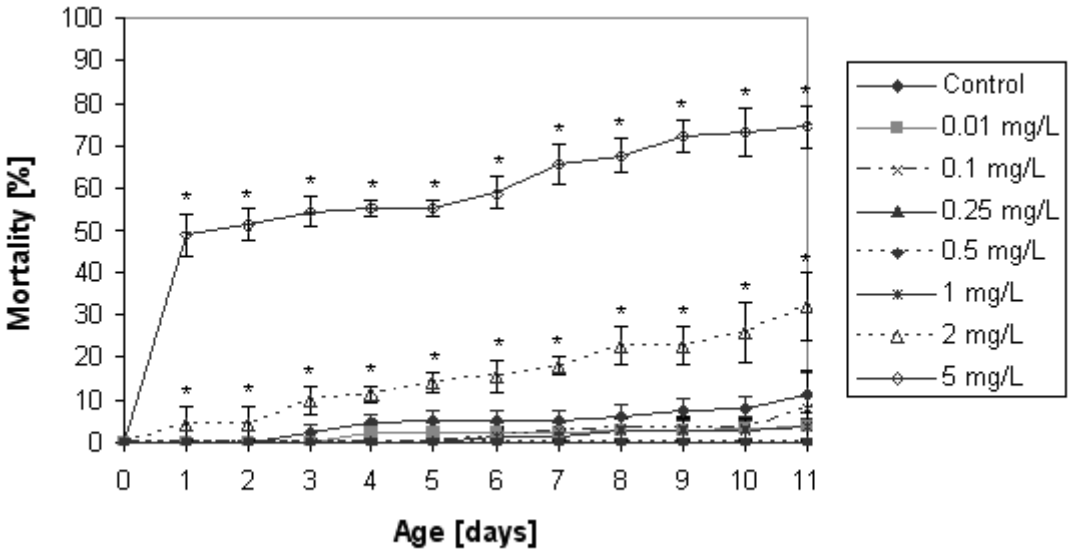

Fig. 6 Cumulative mortality [%] of *D. rerio* larvae exposed to different diazinon concentrations [mg/L] (means ± SD; number of larvae per replicate: 30 (days 0 - 5); 26 (days 6 - 8); 22 (days 9 - 11), 3 replicates for each experiment). * Significantly different to control treatment at $P < 0.05$

Diazinon in a concentration of 2 mg/L decreased the locomotor activity in 5- and 8-days-old *D. rerio* larvae (Fig. 7).

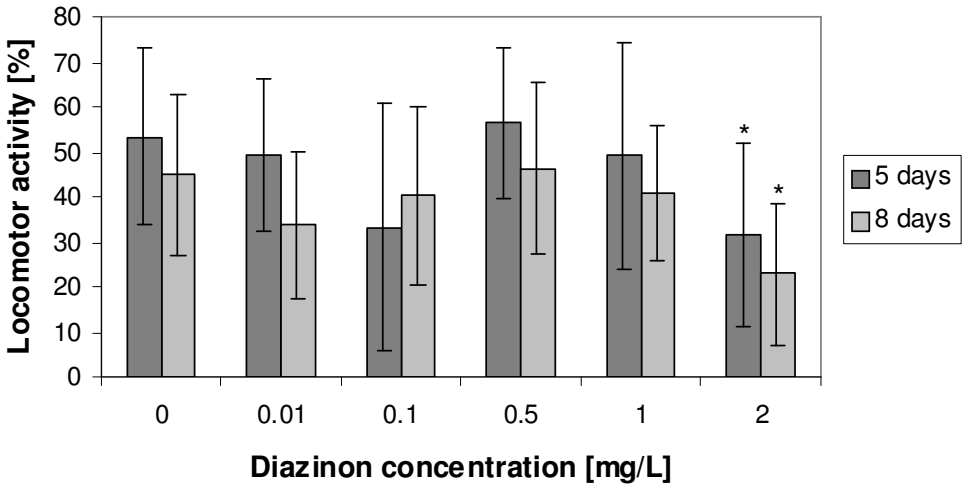

Fig. 7 Locomotor activity (% time spent on locomotion) of 5- and 8-days-old *D. rerio* larvae exposed to different diazinon concentrations [mg/L] (n = 10-12, means ± SD)

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).

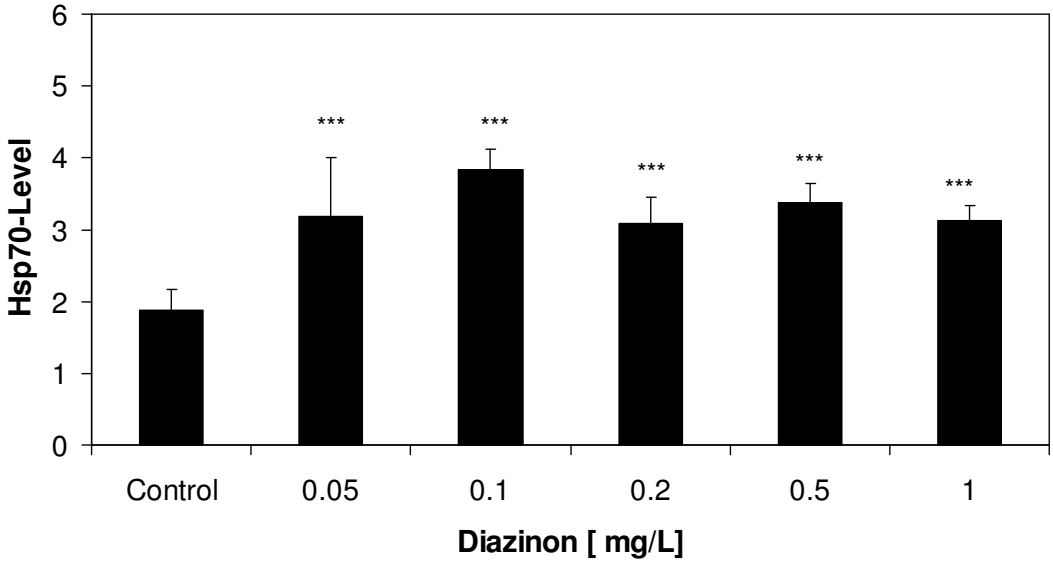

Fig 8 Hsp70 levels of zebrafish larvae exposed to diazinon (n=10, means ± SD). *** Significantly different compared to the control at $P < 0.001$

Binary mixtures of 3,4-dichloroaniline and diazinon

In contrast to the single substance tests, oedemas occurred at the end of the prolonged embryo test (96 h) only. In single substance test, edema occurred early if animals were exposed to diazinon (72 h post fertilisation, most animals with edema died during the

following 24 h) and later if exposed to 3,4-DCA (96 h post fertilisation, see Fig. 2). Due to the different time points of occurrence of edema, 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), indicated by the straight isoboles (lines of equal effect). 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 1.

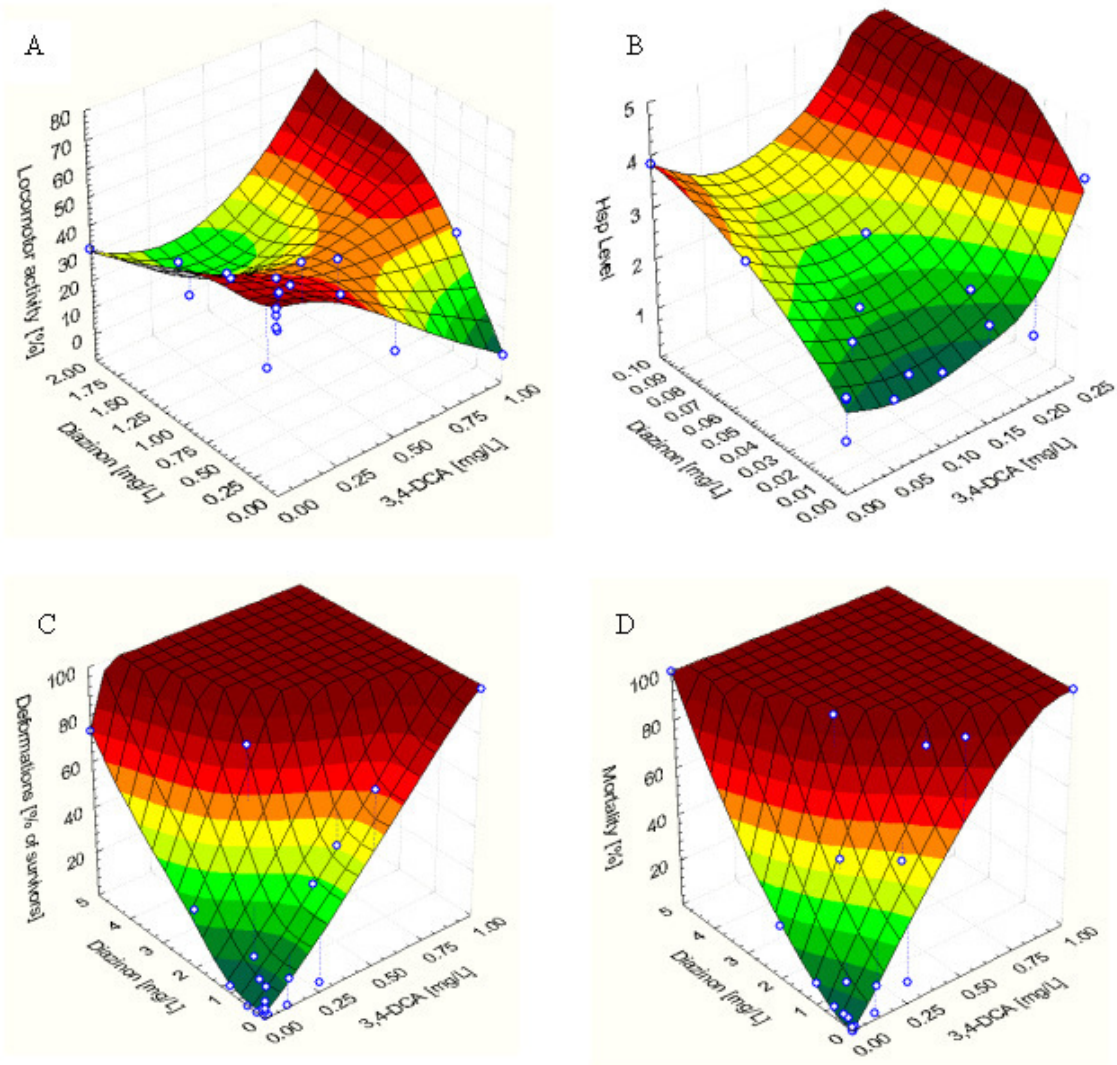

Fig. 9 a Locomotor activity (percent of total time spent in locomotion) of 5-days-old *D. rerio* larvae**, b** Hsp70 levels (b) of 7 days old *D. rerio* larvae, **c** deformations [%] and **d** mortality [%] of 10 days old *D. rerio* larvae exposed to different 3,4-dichloroaniline and diazinon concentrations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

4. Discussion

In the present study, a pesticide and a pesticide degradation product were investigated concerning their ecotoxicological impact on a wide 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.

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 and 0.2 mg/L for survival rates in early life stages of zebrafish after 4 and 2 weeks of exposure to 3,4-DCA respectively. So far reported LOECs for survival and sublethal effects in 48 h old embryos are 12.4 µM (equivalent to 2.01 mg/L) (Voelker et al. 2007). Those values are in good accordance with the results for the respective parameters in the present study (LOEC for deformations: 1 mg/L in the 96 h embryo test and 0.25 mg/L in the subchronic test; LOEC for mortality: 0.5 mg/L in the subchronic test). 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 96 h LC_{50} is 8 mg/L for adult zebrafish (Keizer et al., 1991). For zebrafish embryos and larvae no LC_{50} value could be obtained from the literature, but in the present study ≥50 % mortality at 5 mg/L diazinon already occurred in ≥1 day old fish, which indicates a higher sensitivity of embryos and larvae to diazinon exposure compared to adult fish. Mixture toxicity tests with these two substances have not been conducted 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 1 for both).

Table 1 Comparison of the LOECs of the exposure of zebrafish embryos and larvae to 3,4-dichloroaniline and diazinon.

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

CA concentration addition

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 as 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 4.5-6 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 exerted

by diazinon concentrations which were about ten times lower than the corresponding 3,4-DCA concentrations. Taking into account that Hertl and Nagel (1993) found bioconcentration factors of 86 in 4 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 (oedemas) 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 oedemas and mortality) showed reactions to diazinon exposure exclusively (for details see Osterauer and Köhler, 2008).
The investigated behavioural endpoints were less sensitive than the biochemical parameter Hsp70, but responded at 5 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 acetylcholinesterase inhibitor chlorpyrifos, locomotor activity has been shown to be a very sensitive parameter in zebrafish (Kienle et al. 2008a, b). 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 diazinon completely and for 3,4-dichloroaniline with respect to exposure time but not to exposure level (LOEC 0.25 mg/L for Hsp70 (7 days) and deformations (≥9 days)). Hypothesis 2 ("The acetylcholinesterase 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 and mortality during the first 96 h post fertilisation) exclusively occurred after exposure to diazinon.
Hypothesis 3 ("3,4-Dichloroaniline and diazinon should act independently in equitoxic mixtures for all investigated endpoints."), 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. Concentration addition has been found to occur in mixtures of independently acting substances before (Altenburger et al., 2004, Schwarzenbach et al., 2006).

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 as possible.

Acknowledgements

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<u>Kapitel 4</u>: Linking behaviour to acetylcholinesterase inhibition in embryos and larvae of zebrafish (*Danio rerio*) exposed to pesticides

Cornelia Kienle[a], Eberhard Küster[b], Almut Gerhardt[c], Heinz-R. Köhler[a]

[a]Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b]UFZ, Helmholtz Centre for Environmental Research, Department of Bioanalytical Ecotoxicology, Permoserstr. 15, D-04318 Leipzig, Germany

[c]LimCo International, Oststrasse 24, D-49477 Ibbenbüren, Germany

Abstract

Many insecticides act on the nervous system by inhibiting the enzyme acetylcholinesterase, which may result in severe effects on different levels of biological organisation, e.g. behaviour and development of both target and non-target species in the ecosystem. To link behavioural to suborganismal alterations, the effects of two insecticides with the same mode of action on the endpoints enzyme activity, behaviour, deformations and mortality of zebrafish embryos and larvae have been investigated in subchronic exposures up to 11 days post fertilisation (dpf).

The activity of the enzyme acetylcholinesterase (AChE) increased significantly with the age of the zebrafish. The most prominent effects on enzyme activity occurred at an age of 120 and 196 hours post fertilisation (hpf) where the acetylcholinesterase was inhibited significantly already at 0.01 mg CHP/L. At the same concentrations, effects on locomotor activity were obvious as well. Effects on those two parameters hence occurred at much lower concentrations than increased morphological abnormalities and mortality (0.25 and 0.5 mg/L respectively). Diazinon was much less toxic concerning behavioural alterations and enzyme inhibition compared to chlorpyrifos, although both substances are likely to affect behaviour because of their specific mode of action. In binary mixtures, concentration additivity was detected for all the observed parameters.

The present study shows that, despite their similar mode of action, the neurotoxic insecticides chlorpyrifos and diazinon lead to different responses in zebrafish embryos and larvae on organismal and on suborganismal level with respect to the effect concentrations. We recommend combining behavioural parameters with the

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measurement of acteylcholinesterase activity, as both parameters are closely linked and provide information about the exposure of an organism to neurotoxic chemicals (AChE inhibition) as well as about effects on the organism level (behaviour).

Keywords: behavioural alterations; Multispecies Freshwater Biomonitor®, MixTox model

1. Introduction

Pollutants act on different levels of biological organisation. Effects on the target site at the biochemical level may entail effects on higher levels of biological organisation, on the organism as well as on populations and ecosystems. To elucidate the coherences between those levels, it is essential to combine biochemical markers with higher level effects, e.g. on the behaviour and development of organisms.

In this context, an important mode of toxic action is the inhibition of the enzyme acetylcholinesterase, which cleaves and thus inactivates the neurotransmitter acetylcholine (Kamrin, 1997; Pesando et al., 2003). An inhibition of the enzyme thereby interferes with neurotransmission in cholinergic synapses and neuromuscular junctions. In developing zebrafish embryos, AChE also is remarkably important for the neuronal and muscular development (Behra et al., 2002; Hanneman, 1992) or the axon outgrowth (Hanneman and Westerfield, 1989). The inhibition of cholinesterases by pesticides is accepted as a biomarker of exposure (Walker, 1995) and has already been successfully applied to zebrafish embryos exposed to various organophosphates such as paroxon-methyl, aldicarb and aldicarb-sulfoxide, where it was also suggested as a biomarker of effect, due to the clear concentration-dependent effect on acetylcholinesterase activity (Küster, 2005; Küster and Altenburger, 2006, 2007). Our study represents the first effort to examine this biomarker on zebrafish larvae and to relate it to higher level effects, such as behavioural alterations and developmental impairment in the same organisms.

Due to their specific mode of action as inhibitor of the enzyme acetylcholinesterase, the insecticides chlorpyrifos and diazion were chosen for exposure experiments. Both pesticides have often been found together in mixtures in several surface waters in the United States (Gilliom et al., 2006).

Chlorpyrifos (CHP) is a broad-spectrum organophosphate compound (Kamrin, 1997). It is the active ingredient in a variety of insecticides (e.g. Dursban™ and Lorsban™), which are some of the most widely used insect control products in the world (Dow

AgroSciences, 2008). Chlorpyrifos acts on pests primarily as a contact poison and additionally as a stomach poison. It is considered very highly toxic to freshwater fish with 96h LC_{50} values ranging from of 0.009 mg/L in adult rainbow trouts to 0.331 mg/L in fathead minnow (Kamrin, 1997; U.S.EPA, 1986). The highest concentrations measured in the environment were about 0.3 µg/L in several surface waters in the United States (Gilliom et al., 2006).

Diazinon is a non-systemic organophosphate insecticide which is extensively used for pest control in home gardens and farmland, as well as in veterinary treatments (Kamrin, 1997). In ecotoxicity tests *Ceriodaphnia dubia* was most sensitive to diazinon exposure (96 h LC_{50}: 0.32-0.35 µg/L (Bailey et al., 1997), whereas adult zebrafish and fathead minnows (*Pimephales promelas*) reacted at higher concentrations (96 h LC_{50}: 2.21 – 8 mg/L and 10.3 mg/L respectively; (Ansari et al., 1987; Keizer et al., 1991; Meier et al., 1979). In the environment, concentrations of 1.5 µg/L have been detected in urban waterways in California (Bailey et al., 2000).

The aim of this study was to investigate whether two similarly acting compounds (diazinon and chlorpyrifos) exposed individually and in binary mixtures exert effects on the biochemical level (acetylcholinesterase activity) which can be linked to effects on the organism level (development and behaviour) in zebrafish embryos and larvae.

By comparison of responses at different age stages, the appropriate age for the application of the biomarker acetylcholinesterase activity was determined. The measurement of acetylcholinesterse activity was proposed by Küster and Altenburger (2007) as a useful supplement to the zebrafish embryo test established by Nagel (2002), due to its higher sensitivity.

The following hypotheses were tested for juvenile zebrafish.

1. The enzyme activity as well as the degree of enzyme inhibition increases with the age of the zebrafish.
2. Inhibition of the enzyme acetylcholinesterase results in behavioural impairment in juvenile zebrafish.
3. In mixtures of chlorpyrifos and diazinon, both substances act additively as expected by their similar mode of action.

2. Materials and Methods

2.1 Test animals and acquisition of eggs

Adult male and female zebrafish (*Danio rerio*, strain: WIK, ZFIN ID: ZDB-GENO-010531-2) were kept as described in Kienle et al. (2008) in aerated and filtered aquaria at 26 ± 1°C, a conductivity of 400 µS/cm and a 12:12 hour light:dark cycle. The fish were fed twice daily with dry flake food (Nutrafin Max, Hagen, Germany) and frozen small crustaceans (Bosmidae, *Moina* sp.), *Tubifex* or midge larvae (MM Aquaristik, Germany), respectively. Eggs were retrieved using spawn traps with a spawning substrate, which were placed in the aquaria the evening before spawning. In the morning, sixty minutes after spawning had begun (triggered by sudden illumination of the aquaria), the spawn traps were removed and the eggs were collected. All studies involving experimental animals were conducted in accordance with national and institutional guidelines for the protection of animal welfare.

2.2 Test substance

Chlorpyrifos (Pestanal, analytical standard, Sigma-Aldrich, Germany) was dissolved in reconstituted water (OECD, 1992, Guideline 203). In order to prepare a stock solution, it was stirred continuously for at least 4 h at a water temperature of about 45 °C and a pH of 8.0. Subsequently, the solution was kept at 35 °C overnight until use with continuous stirring. From this stock solution the test solutions were prepared directly before use. Nominal test concentrations for exposure experiments were 0.01, 0.1, 0.25, 0.5 and 1 mg CHP/L (1 mg CHP/L corresponds to 0.0029 mmol/L) and one negative control with pure reconstituted water. The test solution was exchanged every second day to keep the concentrations as constant as possible.

Diazinon (Pestanal, analytical standard, Sigma-Aldrich, Germany) was dissolved in reconstituted water in order to prepare a stock solution of 10 mg/L while constantly stirring. Test solutions were prepared from this stock solution directly before use. Test concentrations for exposure experiments were 0.01, 0.1, 0.25, 0.5, 1, 2 and 5 mg Diazinon/L (1 mg Diazinon/L corresponds to 0.0033 mmol/L).

The test design for the mixture experiments is given in Fig. 1. Selection of the mixtures was based on the results of the single substance tests. Calculation of mixture concentrations was based on the LOECs (lowest observed effect concentrations = 1 toxic

unit, 1 TU) obtained in the single substance tests. The two substances were combined in concentrations equal to either 0.25, 1, or 1.5 TU. A negative control was run in parallel.

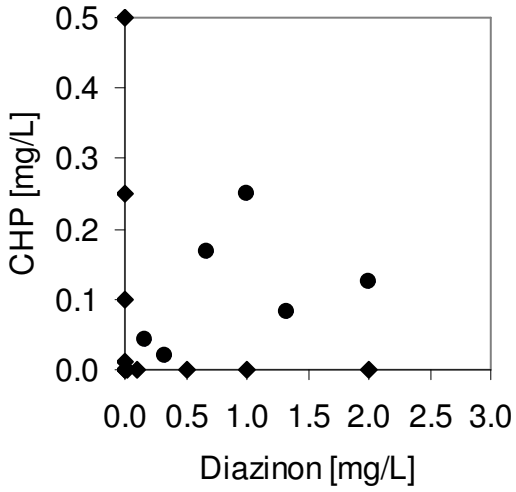

Fig. 1. Test design for the mixture experiments with chlorpyrifos [mg/L] and diazinon [mg/L]. x and y axis: data points for single substance tests, in between: data points for mixtures. Calculation of mixture concentrations was based on the LOECs (lowest observed effect concentrations = 1 toxic unit, 1 TU) obtained in the single substance tests. The two substances were combined in concentrations equal to either 0.25, 1, or 1.5 TU. A negative control was run in parallel.

Analytical conformation of chlorpyrifos and diazinon concentrations was performed with gas chromatography–mass spectrometry (GC/MS) (HP5890 series II, Hewlett-Packard, Waldbronn, Germany) using a chlorpyrifos standard at a concentration of 1 mg/L and a diazinon standard at a concentration of 10 mg/L in reconstituted water (both Dr. Ehrenstorfer, Augsburg, Germany). The substances were extracted from the aqueous, acidificated solution with dichloromethane as a solvent by shaking in a separating funnel for 3 minutes. After the thorough separation of the two phases, the solvent phase was dried using Na_2SO_4, and was then filled in a 50 mL rotovap bulb. Thereafter the solvent was ablated to a volume of 1 mL in a rotating evaporator. This volume was filled in GC/MS sampling vials and the concentration determined via GC/MS. For chlorpyrifos, the stock solution and highest test concentration of 1 mg/L and for diazinon the stock solution of 10 mg/L was measured, using an injection volume of 1 µL respectively.

2.3 Exposure experiments

A subchronic test with a duration of eleven days was conducted according to the VMD Guidance Note "Ecotoxicity testing of medicines intended for use in fish farming" (VMD, 1996). Freshly fertilized zebrafish eggs were exposed to the test substances in glass Petri dishes up to an age of eleven days post fertilization (dpf). No food was provided during the experiments.

The tests for the acquisition of developmental and behavioural parameters were performed as described in Kienle et al. (2008). 30 eggs per Petri dish were exposed with three replicates per concentration. Several endpoints, such as hatching rate (up to an age of 96 hours), deformations and mortality, were recorded daily up to an age of eleven days post fertilization (dpf). From each replicate, four larvae were randomly removed at 5, 8, and 11 dpf for behavioural measurements. Those were carried out 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).

For enzyme measurements, in a separate experiment, 50 eggs per Petri dish were exposed with eight replicates per concentration. At an age of 48 hours post fertilization (hpf), 20 embryos were removed and rinsed thoroughly in fresh reconstituted water. Subsequently the animals were introduced in 2 mL microcentrifuge tubes and, after removing excess water by pipetting, they were snap-frozen in liquid nitrogen and stored at -20°C for enzyme measurements. Storage never exceeded six weeks. At 120 and 192 hpf, the same method was applied to the remainder, only the number of animals per replicate was reduced to 10 due to their older age and the therefore presumably higher enzyme content.

2.4 Biochemical analyses

The analysis of the enzyme activities of cholinesterase were done as described in Küster (2005). Here 20 snap frozen embryos were homogenised on ice in 0.4 ml ice cold phosphate buffer (pH 7.5, 0.1 M $NaH_2PO_4 \times H_2O$-containing 0.1% v/v Triton X-100) and centrifuged at 4 °C for 15 min (10 000 g). The supernatants were used either directly for enzyme analysis or stored at −20 °C until analysis. Storage of the samples never exceeded 1 week. Enzyme assays were carried out in quadruplicate per sample at 22 °C.

The assays followed the method described by Ellman et al. (1961) adapted to microtitre plates (Küster, 2005) using DTNB as the chromogenic reagent and Acetyl-Thiocholinjodid (ATC) as the substrate. The specific enzyme activity is expressed as units (U) per mg of protein, with 1 U defined as the amount that hydrolysed 1 µmol of substrate per minute. Protein concentration of the samples was determined in quadruplicate at 750 nm using a commercial kit (DC Protein Assay, BioRad, München, Germany) based on the Lowry assay (Lowry et al., 1951). Five microliters of supernatant gained from 20 (48 hpf) or 10 embryos (120 and 192 hpf) were sufficient with maximum protein concentrations of 10 mg/L. Bovine serum albumin, fraction V served as the standard protein. A standard curve using the quadratic formula $y = a + bx + cx^2$ was generated to correct for the folin reagent reaction (Peterson, 1979; Peterson et al., 1983). Each calibration curve was generated with five different protein dilutions. The percentage of AChE inhibition was derived by expressing the activity levels of exposed animals as the percentage of the activity in controls.

2.5 Behavioural measurements using the MFB®

The Multispecies Freshwater Biomonitor® is an online biomonitor for quantitative and continuous recording of the behavioural pattern of animals (Gerhardt et al., 1994). The activity of the animals is measured in flow-through sensor chambers with quadropole impedance conversion as the measuring principle (Gerhardt, 2000).

Behavioural measurements were performed as described in Kienle et al. (2008) and Scheil et al. (2009). In short, the locomotor activity of the fish larvae was analysed in measurement chambers (length: 4 cm, diameter: 1 cm), which were placed into glass aquaria (15*20*20cm) containing 1.5 L of the respective solution. Those were arranged in duplicate in a surrounding black basin filled with temperature adjusted water (26 ± 1°C) and illuminated from above during the measurements (58 Watt neon light, distance to chambers: 145 cm). In order to record the locomotor activity, the larvae were transferred carefully into the chambers (one larva per chamber) as described in Kienle et al. (2008). After a 10 min acclimation time, the behaviour of 11 - 12 larvae per treatment was continuously recorded for a duration of 2 h in 10 min intervals with a duration of 4 min per measurement.

2.6 Data analysis

The data on enzyme inhibition were all normally distributed (Shapiro-Wilk test, JMP 7.0.1, SAS systems, USA). Therefore we conducted an univariate Anova with Dunnett´s Test as a Post-hoc test (JMP 7.0.1, SAS systems, USA), to compare the exposure treatments with the control. Tukey´s HSD test (JMP 7.0.1, SAS systems, USA) was used for comparison of the control treatments at different age stages.

Data for behavioural and developmental parameters were analysed as described in Kienle et al. (2008) with nonparametric methods due to the only partial normal distribution. The significance of the data of all tests was tested using a Friedman's ANOVA (Statistica 5.0, StatSoft, USA) with a subsequent pairwise comparison (Wilcoxon two group test, JMP 4.0, SAS systems, USA) to detect differences between control and exposure treatments. Significance levels were defined as follows: $p < 0.001$ highly significant: ***, $p < 0.01$ strong significance: **, $p < 0.05$ significant: *. Concerning behavioural measurements, means of locomotor activities (percent of total time spent on locomotion) for each larva were calculated separately for the first and the second hour. For statistical evaluation, the data on "percentage time spent on locomotion" were arcsin transformed from proportional values. Calculation of the response surfaces for mixture data of chlorpyrifos and diazinon was performed with Statistica 5.0 (StatSoft, USA). Modelling of mixture responses was performed using the MixTox Model (Jonker et al., 2005).

3. Results

3.1 Abiotic parameters and measured concentrations

In the exposure experiments, optimal conditions for the larvae were provided with a temperature of 26.5 ± 0.5 °C, an oxygen concentration of 7.71 ± 0.14 mg O_2/L (99.3 ± 1.6 % oxygen saturation), a pH of 8.08 ± 0.09 and a conductivity of 650 ± 2 µS/cm (means ± SD of control treatments, n = 6). When measuring the stock solutions of the pesticides from which the respective test solutions were prepared, the retrieval rate of chlorpyrifos was 51.6 % (nominal concentration 1 mg/L) and that of diazinon 123 % of the nominal concentration (10 mg/L) compared to the respective standard.

3.2 Enzyme activity and protein content at different age stages

Enzyme activity increased significantly with increasing age of the zebrafish ($p < 0.001$), being lowest at 48 hpf with 25.9 ± 3.1 µmol*min^{-1}*mg^{-1} protein, followed by 302.5 ± 27.4 µmol*min^{-1}*mg^{-1} for 120 h old zebrafish and 425.8 ± 33.0 µmol*min^{-1}*mg^{-1} in 192 h old larvae. No significant differences were detected between the control treatments of the different tests at the same age stages.

3.3 Exposure experiments

Chlorpyrifos

When exposing zebrafish embryos and larvae to chlorpyrifos, enzyme activity was significantly decreased at 48, 120 and 192 hpf, but to a much higher extent at 120 and 192 hpf and at concentrations as low as 0.01 mg/L (Fig. 2). Comparing those results to developmental effects, here first effects occurred from an age of 4 and 5 days onwards at concentrations of 0.25 and 0.5 mg CHP/L, with a significant increase in the percentage of individuals with morphological deformations such as an abnormal bending of the spine and heart edema ($p < 0.05$). Mortality was significantly increased from an age of eight days onwards at 0.5 mg CHP/L ($p < 0.05$) with a calculated LC_{50} of 0.38 mg CHP/L at an age of nine days.

At 120 hpf the significant inhibition of enzyme activity from 0.01 mg CHP/L onwards was mirrored in the behavioural measurements, where the locomotor activity decreased significantly at ≥0.01 mg CHP/L as well (Fig. 3). In higher concentrations (≥0.25 mg/L at 4-5 dpf), the larvae displayed jerky movements with many pauses and muscular cramps.

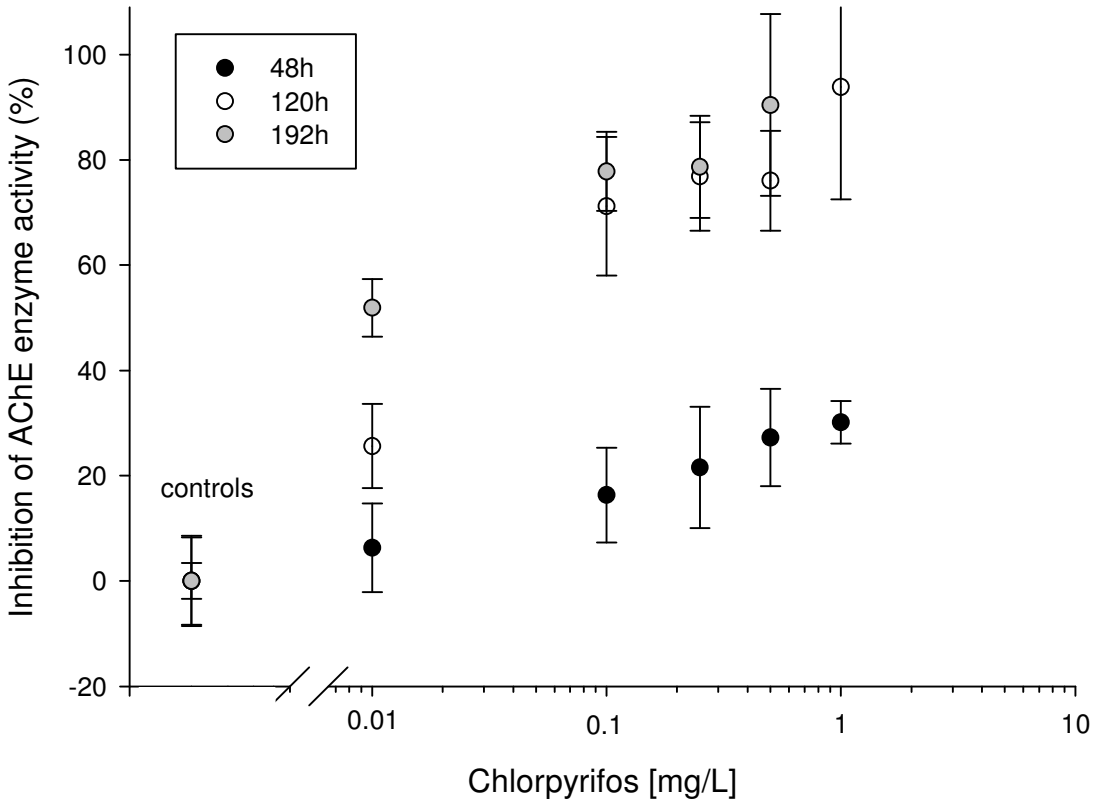

Fig. 2. Chlorpyrifos: Inhibition of AChE enzyme activity (%) in zebrafish embryos and larvae at 48, 120 and 192 hpf, exposed to chlorpyrifos from the time of fertilization onwards. Significant difference to the control: $p < 0.001$ at ≥0.25 mg/L (48 h), ≥0.01 mg/L (120 h, 192 h), n = 6-9, data points represent means ± SD. 1 mg/L corresponds to 0.0029 mmol/L chlorpyrifos.

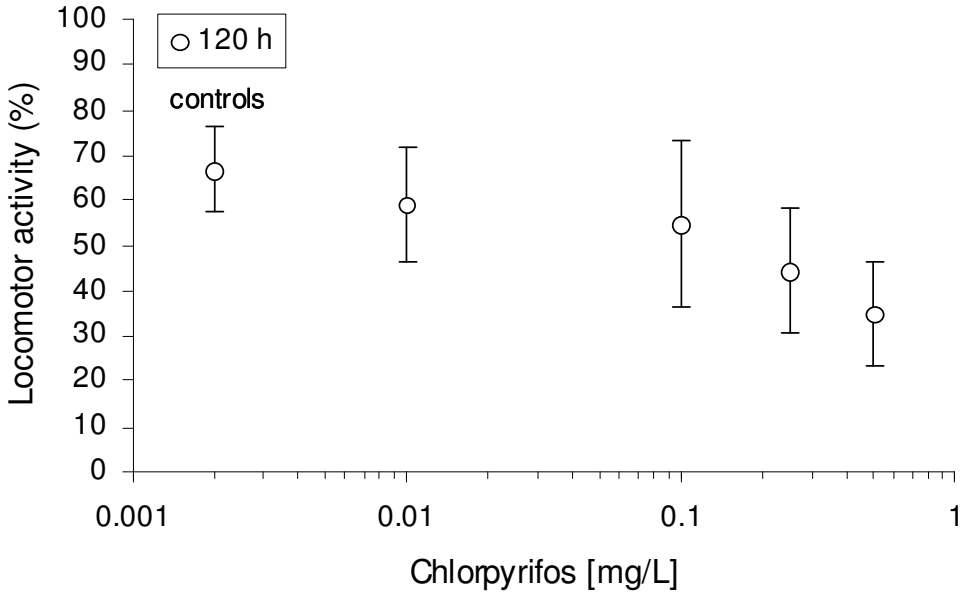

Fig. 3. Chlorpyrifos: Locomotor activity (percent of total time spent in locomotion) of *D. rerio* larvae 120 hpf exposed to different chlorpyrifos concentrations [mg/L]. Significant difference to the control: $p < 0.05$ (0.01 and 0.1 mg/L), $p < 0.001$ (0.25 and 0.5 mg/L), n = 10-12, data points represent means ± SD. 1 mg/L corresponds to 0.0029 mmol/L chlorpyrifos.

Diazinon

Being exposed to diazinon, the enzyme activity in zebrafish embryos and larvae was significantly decreased in 48, 120 and 192 h old animals (Fig. 4). At 48 hpf this was obvious at 2 and 5 mg/L, where the deformation rate, namely edema and an abnormal bending of the spine (at 5 mg/L from 24 hpf onwards) and the mortality rate (at 2 and 5 mg/L from 24 and 48 hpf onwards) were significantly elevated as well ($p < 0.05$).
At 120 and 192 hpf diazinon led to significantly decreased locomotor activity at 2 mg/L (Fig. 5). Here enzyme activity decreased at concentrations as low as 0.5 mg diazinon/L.

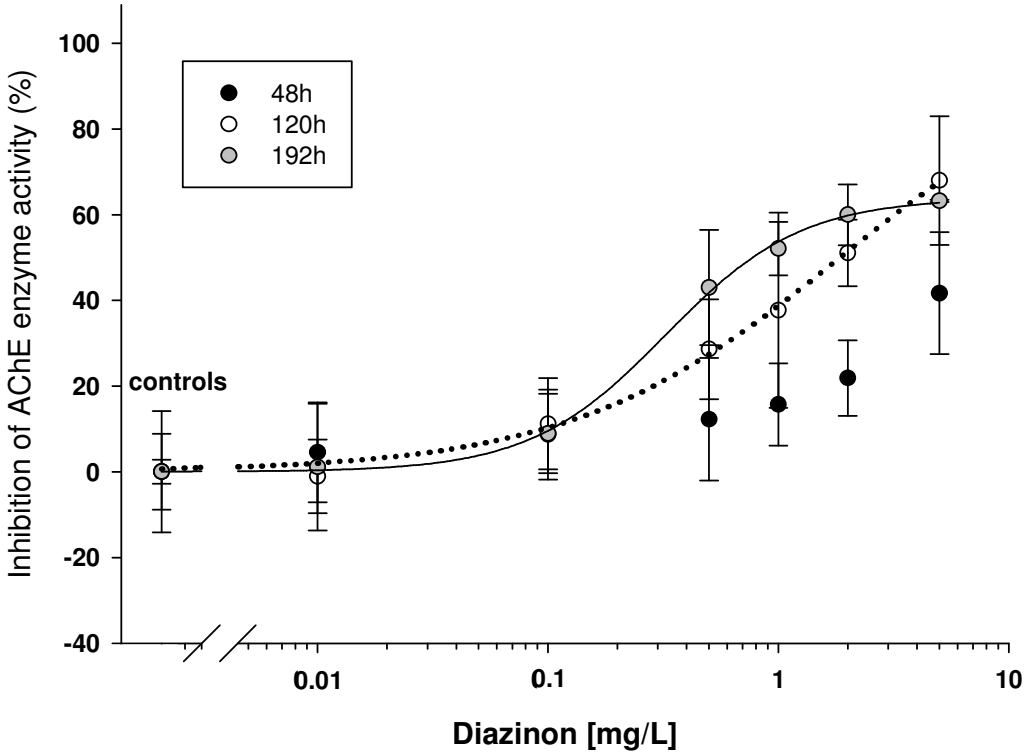

Fig. 4. Diazinon: Inhibition of AChE enzyme activity (%) in zebrafish embryos and larvae at 48, 120 and 192 hpf, exposed to diazinon from the time of fertilization onwards. Significant difference to the control: $p < 0.001$ at ≥2 mg/L (48 h) and ≥0.5 mg/L (120 h, 192 h), n = 5-9 (n = 2 for 5 mg/L at 192 hpf), data points represent means ± SD. For 48 and 120 h data concentration-response-relationships could be fitted. 1 mg/L corresponds to 0.0033 mmol/L diazinon.

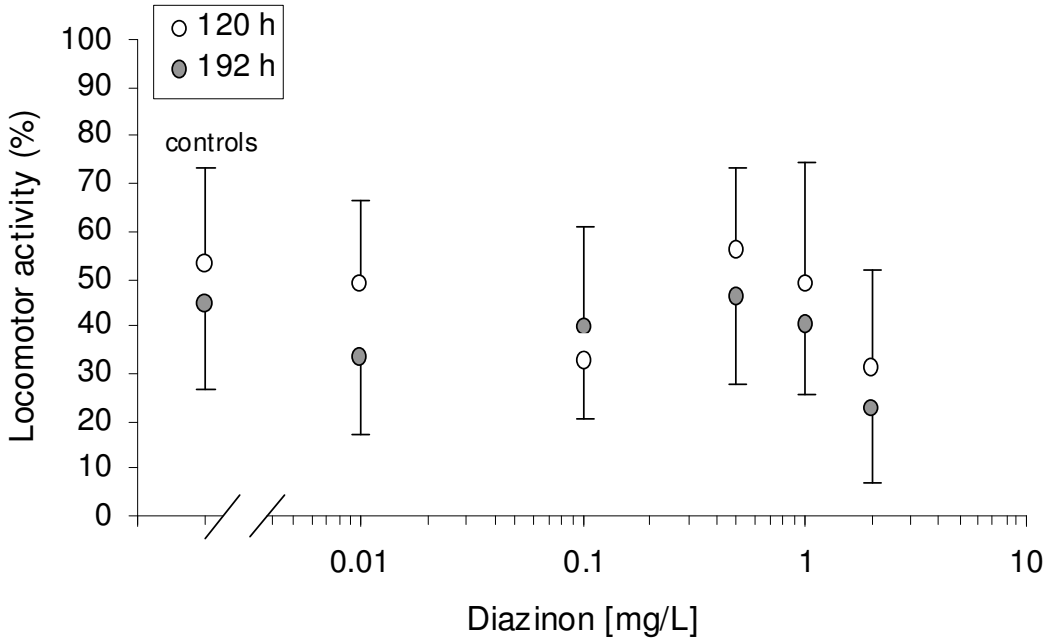

Fig. 5. Diazinon: Locomotor activity (percent of total time spent in locomotion) of 5 and 8-day-old *D. rerio* larvae exposed to different diazinon concentrations [mg/L]. Significant difference to the control: $p < 0.05$ at 2 mg/L, n = 10-12, data points represent means ± SD. 1 mg/L corresponds to 0.0033 mmol/L diazinon.

Mixture toxicity

Mixtures of diazinon and chlorpyrifos caused a decrease in enzyme activity with the single compounds acting additively in the mixtures at an age of 48, 120 and 192 hpf ($p < 0.001$ respectively), as observed for locomotor activity (120 hpf: $p < 0.05$), deformations (240 hpf: $p < 0.001$) and mortality (240 hpf: $p < 0.001$) as well (Fig. 6 A-D).

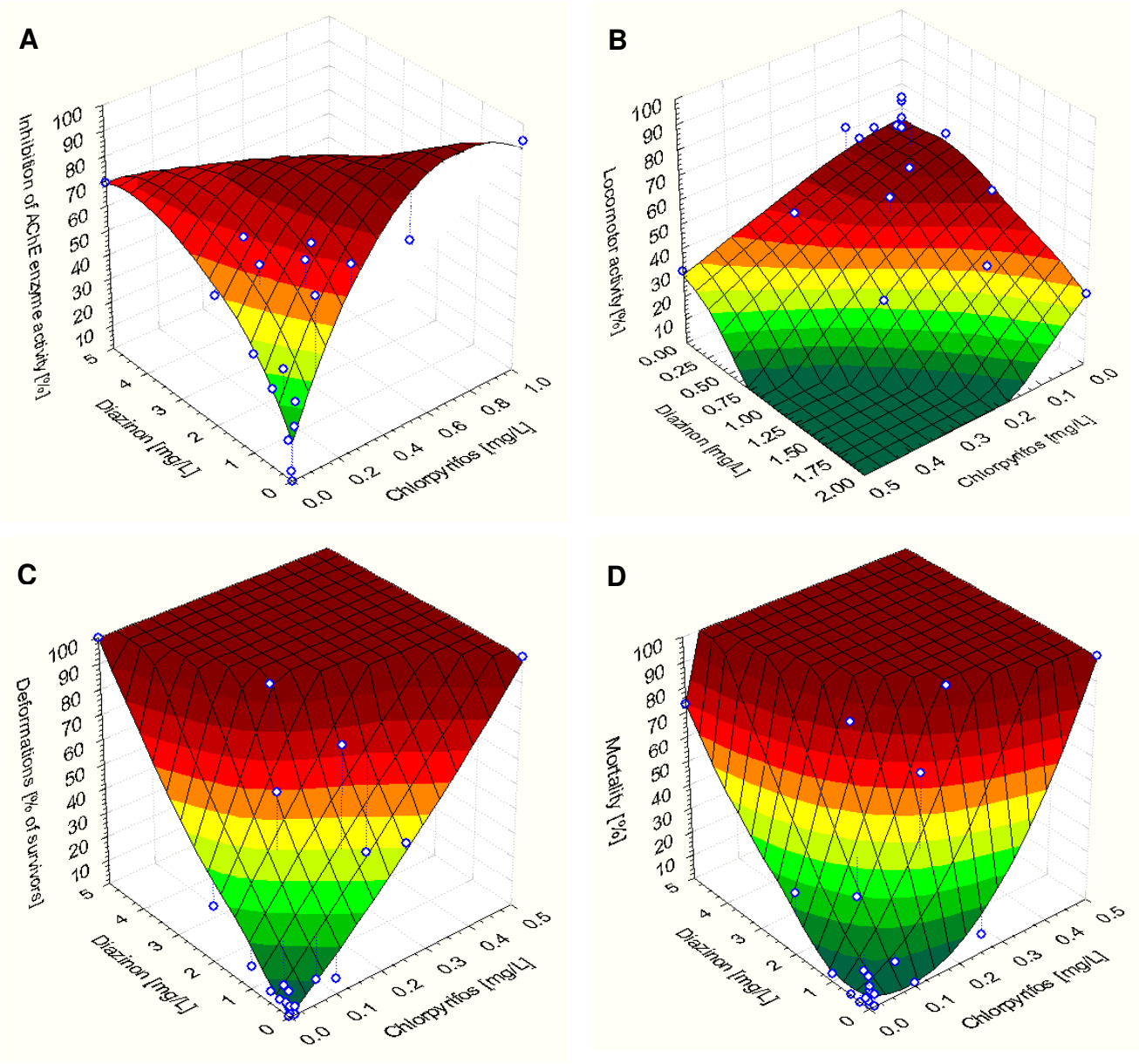

Fig. 6. Mixtures of chlorpyrifos and diazinon: **(A)** Enzyme inhibition [%] in zebrafish embryos and larvae at 120 hpf **(B)** Locomotor activity (percent of total time spent in locomotion) of 5-day-old *D. rerio* larvae**, (C)** deformations [%] and **(D)** mortality [%] of 10-day-old *D. rerio* larvae exposed to different chlorpyrifos and diazinon concentrations [mg/L], single and in binary mixtures (surface plots with isobolic lines calculated on the basis of means).

4. Discussion

In the present study, the effects of two neurotoxic pesticides were investigated concerning their ecotoxicological impact on enzyme activity, behaviour and development of early life stages of zebrafish. In previous studies the influence of acetylcholinesterase inhibitors on enzyme activity and development of zebrafish embryos has been investigated with single substances (Küster, 2005; Küster and Altenburger, 2007). However, studies on the combined effects of pollutants on this parameter and subsequent behavioural effects as well as studies on effects on older age stages have been missing until now.

A clear increase in the activity of the enzyme with increasing age was observed as well as an increase in the degree of enzyme inhibition of exposed animals at 120 and 192 hpf compared to activities at 48 hpf (see Fig. 2 and 4). This was most prominent for chlorpyrifos, but also observable for diazinon. Our first hypothesis ('The enzyme activity as well as the degree of enzyme inhibition increases with the age of the zebrafish.') can therefore be verified. A reason for the low degree of enzyme inhibition at 48 hpf might be that the chorion represents a barrier for the toxicant, which was observed for zebrafish embryos exposed to lindan (Görge and Nagel, 1990). Another potential reason for that result might be that zebrafish embryos are not able to metabolize substances yet (Mattingly and Toscano, 2001) or that the enzyme acetylcholinesterase has not yet fully developed in neurotransmitting processes in the embryonic stage (or is not as important as in older embryos for neurotransmitting processes) as the low enzyme concentration may suggest; therefore, the substances may not be able to inhibit the enzyme substantially. The insecticide chlorpyrifos excerts toxicity mainly by metabolic transformation to clorpyrifos-oxon, which poses a much more potent inhibitor of the enzyme acetylcholinesterase (Chambers and Chambers, 1989; Lech and Bend, 1980); the same is true for diazinon which is metabolized to diazoxon and other metabolites such as 2-isopropyl-6-methyl-4-pyrimidinol (pyrimidinol) by the NADPH-cytochrome P450 mixed function oxidase system (MFO) (Hogan and Knowles, 1972; Keizer et al., 1993; Keizer et al., 1991). Given the substantial difference in the degree of acetylcholinesterase inhibition, an optimal use of this promising parameter requires the zebrafish embryo test be prolonged after the chorion stage, e.g. up to 96 or 120 hpf, to get clearer and more reliable results.

The second hypothesis ('Inhibition of the enzyme acetylcholinesterase results in behavioural impairment in juvenile zebrafish.') could be verified by the measurements of enzyme activity as well as locomotor activity. The larvae showed jerky swimming movements with pauses at concentrations of ≥250 µg chlorpyrifos/L in acute measurements (Kienle et al., in press) as well as in the subchronic test with chlorpyrifos. Here locomotor activity as well as enzyme activity was decreased at ≥10 µg/L at an age of 5 days. Cramping could be observed as well. For diazinon similar effects could be observed, however in a much higher concentration range (≥2 mg/L) than for chlorpyrifos and not in environmentally relevant concentrations.

These movements are amongst the typical effects resulting from the mode of action of chorpyrifos as inhibitor of the enzyme acetylcholinesterase (AChE) (Kamrin, 1997; Kegley et al., 2007). AChE inhibitors are leading to depression with acute intoxication. This results in the reduction of a variety of behavioural responses, innate as well as learned (Bignami et al., 1975). In low doses the activity can be stimulated in some cases (Brunet et al., 1997), in others periods of hyperactivity can precede or follow periods of reduced activity (Fryday et al., 1995; Hart, 1993). AChE inhibitors are capable of altering daily activity patterns of animals (Brunet and Cyr, 1992). Such alterations induced by chemicals can eventually have effects on more specific behaviour patterns such as predator avoidance (Dell`Omo, 1997).

In the only other available study relating acetylcholine esterase inhibition directly with behavioural measurements, Sandahl et al. (2005) found a coherence between the inhibition of acetylcholinesterase and behavioural impairment in 4-5 month old coho salmon (*Oncorhynchus kisutch*) exposed to chlorpyrifos at concentrations of 0.6 to 2.5 µg CHP/L. Here a concentration-dependent inhibition of the AChE activity in brain and muscle tissue occurred, as well as the inhibition of the behavioural patterns investigated (spontaneous swimming and feeding behaviour). Therefore, the authors concluded that there must be a close relationship between the degree of AchE inhibition in the brain and behavioural impairment. With an effect concentration as low as 10 µg/L in the present study, the results for zebrafish and coho salmon are in the same order of magnitude.

For diazinon behavioural impairment has also been reported for adult medaka (*Oryzias latipes*) in concentrations of 0.1 mg/L diazinon and also for larval zebrafish exposed to 1,

15 and 30 µg/L diazinon (Chon et al., 2005; Wall, 2000). In this case, the effect concentrations of our study are much higher as compared to the previous results.

A possible reason for the much lower toxicity of diazinon as compared to chlorpyrifos could be a difference in the octanol-water partition coefficients (K_{ow}). However with log K_{ow} values in the range of 3.02 (Suntio et al., 1988) and 3.81 (Ladaa et al., 1998) found in the literature for diazinon and 4.99 (Kamrin, 1997) and 5.11 (Ladaa et al., 1998) for chlorpyrifos, the differences in toxicity between those substances might partly have been caused by differences in the log K_{ow}, which might indicate a faster adsorption of chlorpyrifos compared to diazinon as well as a slower elimination and a higher bioaccumulation.

In binary mixtures the substances acted additively for all of the observed parameters in the tested concentration range and the concentration ratios tested. Therefore our last hypothesis ('In mixtures of chlorpyrifos and diazinon both substances act additive as expected by their similar mode of action.') could be verified. However the differences in toxicity of the two substances led to equipotent effects for diazinon at much higher concentration levels than that observed for chlorpyrifos. In the few papers available, this could also be observed for *Ceriodaphnia dubia* exposed to both substances (Bailey et al., 1997) as well as for rats (Timchalk et al., 2005). Increased toxicity compared to the single substances was observed, when the invertebrates *Hyalella azteca* and *Musca domestica* were exposed to a mixture of atrazine and several organophosphate insecticides (chlorpyrifos, methyl parathion, and diazinon) (Anderson and Lydy, 2002). No data on fish concerning diazinon/chlorpyrifos mixtures were available. Chinook salmon exposed to binary mixtures of several organophosphate and carbamate insecticides the substances acted additively on brain acetylcholinesterase (Scholz et al., 2006). Our results therefore support the previous studies. Concerning risk assessment for organophosphate and carbamate pesticides acting as acetylcholinesterase inhibitors, toxicity might be underestimated when only looking at the single toxicants (Scholz et al., 2006) and also low toxicant concentrations in the NOEC range may sum up to a toxic effect, when occurring in mixtures (Silva et al., 2002). Also, exposure occurring in the embryonic and larval stages can, although only a short time period, influence the behaviour up to the adult stage as shown by Levin et al. (2003). Exposure of *D. rerio* to 10 and 100 ng CHP/L in the larval stage (1-5 dpf) was capable of altering spatial

discrimination and response latency up to the adult stage as well as leading to elevated mortality at 100 ng/L in 20-38 week old zebrafish. Therefore chlorpyrifos might pose a clear risk for fish in the environment and should be addressed further. For diazinon, the risk might be somewhat lower as the effect concentrations are quite high; however, in hotspots or in acute pollution, a risk might be present as well.

Conclusions

The parameter acetylcholinesterase inhibition has been proven to be a reasonable and reliable parameter for exposure of zebrafish to organophosphate pesticides. For chlorpyrifos the effect concentrations were much lower than the effect concentrations for morphological abnormalities or survival, but equal to the effect concentrations for behavioural impairment. Therefore those parameters seem to be closely related. The reasons for the low diazinon toxicity might be differences in the log K_{ow}.
Mixtures of both substances exhibited, as expected, additive toxicity on the parameters acetylcholinesterase inhibition, locomotor activity, deformations and survival. Regarding risk assessment for the investigated compounds, this also means that low concentrations of the single compounds in mixtures may sum up to a toxic effect.

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Kapitel 5: Impairment of trophic interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) by chlorpyrifos

Cornelia Kienle[a], Miriam E. Langer[a], Almut Gerhardt [b], Heinz-R. Köhler[a]

[a]Department of Animal Physiological Ecology, University of Tübingen, Konrad-Adenauer-Str. 20, D-72072 Tübingen, Germany

[b]LimCo International, Oststrasse 24, D-49477 Ibbenbüren, Germany

* Both authors contributed equally to this paper and share first authorship.

Abstract

This paper presents a new approach for the investigation of predator-prey interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) impaired by chlorpyrifos, a neurotoxic insecticide. With a simple experimental design including four different treatments: (1) control, (2) predator exposed, (3) prey exposed and (4) both, predator and prey, exposed, we were able to detect an increase in the feeding rate of zebrafish preying on exposed chironomids after acute (2 h) exposure to 6 µg/L CHP. Previous a decrease in the burrowing behaviour of exposed chironomid larvae was observed. However when pre-exposing simultaneously both predators and prey, no significant differences in the feeding rate of zebrafish were observed. This suggests an impairment in prey recognition of the exposed zebrafish. At a lower CHP concentration (1 µg/L), no differences in feeding rate of zebrafish were observed. We propose the use of trophic interactions as parameters in higher tier studies for chemical testing and evaluation of ecotoxicological risk assessment.

Keywords: feeding depression, pesticide, non-biting midge, fish, interspecific interaction

unpublished manuscript

1. Introduction

Behavioural responses often occur rapidly after exposure to environmental pollutants. Therefore they represent a sensitive indicator of the influence of pollutants on non target organisms. Pollutant-induced alterations in behaviour are acting not only on individuals, but also on the viability of populations and the structure of ecosystems (Dell`Omo, 2002). Up to now, ecotoxicological studies have focused mainly on the direct effect of pollutants on organisms; although indirect effects, such as an impairment of inter- and intraspecific interactions, are also likely to occur following an exposure event. In this context, predator-prey relationships are important interactions between species and may be susceptible to pollutant exposure.

To date, studies of predator-prey interactions have concentrated mainly on either the predator or the prey. This topic has been addressed in a number of studies up to now, with most investigations aiming at the prey (e.g. Baker and Ball, 1995; Brown, 2003; Goyke and Hershey, 1992; Hershey, 1987; Hölker and Stief, 2005; Macchiusi and Baker, 1992; Schulz and Dabrowski, 2001; Sih, 1982; Tseng, 2003). But as predator and prey live in the same biocoenosis it is quite likely, that both groups of organisms will be affected by pollution either directly or indirectly. Only a few studies are available which focus on the predator (e.g. Hamers and Krogh, 1997; Power, 1990). Grippo and Heath (2003) detected the effects of mercury on the foraging efficiency and capture speed of fathead minnows (*Pimephales promelas*) exposed to 13 and 57 µg/L $HgCl_2$. The prey capture rate of mummichogs (*Fundulus heteroclitus*) in the laboratory was closely related to the diet of the fish in the field, thus representing a biomarker with high ecological relevance. However, due to great variability at the different test sites it was not especially sensitive (Weis et al., 2001).

As proposed by Lima (2002), important conclusions can be drawn about ecological consequences if predators and prey are regarded equally in the investigation of predator-prey interactions. This approach has been focused in a few field and laboratory studies with aquatic invertebrates, amphibians, fish and terrestric organisms (Bridges, 1999; Hamers and Krogh, 1997; Rahel and Stein, 1988; Taylor et al., 1995; Thorp and Bergey, 1981). Therefore, in our study, we aimed at including both, predator and prey, in the pollution scenario.

As “model” predator we chose the zebrafish (*Danio rerio*), which originally lives in

stream habitats rich in macrophytes in South East Asia (Börries, 2006). Ecologically, fish represent a very important group of secondary consumers and in part of top predators. Besides they serve as a food basis in these ecosystems. In many studies and husbandry instructions chironomids have been used as prey objects for *D. rerio* (Béchard et al., 2008; Lawrence, 2007; Nyholm et al., 2008).

Our "model" prey organisms were larvae of the non-biting midge *Chironomus riparius*. This organism was chosen because of its ecological importance as food item for fish (Pinder, 1986). As sediment-dwelling organisms, they are particularly susceptible to sediment bound pollutants.

In the literature, studies showed that larvae of *C. riparius* burrowed significantly deeper when exposed to the fish kairomones, simulating increasing predator density by *Rutilus rutilus* (Hölker and Stief, 2005). A predatory damselfly, which oriented visually as do fish, fed mostly on chironomids which spent more time out of the tube, i.e. on the sediment surface (Hershey, 1987).

As a "model" for an environmentally relevant pollutant we chose chlorpyrifos (CHP), a broad-spectrum organophosphorus insecticide (Richardson, 1995). It is one of the most common active compounds in pest control products worldwide (Dow AgroSciences, 2008) and is applied in high amounts to agricultural areas of corn, cotton, apples and other orchard crops (Gilliom et al., 2006). In 1990, approx. 1.4 million pounds of this insecticide were applied in the Central Valley of California (Sheipline, 1993). In urban streams in the United States, the chlorpyrifos concentration exceeded water quality benchmarks in 37 % of the sites (2nd highest exceedance rate after diazinon) and in 21% of the sites in agricultural streams (highest exceedance rate) during 1992-2001 (Gilliom et al., 2006). Environmental concentrations of 0.19 – 0.3 µg/L were detected in urban waterways in California and in several surface waters in the USA (Bailey et al., 2000; Gilliom et al., 2006). CHP acts on the nervous system as an inhibitor of the enzyme acetylcholinesterase (Kamrin, 1997). The toxicity of chlorpyrifos has been mainly assessed during the early life stages of zebrafish (e.g. Kienle et al., in press; Levin et al., 2003; Levin et al., 2004; Roex et al., 2002; Scheil and Köhler, 2009), where effective imparing concentrations were 10 µg/L for locomotor activity and 250 µg/L for morphological abnormalities (Kienle et al., in press). For adult freshwater fish, after 96 h LC_{50} values ranged from 9 µg/L for adult rainbow trout to 331 µg/L for fathead minnow

(Kamrin, 1997; U.S. EPA, 1986).

The effects of chlorpyrifos on chironomids have been assessed in various studies (Ankley et al., 1994; Belden and Lydy, 2000; Callaghan et al., 2001; Fisher et al., 2000; Jin-Clark et al., 2002; Lydy et al., 1999; Moore et al., 1998; Schuler et al., 2005). For *Chironomus tentans* effective concentrations, for the single substance, were found to be at 0.3 µg/L (48 h LC_{50}) (Moore et al., 1998) and 0.07 µg/L (10 d LC_{50}) (Ankley et al., 1994), The EC_{50} for abnormal swimming movements was 0.39 – 0.49 µg/L for chlorpyrifos (Belden and Lydy, 2000; Jin-Clark et al., 2002).

The above studies mainly investigated the effects of chlorpyrifos alone and in mixtures on the acute toxicity to *C. tentans* and on early-life stage toxicity to zebrafish with different parameters. These included abnormal swimming movements and mortality, among others. However, studies regarding predator-prey interactions with this widely used insecticide are lacking for invertebrates as well as for fish.

In the present study the effects of chlorpyrifos on predator-prey interactions between zebrafish (*D. rerio*) and chironomids (*C. riparus*) were investigated. The following hypotheses were tested for predator-prey interactions between zebrafish and chironomids.

1. Exposed chironomids are burrowing less than control animals, and are therefore more susceptible to predation by fish.
2. Predation by fish leads to increased burrowing behaviour in exposed as well as control chironomids.
3. When exposing predator and prey, the decreased ability of the predator to recognize the prey and of the prey to burrow are outweighed, resulting in no significant differences in feeding rate compared to the control.

2. Materials and Methods

In the following experiment the zebrafish *Danio rerio* was used as the predator, and the larvae of the non biting midge, *Chironomus riparius* as the prey. The animal maintenance and the experiments were conducted in an acclimatized chamber at 25±0.5°C.

2.1. Animal culturing and keeping

Chironomus riparius

Egg ropes of *C. riparius* have been collected from a breeding stock of the University of Tübingen, and kept at 21±0.5°C. After hatching, chironomids in the first larval stage (L1) were reared in plastic containers containing dechlorinated tap water and a two centimetre thick layer of quartz sand (particle size 0.1-0.3 mm, burned for 3 h at 500°C to remove organic matter; Dehner, Germany) under constant aeration. Every day the chironomid larvae of each stock vessel were fed *ad libitum* with fine powderized ground fish flakes (50% Tetramin, 50% Tetraphyll, Tetra, Germany). Dechlorinated tap water was exchanged once a week. For acclimation to the final test conditions, *C. riparius* larvae (L1) have been kept in a climate chamber at 25 ± 0.5°C for ten days until they reached the L4 stage. After 10 days larvae reached the L4 stage and were used for the predator-prey experiment.

Danio rerio

The four to six month old *Danio rerio* (total length: 27.93 ± 3.95 mm) used in our experiments were partly the offspring of wild-type zebrafish from the strain WIK (ZFIN ID: ZDB-GENO-010531-2) and wild-type zebrafish from the strain Tue.G14 (generously provided by the Max-Planck-Institute for Developmental Biology in Tübingen). The fish were kept in aerated and filtered aquaria with a minimum of 1 litre of water per fish. Culture conditions were 25 ± 0.5°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 had up to one month time for acclimatisation to the new environment. To become acquainted with the prey objects, *D. rerio* was fed with living *C. riparius* larvae several times before the start of the experiment.

2.2 Test substance

Chlorpyrifos (Pestanal, analytical standard, Sigma-Aldrich, Germany) was dissolved in reconstituted water (OECD, 1992). In order to prepare a stock solution it was constantly stirred for a minimum of 4 hours at a water temperature of about 45°C and a pH of 8.0. Subsequently, the solution was kept at 35°C overnight until use with constant stirring. From this stock test solutions were prepared directly before use with dechlorinated tap water. Nominal test concentrations for exposure experiments were 1 µg and 6 µg CHP/L. The retrieval rate for chlorpyrifos in an earlier study with the same experimental setup for stock solution preparation was 51.8 % in analytical measurements (Kienle et al., in press). Therefore concentrations of 0.5 and 3 µg/L CHP have to be expected in our exposure experiments.

2.3 Experimental design

Preliminary tests

In a preliminary test, the burrowing behaviour of *C. riparius* in the L4 stage has been observed (unpublished data). The burrowing behaviour of 3×50 *C. riparius* L4 larvae was examined every 20 minutes and the part of *C. riparius* totally and partly visible has been investigated by visual observation. Due to those experiments, a two hour period was determined as the adequate time for healthy *C. riparius* to dig entirely into the sediment and to show natural behaviour.

In a preliminary test, the recapture rate of *C. riparius* L4 larvae burrowed in quartz sediment was observed (replicated 9 times) as well. The recapture rate was 97.22%.

Additionally, in a preliminary test, the feeding rate of *D. rerio* with 100 introduced *C. riparius* larvae was determined. After 2 hours, the average number of surviving chironomids was between 50 and 60 individuals. With this medial number of surviving chironomids in the control treatment it is possible to detect both, an increase or decrease in feeding rate/survival rate. Therefore in the main experiment we chose 100 chironomids as adequate number for the predator-prey experiments.

Main experiments

In this study, three different treatments and one negative control have been investigated

1. Predator pre-exposed (Dc *D. rerio* contaminated)
2. Prey pre-exposed (Cc *C. riparius* contaminated)
3. Predator and prey pre-exposed (Bc Both (fish and chironomids) contaminated)

All treatments were replicated three times. In every replicate 5 *D. rerio* as predator and 100 *C. riparius* as prey were introduced.

100 chironomids per replicate have been collected randomly and transferred for exposure into large dishes (15 cm diameter, depth 8 cm) containing 50 g of quartz sediment and the corresponding chlorpyrifos solution made from dechlorinated tap water. After two hours the chironomids were transferred into 10 L aquaria containing 400 g quartz sediment (corresponding to a 1-2 cm thick layer) and 8 L of a mixture of tap water and distilled water to obtain a conductivity of 400-450 µS/cm. During the transfer of the test organisms into the feeding aquaria special attention was paid to make sure that no contaminated sediment and water were transferred. In the two hours following the transfer the chironomids were able to burrow into the sediment.

In the meantime, 5 *D. rerio* per replicate were transferred into 4 L aquaria containing 3 L of the respective chlorpyrifos solution or control water. All aquaria have been wrapped with a black cover to avoid disturbances from human presence. The fish were exposed for two hours to the CHP contaminant.

Before the transfer of *D. rerio* into the 10 L aquaria with the *C. riparius* larvae, the numbers of chironomids completely visible at the surface and those partly visible were counted. After the transfer, the fish had two hours to forage and feed on *C. riparius*.

After these 2 hour period, the number of chironomids completely at the surface and those partly visible was re-counted. The fish were removed and anaesthetised with benzocain. The total length of each fish was measured with a sliding calliper (powerfix EMC, model number Z11155, resolution 0.01 mm). Water was removed by dabbing the fish on paper towels. Each fish was shock frozen in liquid nitrogen and stored for further AChE inhibition analyses.

Subsequently, surviving chironomids were searched in the 10 L aquaria and the sediment. The surviving organisms were also dried on paper towels and if possible 5

samples containing 5 chironomids were shock frozen in liquid nitrogen and stored for later AChE inhibition analysis.

Several abiotic parameters (i.e. pH, conductivity, and temperature) have been measured at the beginning and the end of the feeding trial.

2.4 Data analysis

Nonparametric methods were chosen for the analysis because the data were only partially normally distributed (Shapiro-Wilk Test, JMP 4.0, SAS systems, USA). The data from all tests were analysed for significance using Friedman's ANOVA (Statistica 5.0, StatSoft, USA), followed by a Wilcoxon two group test (equivalent to Mann-Whitney test, JMP 4.0, SAS systems, USA) to examine differences between control and exposure treatments.

3. Results

The average total length of the zebrafish was 27.93 ± 3.95 mm (see Table 1). There were no significant size differences between the various treatments (Friedman`s Anova n.s.).

Table 1: Total length of *D. rerio* (mm) (mean ± SD). No significant difference was found in *D. rerio* size between the different treatments.

	Treatment			
	control	**CK**	**DK**	**BK**
Mean	28.42	27.60	28.34	27.36
SD	3.47	4.13	4.60	3.64

At a concentration of 1 µg/L of CHP no significant difference between the treatments was observed (Fig. 1).

No changes in the numbers of burrowed chironomids, of chironomids partly at the surface and of chironomids remaining at the sediment surface occurred neither before the introduction of the fish into the feeding aquaria nor after the removal of the fish (Friedman`s Anova n.s., respectively; data not shown). Also no significant difference was found in the feeding rate of *D. rerio* preying on *C. riparius* exposed to 1 µg/L CHP in neither of the treatments (Fig. 1).

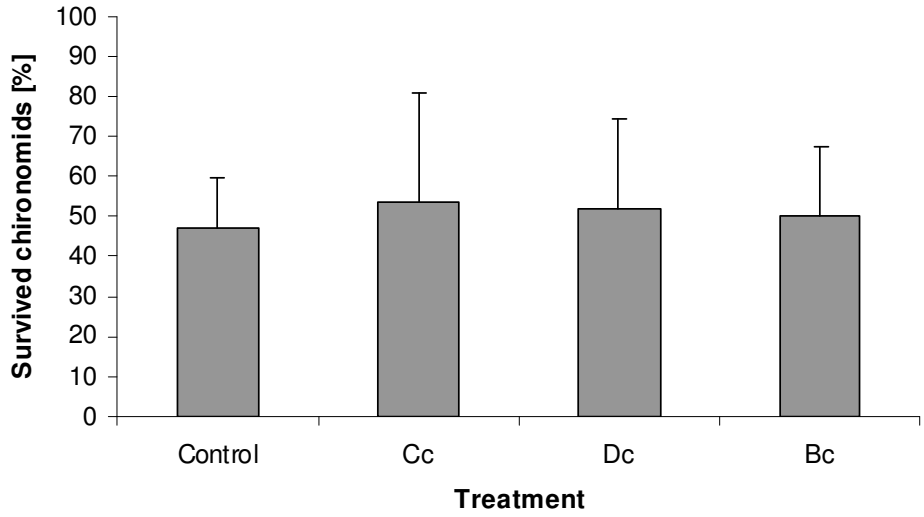

Fig. 1. Feeding rate [%] of *D. rerio* on larval chironomids after two hours. Fish and/or chironomids were exposed to 1 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. n = 3, bars represent means ± SD.

At a concentration of 6 µg/L CHP the burrowing behaviour of exposed *C. riparius* was significantly changed compared to the burrowing behaviour of nonexposed chironomids before the introduction of the fish. Here the number of *C. riparius* remaining completely at the sediment surface was significantly increased (Wilcoxon Cc p=0.0495, Bc p=0.0495) (Fig. 2).

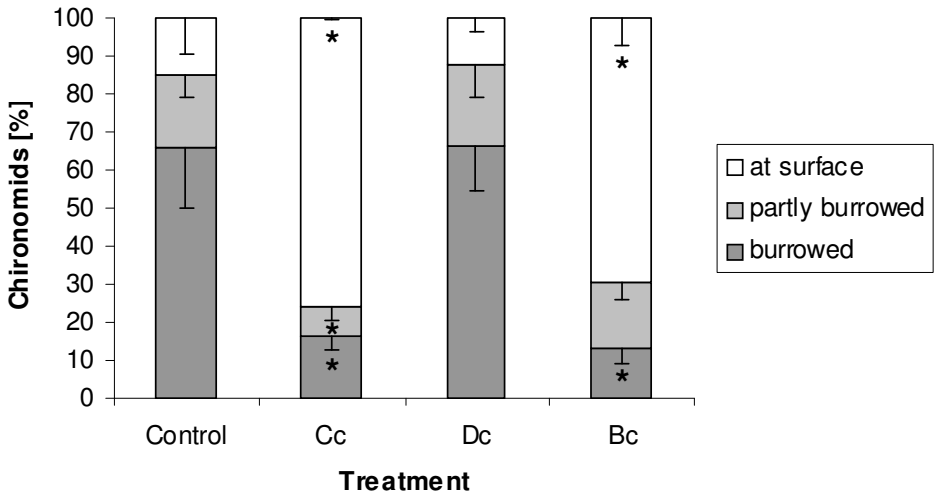

Fig. 2. Percentage of chironomids at the sediment surface, partly burrowed and totally burrowed before introducing the zebrafish. Fish and/or chironomids were exposed to 6 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. * Significantly different to the control, $p < 0.05$. n = 3, bars represent means ± SD.

Consequently, a significantly decreased number of larvae were partly and fully burrowed compared to the number of unexposed chironomids. No significant difference occurred between the burrowing behaviour of unexposed (Control and Dc) or exposed chironomids (Cc and Bc), respectively. After being preyed on by zebra fish, a majority of the surviving *C. riparius* in the control and the Bc treatment were burrowed in the sediment (Fig. 3). Compared to that, the number of exposed *C. riparius* (Cc and Bc) at the surface was significantly increased (Fig. 3) (Wilcoxon Cc p=0.037, Bc p= 0.037).

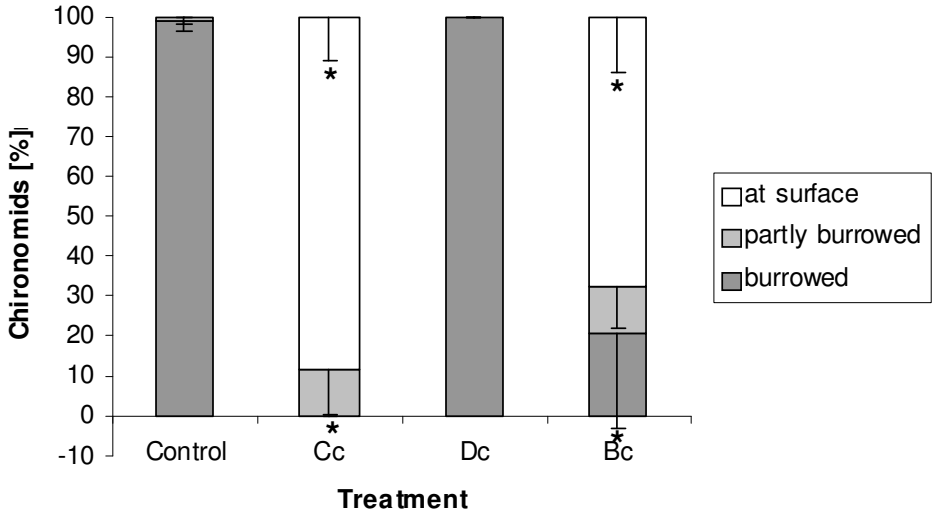

Fig. 3. Percentage of chironomids at the surface, partly burrowed and totally burrowed after being preyed on by zebrafish. Fish and/or chironomids were exposed to 6 µg/L CHP prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. * Significantly different to the control, $p < 0.05$. n = 3, bars represent means ± SD.

Comparing the number of burrowed chironomids before and after the introduction of the fish, significantly more animals were completely burrowed in the control treatment, as well as in the treatments where only the zebrafish were contaminated (Wilcoxon, $p = 0.046$ and $p = 0.046$, respectively) (Fig. 2 and 3). However, in the treatment where only the chironomids were exposed a significantly less number of animals was burrowed (Wilcoxon, $p = 0.037$) and when both, predator and prey, were exposed, no significant difference in the number of burrowed chironomids before and after the introduction of the fish was observable.

At the 6 µg/L CHP level the feeding rate of nonexposed *D. rerio* on exposed *C. riparius* was significantly increased compared towards the control (Wilcoxon $p = 0{,}0495$) (Fig. 4).

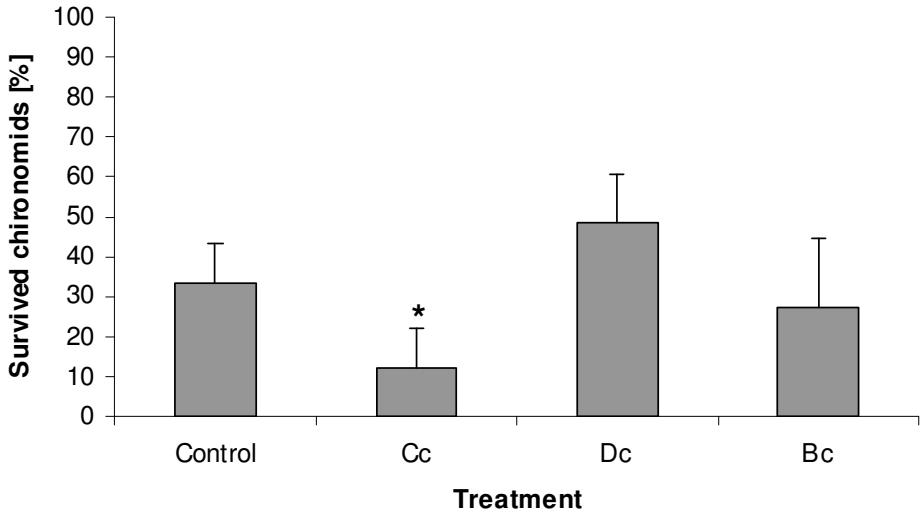

Fig. 4 Feeding rate [%] of *Danio rerio* on larval chironomids after two hours. Fish and/or chironomids were exposed to 6 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. * Significantly different to the control, $p < 0.05$. n = 3, bars represent means ± SD.

4. Discussion

The integrity of ecosystems can be influenced by stressors on many different levels. Most studies have focused on the direct effects of contaminants on single species. In the present study interactions between representatives of two trophic levels and different habitats, chironomids as benthic detritus feeders and fish as pelagic secondary consumers, were investigated. Contaminants may unbalance predator-prey relationships in a way that the hunting ability of fish and the predator avoidance behaviour of chironomids could be impaired.

The main exposure route for aquatic ecosystems is spray drift or runoff after a rain event following pesticide application. Therefore in stream systems, mainly short-time pollutant pulses occur. Regarding sediment exposure, chlorpyrifos exhibits a high affinity to sediments and a potential adsorption to sediment particles should not be omitted (Gilliom et al., 2006; Kamrin, 1997). In such a situation, chironomids might be

exposed even longer. Our study simulated realistic pulse exposures (both as a low and as a high dose), as concentrations of up to 0.3 µg CHP/L water have been measured in aquatic systems (Gilliom et al., 2006). Schulz (2001) detected maximum chlorpyrifos concentrations of 924 µg/kg CHP in the sediment after a single rainstorm event in the Lourens River, South Africa, whereas concentrations in the water were only 0.2 µg/L CHP.

When examining the burrowing behaviour of chironomids and the foraging behaviour of zebrafish exposed to 1 µg/L CHP, neither the natural behaviour of *C. riparius* nor the feeding rate of the fish seemed to be impaired by the pollutant.

This might result from the low concentration and short exposure time of these organisms to CHP. The highest tested CHP concentration of 6 µg/L could occur in water after a rain event following pesticide application, as high concentrations of CHP can be expected over a short period of time. At this concentration (6 µg/L), CHP impaired the ability of the exposed chironomids to show natural burrowing behaviour. In these treatments a major part of the chironomids stayed at the sediment surface instead of burrowing. Therefore, they seemed to be better detectable and more easily preyed upon by the unexposed *D. rerio* (Cc) (Fig. 4). Accordingly, our first hypothesis ('Exposed chironomids are burrowing less than control animals, and are therefore more susceptible to predation by fish.') was verified. In choice-experiments, Hershey (1987) found that predators consistently selected chironomids which spent more time out of their tube.

In the treatments with non-exposed chironomids (control and Dc), the introduced fish seemed to trigger an increase in burrowing behaviour. This could be due to the fact that the proportion of chironomids at the sediment surface was almost reduced to zero (Fig. 3). Such a behaviour has been observed with chironomid larvae exposed to fish-borne chemical cues (kairomones) simulating increasing predator densities (Hölker and Stief, 2005). It can be assumed that those chironomids which had burrowed survived. In the treatment with both, chironomids and zebrafish, being exposed, the feeding rate as well as the number of burrowed chironomids resembled that in the control treatment (Fig. 3). Thus, our second hypothesis ('Predation by fish leads to increased burrowing behaviour of chironomids in exposed as well as control animals.') was, in part, proven true. This is due to the fact that significantly more chironomids were burrowed in the

control and the Dc treatment after being preyed upon by fish, compared to the situation without fish (Fig. 2 and 3). The significantly reduced number of animals burrowed in the Cc treatment, after fish predation, indicates an easier capture of those animals by fish. This could be interpreted as a result of a reduced ability to burrow and increased convulsions. In the Bc treatment the chironomids did not or were not able to change burrowing behaviour due to fish predation as no significant difference in burrowed animals could be observed. Therefore finally, our third hypothesis ('When exposing predator and prey, the decreased ability of the predator to recognize the prey and of the prey to burrow are outweighed, resulting in no significant differences in feeding rate compared to the control') could be proven. This was achieved by the similar feeding rate of zebrafish in control treaments and in the Bc treatment. Similar results were obtained when investigating predator-prey relationships between two amphibian species under insecticide exposure (Bridges, 1999). Here predation rates did not differ from those under natural conditions when pre-exposing both, predator and prey, simultaneously.

In the literature, chironomids have been found to be an important prey object to several fish species (Forsyth and James, 1988). It is known that the densities of chironomids can respond to fish predation (Gilinsky, 1984). In conclusion, the results from our study imply that the biocoenosis of aquatic ecosystems might be indirectly affected due to pollutant exposure.

The effect concentration in our study is in the range of earlier studies with *C. tentans* exposed to chlorpyrifos, where effective concentrations of 0.3 µg/L (48 h LC_{50}), 0.07 µg/L (10 d LC_{50}) and 0.39 – 0.49 µg/L (EC_{50} for abnormal swimming movements) were observed (Ankley et al., 1994; Belden and Lydy, 2000; Jin-Clark et al., 2002; Moore et al., 1998). However, our results can be considered as even more relevant due to the short exposure time and the integrative parameters observed. The same is true for chlorpyrifos exposure to zebrafish, where subchronic effects on locomotor activity were visible at 10 µg/L (Kienle et al., in press) and chronic effects on response latency and spatial discrimination of adult zebrafish occurred after early life stage exposure to 0.1 µg/L chlorpyrifos (Levin et al., 2003). Our effective concentration is slightly higher than the one mentioned above. In a previous study, the predator avoidance behaviour of chironomids in reaction to kairomones of predatory fish (*Rutilus rutilus*) did influence mineralization processes of organic matter (Stief and Hölker, 2006). This indicates that

predator-prey interactions have an impact on basic ecosystem processes. Alterations in predator-prey relations due to environmental pollutants may therefore affect these processes.

Our results implicate that simple single species ecotoxicity tests do not reflect adequately the possible effects of a toxin in an ecological context. Up to now the relevance of predator-prey interactions has not been considered in chemical risk assessment. Our study shows the relevance of the mentioned problem and also gives a simple method to quantify the effects of a toxic compound, CHP, on interactions between predator and prey organisms.

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Kapitel 6: Behaviour of *Corophium volutator* (Crustacea, Amphipoda) exposed to the water-accommodated fraction of oil in water and sediment

Cornelia Kienle and Almut Gerhardt

LimCo International, An der Aa 5, 49477 Ibbenbüren, Germany

Abstract

We investigated the short-term effects of the water accommodated fraction (WAF) of weathered Forties crude oil on the behavior of *Corophium volutator* in the Multispecies Freshwater Biomonitor® (MFB). When exposing *C. volutator* to 25 and 50 % WAF in aqueous exposures, hyperactivity with an additional increase in ventilation was detected, whereas exposure to 100 % WAF led to hypoactivity (narcosis). In a sediment exposure with 100 % WAF, there was an increased tendency toward hyperactivity. In a pulse experiment, hyperactivity appeared at and after a 130-min exposure to 50 % WAF in a majority of cases. Our experiments suggest that the behavior of *C. volutator* as measured in the MFB may be an appropriate parameter for coastal monitoring.

Keywords: Mud shrimp, Oil, Pulse pollution, Locomotor activity

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1. Introduction

Intertidal communities are highly vulnerable to oil spill incidents. This is a consequence of their location at the shoreline interface between water and land where floating oil is deposited by the waves [1]. The water-accommodated fraction (WAF) of oil is a combination of single-phase homogenous mixtures (water-soluble fractions) of hydrocarbons and dispersions of fine oil droplets in water [2]. It is this fraction that often represents the greatest risk to aquatic organisms.

The mud shrimp *Corophium volutator* is one of the most abundant organisms in estuarine mudflats of the North Atlantic, American, and European coasts, extending from western Norway to the Mediterranean and into the Black Sea and Azov Sea (http://www.marlin.ac.uk/species/Corophiumvolutator.htm). It can attain a size of approximately 10 mm and lives in self-constructed tubes in intertidal mudflats, salt-marsh pools, and brackish ditches (http://ip30.eti.uva.nl/bis/crustacea.php). It has the habit of swimming when in open water (http://www.marlin.ac.uk/species/ Corophiumvolutator.htm) but generally shows low motility and burrows in the sediment most of the time [3]. *Corophium volutator* tolerates a wide range of salinity from near fully saline to almost freshwater and is locally abundant (http://ip30.eti.uva.nl/bis/ crustacea.php). It has already been used in several marine bioassays to assess acute as well as chronic toxicity [4–8]. For behavior measurements with *C. volutator*, test parameters have been burrowing time, re-emergence from the sediment and activity prior to burrowing (exposure to WAF) [6], changes in swimming behavior, and locomotor and ventilatory activity (exposure to the pesticide Bioban [Brenntag, Deerlijk, Belgium]) [7]. Behavior is considered to be a sensitive indicator for effects of contaminants [9].

Until now, only one behavioral study regarding WAF sediment exposure has been conducted [6]. The present study represents the first effort to investigate the effects of WAF (aqueous and in sediment) on behavior and survival of *C. volutator* using the Multispecies Freshwater Biomonitor (MFB; LimCo International, Ibbenbüren, Germany), an online biomonitor that continuously and quantitatively records the behavior pattern of animals in both aqueous and sediment exposures.

The aims of the present study were to examine the suitability of the MFB for detecting effects of WAF (aqueous and in sediment) on *C. volutator*. The effects of several dilutions

of WAF on the locomotor and ventilatory activity of *C. volutator* were to be assessed, as were any differences between behavior in aqueous and in sediment exposures. An additional objective was to examine the ability of *C. volutator* to recover from aqueous WAF exposure.

2. Materials and Methods

2.1 Maintenance of test animals

Adult *C. volutator* and sediment were collected as described in Smith et al. [8] from an intertidal area of the Avon estuary near Aveton Gifford, South Devon, United Kingdom. Amphipods were separated from the sediment via sieving through a 500-µm sieve so that neonates passed through, while midsize individuals, which should be used for the tests, remained in the sieve. The animals were put into 5-L culture tanks holding field-collected and sieved (<300 µm) sediment as well as aerated and filtered seawater (25 ± 1 ‰). The tanks were maintained at 15 ± 1°C with a 12:12-h light:dark cycle. After an acclimation period of 7 d, sediment with embedded *C. volutator* was sieved again to extract the individuals. Size-matched specimens of medium size (~3–4 mm) were used for behavior measurements.

2.2 Preparation of WAFs and spiking of sediment

The WAFs were prepared using weathered Forties Blend crude oil consisting mainly of paraffines, naphthenes, and aromatics. They were prepared as described in Smith et al. [8]. For exposure preparations, 5-L Pyrex bottles were used. Twenty-five milliliters of crude oil and 2,475 ml of 25 ‰ sea water were slowly vortex mixed in a temperature-controlled room (15 ± 1°C) for 24 h and then left to re-equilibrate for 1 h. Sampling was carried out by carefully siphoning off the water phase containing WAF by applying a gentle pressure of nitrogen to the top of the oil/seawater surface [8]. For the aqueous exposures, 100 % WAF and dilutions of 25 and 50 % WAF in 25 ‰ seawater were prepared.

For the sediment exposures, the sediment was spiked with 100 % WAF as described in Smith et al. [8]. Portions of 160-ml-sieved sediment were placed in 500-ml-wide-neck glass bottles (Schott, Mainz, Germany), and an aliquot of 320 ml WAF (prepared with 25 ‰ seawater; see previous description) was added to each bottle. This mixture was shaken at 15°C for 3.5 h and 200 rpm on an orbital shaker. Afterward, the slurry from

each bottle was transferred to 2-L Pyrex beakers. After allowing for 16 h of settlement, the supernatant was discarded [8]. Before the sediment was transferred to the measurement chambers, it was stirred for approximately 30 s with a spatula for final homogenization. Control treatments were prepared in the same way as the exposure treatments substituting 25 ‰ seawater for WAF.

2.3 Multispecies Freshwater Biomonitor

The MFB, an online biomonitor for continuously and quantitatively recording the behavior pattern of animals, consists of flow-through sensor chambers, a measuring unit, and a personal computer with specific software for data analysis [10]. The measuring principle in the sensor chamber is based on quadrupole impedance conversion. The behavioral signal from the animal is analyzed by a fast Fourier transformation, resulting in a histogram of different signal frequencies. Different frequency ranges can be attributed to different types of behavior, such as locomotion (summarized in frequency band 1: 0.5–2 Hz) and ventilation (summarized in frequency band 2: 2.5–8 Hz) [10]. The MFB nonoptical recording principle based on quadrupole impedance conversion allows for equal signal quality in water and sediment, as has already been shown in several studies [7, 11, 12]. It is presently the only automated biomonitoring system with this capability.

2.4 Experimental setup

Figure 1 shows the experimental setup for the behavior measurements. Experiments were conducted in 100-ml glass beakers filled with approximately 100 ml of test solution. One measuring chamber of the MFB was placed vertically in each beaker. The chambers used for *C. volutator* were 4 cm in length with a diameter of 1 cm, allowing for free movement of the individuals (length of *C. volutator*: ~3–4 mm). The chambers were sealed with a mesh lid at one end (mesh width 0.25 mm), the beaker was partially filled with test solution, and one amphipod was put into each chamber. After sealing the other end of the chamber, the beaker was completely filled with the test solution, and any remaining air bubbles in the chamber were removed.

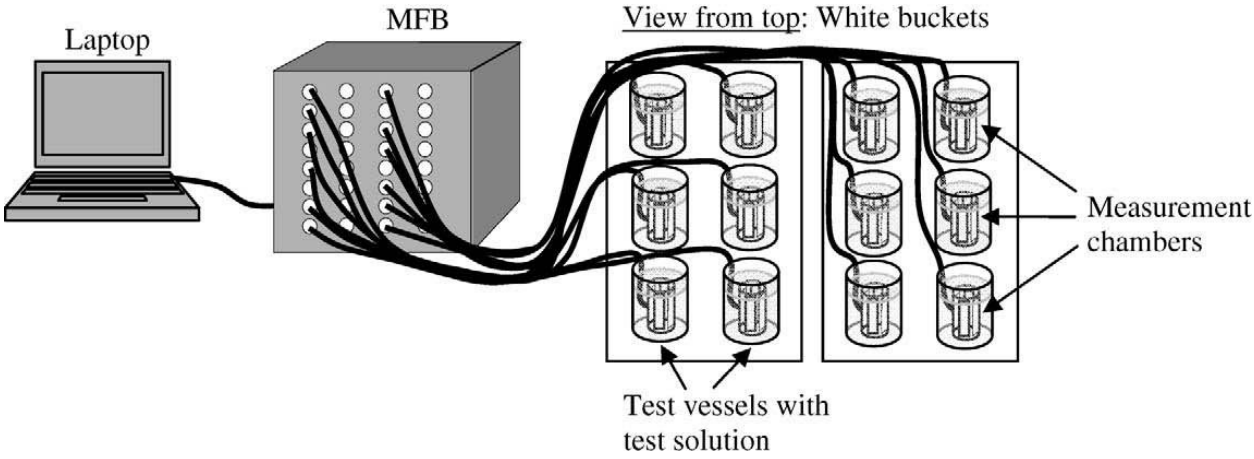

Fig. 1. Experimental setup for the behavior measurements with *Corophium volutator* in aqueous and sediment exposure. MFB = Multispecies Freshwater Biomonitor® (LimCo International, Ibbenbüren, Germany).

Vessels for sediment exposure were also 100-ml glass beakers with one measuring chamber placed within each. The measurement chambers were half filled with sediment and half filled with water in order to give the animals the opportunity to swim in the water column and burrow in the sediment. The sediment was allowed to settle for approximately 1 h. The rest of the procedure was the same as for aqueous exposure.

Subsequently, the locomotor and ventilatory behavior of four to six individuals of *C. volutator* for each treatment was measured for a duration of 2 h in the acute aqueous (0, 25, 50, 100 % WAF) and sediment exposures (0, 100 % WAF). For acute aqueous exposures, three control treatments were conducted in total with each WAF exposure paired with a control in the system. For sediment exposures, the position of the animals in the chambers (in the water column or in the sediment) was noted several times during the exposure period of 2 h (minimum three times).

Moreover, in an additional stress and recovery pulse experiment, the behavior of six *C. volutator* was recorded for 2 h in 50 % WAF and 25 ‰ seawater, respectively, with the previously described experimental setup for the aqueous exposure. After those 2 h, half the solution was removed and replaced with filtered 25 ‰ seawater. After an additional 1.5 h, all of the test solution was removed and replaced by seawater. After approximately 20 h, this pulse experiment was terminated. The animals were kept in a 12:12-h light:dark cycle (light from 8 AM to 8 PM). No food was added.

2.4 Data analysis

For each individual, mean locomotor (0.5–2 Hz, band 1) and ventilatory activity (>2 Hz, band 2) (% time spent on locomotion and ventilation, respectively) were calculated for the exposure time of 2 h. The behavior of four to six individuals of *C. volutator* was measured for each treatment. As the data of the three control treatments for aqueous exposures did not differ significantly, they were summarized for data analysis. For statistical evaluation, the data on time percentage of activity were arcsine transformed from proportional values. Nonparametric methods were chosen because the data were only partially normally distributed (Shapiro–Wilk W test; JMP 4.0, SAS Systems, Cary, NC, USA). Differences between control and exposure treatments were analyzed for significance with a Wilcoxon two-group test (JMP 4.0) followed by a Bonferroni–Holm adjustment [13]. Behavioral data of the stress and recovery experiment were normalized to the reference data (ref, behavioral data of the 25 ‰ seawater treatment) for each data point throughout the whole exposure period ($f(x) = x/ref \times 100$) to flatten out the normal circadian rhythm, while any circadian variation left in the curves could be interpreted as amplification of the rhythmicity due to exposure stress [14]. Afterward, the curve was split into increasing/decreasing and monotonous parts, and a spline run was performed with the data using a linear regression model (JMP 4.0).

3. Results and Discussion

3.1 MFB signals for activity in sediment

Corophium volutator showed almost constant swimming activity in water, as reflected by continuous signals with high amplitude (Fig. 2A), and lower activity in sediment, as reflected by movement signals combined with pauses and a generally lower amplitude (Fig. 2B).

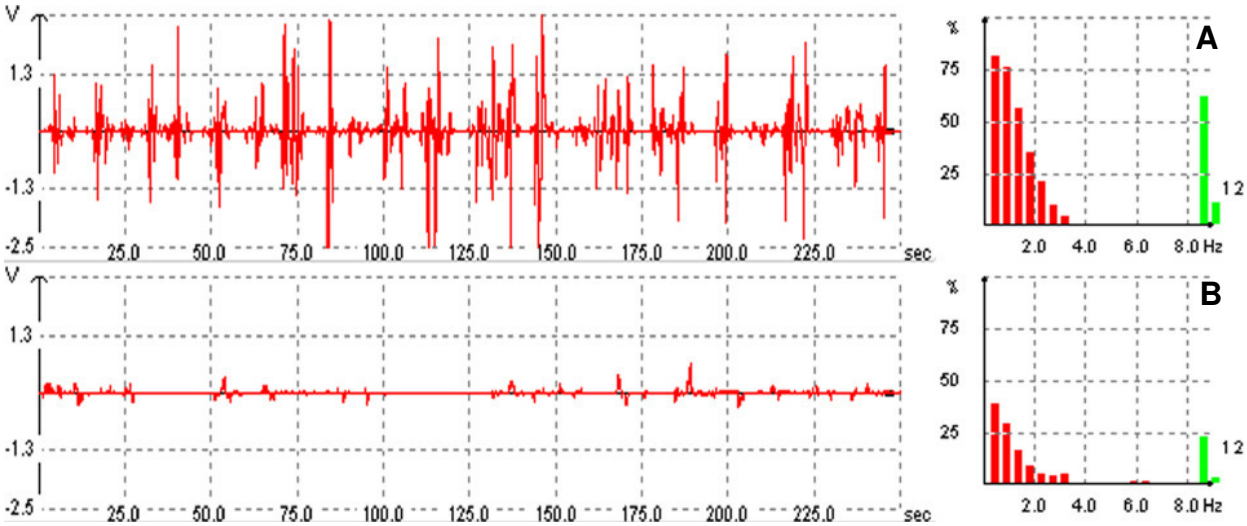

Fig. 2. Different behavior patterns of *Corophium volutator*. Left: Locomotor pattern (amplitude [V] vs time [s]). Right: Corresponding fast Fourier transformation histograms (locomotor activity in % of the time [250 s] vs frequency [Hz]) of *C. volutator* in uncontaminated water (A) and in sediment (B).

In addition to locomotor activity, ventilation signals could be detected, as reflected by regular signals in the relevant frequency range (2.5–8 Hz) (Fig. 3A and C). The signals for locomotion were in the range of 0.5 to 2 Hz, whereas ventilation frequencies lay above 2 Hz (Figs. 2 and 3), similar to the description in Kirkpatrick et al. [7]. With increasing ventilation activity, the histogram changed from low to high frequencies (Fig. 3A).

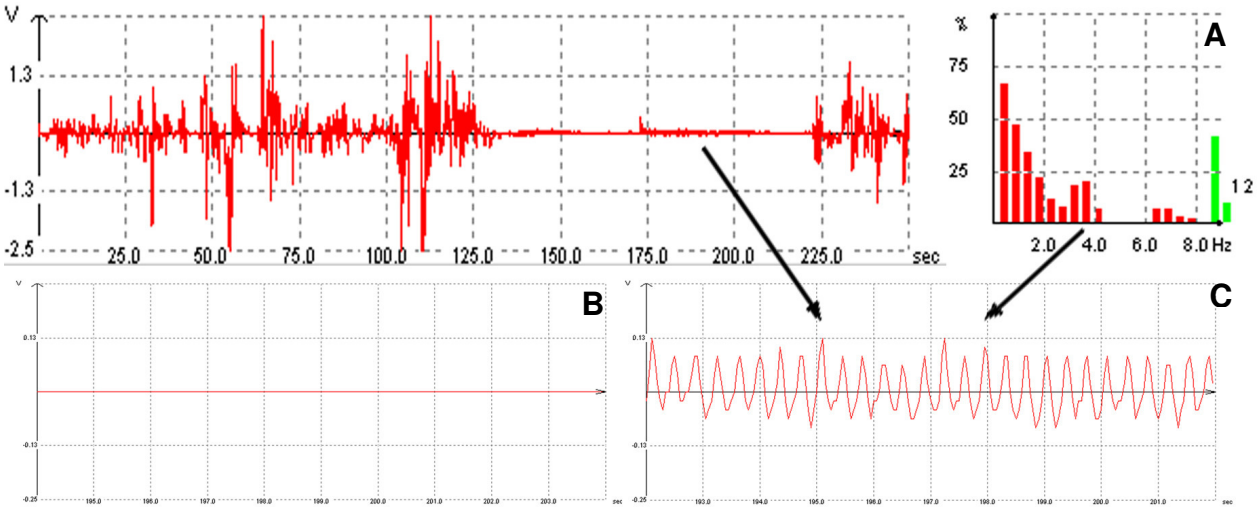

Fig. 3. Different behavior patterns of *Corophium volutator*. Left: Locomotor pattern (amplitude [V] vs time [s]). Right: Corresponding fast Fourier transformation histograms (locomotor activity in percent of the time [250 s] vs frequency [Hz]) of *C. volutator*.(A) Locomotion and ventilation signals in control water. (B) Signal of an empty chamber as baseline. (C) Enlarged view of ventilation signals.

3.2 Acute aqueous exposures to 25, 50, and 100 % WAF

When exposed to 25 and 50 % WAF, *C. volutator* displayed hyperactivity $p < 0.001$ (band 1, 25 % WAF) and $p < 0.01$ (band 1, 50 % WAF) compared to the control treatments. In the treatment with 100 % WAF, the animals showed signs of narcosis and were lying on the bottom for most of the measurement time. In this treatment, variation in locomotor activity was high, three out of five animals showing only low locomotor activity (Fig. 4). Hyperactivity (increased swimming activity) is a common symptom of toxic effects, indicating an avoidance/escape response [14]. This type of locomotor escape behavior has been described for *C. volutator* exposed to Bioban, [7] as well as for other organisms (e.g., *Gambusia holbrooki* [mosquitofish] exposed to acid mine drainage [14] and *Gammarus pulex* [freshwater shrimp] exposed to a simulated Cu-pulse [70 ppb] in situ [15]). Given the stressors (100 % WAF, including oils and polycyclic aromatic hydrocarbons), narcosis would be a consistent response. This was suggested by seeing nonmoving amphipods lying on the sediment. Increased variability in behavior has been observed as a result of toxicity previously [16]. However, in the present study, significant behavioral responses occurred at much lower concentrations than those associated with narcosis (e.g., 25 vs 100 % WAF). The fact that exposure to 100 % WAF does not result in any significant difference to the control may be explained by the high variation in the behavior of the test animals. The differences could be clearly observed,

as the amphipods were not using the whole chamber but only the lower area for swimming and were lying on the bottom most of the time. This increased variation in behavior is presumably due to the fact that some individuals were already affected by narcosis (i.e., less active), while others were not. Hyperactivity has been observed as a first sign of stress before. This behavioral response might present an attempt of the amphipods to escape the toxic area.

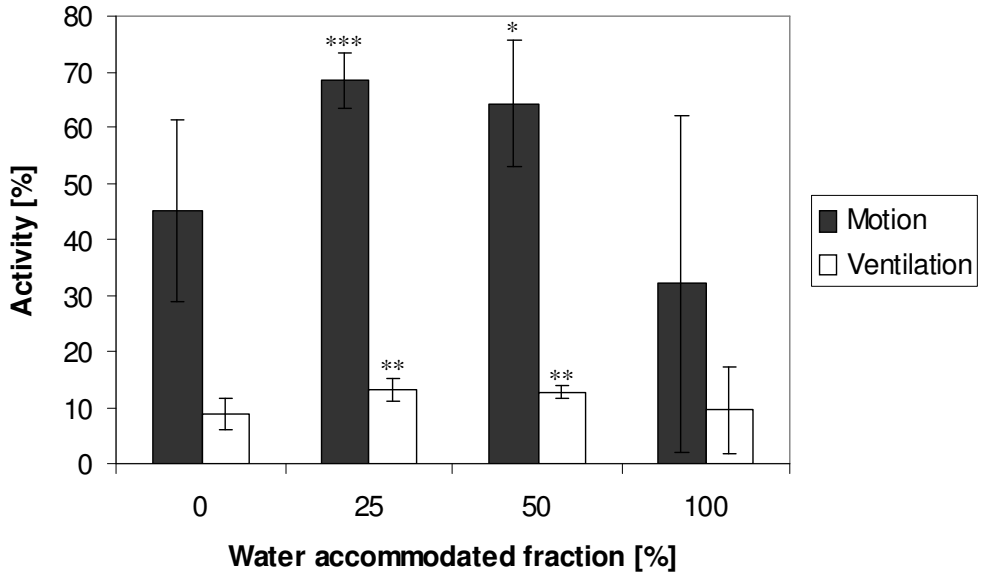

Fig. 4. Activity (%) of *Corophium volutator* in control treatments (n = 4–6 per treatment; summary of three control treatments: 14 individuals in total) and exposed to 25 % (n = 7), 50 % (n = 6), and 100 % (n = 5) water-accommodated fraction in frequency bands 1 (0.5–2 Hz) (■: locomotion) and 2 (2.5–8 Hz) (□: ventilation) (mean ± standard deviation). Significant differences from control treatment: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

With 25 and 50 % WAF, significant effects on ventilation occurred ($p = 0.003$ [band 2, 25 % WAF] and $p = 0.007$ [band 2, 50 % WAF]). A similar response could be observed for *C. volutator* exposed to high concentrations of Bioban [7] as well as for *G. holbrooki* and *Daphnia magna* (water flea) exposed to acid mine drainage [14]. An increase in ventilation might indicate an attempt by the animal to remove the toxins from the body surface [14].

3.3 Sediment 25 ‰ seawater and 100 % WAF exposure

When exposed to sediment spiked with 100 % WAF, *C. volutator* showed a tendency toward hyperactivity, although differences compared to the control were not significant (due to high interindividual variation) (Fig. 5). One of six animals in the WAF treatment did not burrow at all, while five others stayed in the sediment for a certain amount of time. Also, amphipods were swimming, as verified by visual observation and observation of the movement patterns, where swimming activity could be clearly distinguished from locomotor activity in sediment (Fig. 2A and B). In the control treatment, two of six animals frequently alternated between the water and sediment compartments, and four animals were constantly burrowing into the sediment.

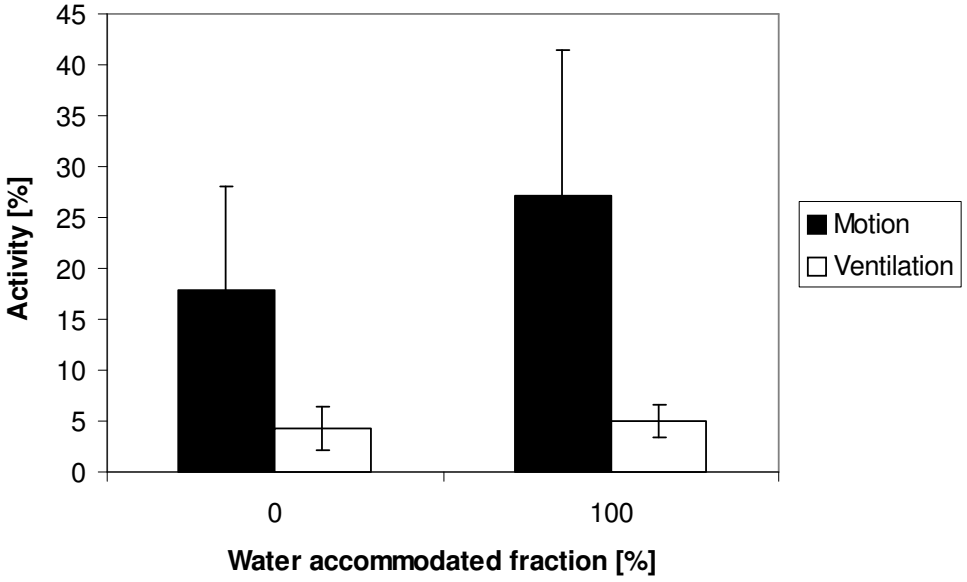

Fig. 5. Activity (%) of *Corophium volutator* in control sediment (n = 6) and in sediment spiked with 100% water-accommodated fraction (n = 6) in frequency bands 1 (0.5–2 Hz) (■: locomotion) and 2 (2.5–8 Hz) (□: ventilation) (mean ± standard deviation).

When examining the burrowing behavior of *C. volutator* exposed to sediment spiked with Forties crude oil, *C. volutator* showed a greater tendency to avoid burrowing and to reemerge from the sediment [6]. In the present study, more animals in the 100 % WAF exposure than in the control treatment spent part of the time swimming (six vs two out of six individuals). This finding supports the results of Scarlett et al. [6] but with differences in exposure conditions (pure oil in Scarlett et al.'s experiments [6] vs WAF in the present study). Moreover, there was less space to burrow in the measurement

chambers in the present study than in the glass beakers in Scarlett et al. [6]. For more detailed sediment studies, it may be useful to give the amphipods more space to burrow (i.e., larger measurement chambers). To be able to distinguish better between behavioral signals in the sediment and in the water compartment in further studies, separate chambers stuck together should be used [7]. In a comparison of laboratory and in situ bioassays with *C. volutator*, the amphipods reacted more sensitively in *in situ* bioassays [17]. This may indicate that observed effects in the present study underestimate in situ effects.

No mortality occurred during any of the experiments of the present study. Also, in chronic studies with WAF (110 d), no significant effects on the survival of *C. volutator* were observed with 100 % WAF [8]. These results demonstrate that behavioral responses may occur at lower concentrations (at 25 and 50 % WAF) than effects to more traditional endpoints like malformations and mortality. This supports the higher sensitivity of behavioral endpoints.

3.4 Aqueous 50 % WAF recovery exposure

In the stress and recovery pulse experiment with a 130-min pulse of 50 % WAF (Fig. 6), *C. volutator* displayed a significant increase in locomotor activity (hyperactivity) compared to the control during the exposure (locomotor activity [normalized] = 97.684 +0.979 min, $r^2 = 0.754$, $p < 0.001$).

After exchanging half the solution with control water, the hyperactive locomotor activity decreased until the full exchange of solution after 240 min and remained near the control level afterward until 420 min. In the recovery period, three activity peaks could be observed. From 420 to 590 min, the locomotor activity increased significantly (locomotor activity [normalized] = -227.938 + 0.873 min, $r^2 = 0.528$, $p < 0.001$) and then decreased below control level (590–670 min, locomotor activity [normalized] = 2,438.827 - 3.655 min, $r^2 = 0.810$, $p < 0.001$). This was followed by a significant increase in activity (670–790 min, locomotor activity [normalized] = -761.004 + 1.179 min, $r^2 = 0.891$, $p < 0.001$), which decreased again afterward. A last small activity peak could be observed from 1,020 to 1,080 min (locomotor activity [normalized] = -1,038.828 + 1.112 min, $r^2 = 0.786$, $p = 0.008$). Afterward, the locomotor activity decreased to the control level and remained similar for the rest of the recovery time (1,110–1,220 min).

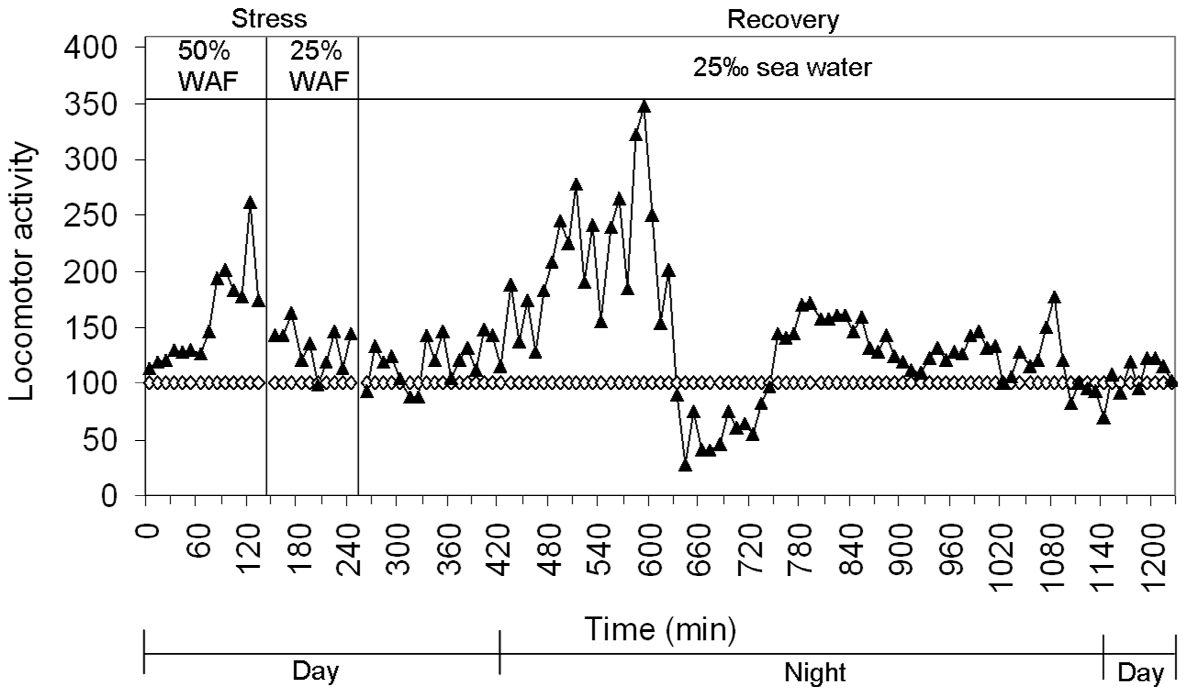

Fig. 6. Locomotor activity (%) of *Corophium volutator* in control treatment (n = 6) (◊ locomotion control) and exposed to 50 % water-accommodated fraction (WAF) (n = 6) (▲: locomotion 50 % WAF) (data normalized to control values, standard deviation [SD] range [control] = 9.31–26.44 %; SD range [exposure] = 6.98–28.65 %) in frequency band 1 (0.5–2 Hz) from 0 to 130 min, to 25 % WAF from 150 to 240 min (data normalized to control values, SD range [control] = 21.55–26.29 %; SD range [exposure] = 6.78–30.69 %) and to control water from 260 to 1,220 min (data normalized to control values; SD range [control] = 12.05–30.20 %; SD range [exposure] = 1.60–30.69 %).

The results show that, if *C. volutator* was first exposed to 50 % WAF (resulting in hyperactivity similar to the 2-h acute exposures), the subsequent 25 % WAF exposure was sufficient to allow the activity to decrease to the control level (dissimilar to the 2-h acute exposures at this concentration level). This indicates that the effects of contaminants on the behavior may differ, depending on the previous exposure conditions. So in the environment, individuals of *C. volutator* that were previously exposed to a certain level of contaminants might be less sensitive than animals living in a noncontaminated environment. This may depend on the frequency of oil spill pulses and their respective concentration levels. The tendency toward higher activity of previously exposed amphipods compared to control animals suggests that effects of exposure to contaminants can continue even though the contaminant is no longer present in the environment. An increase in activity could also be observed for *Crangonyx pseudogracilis* (northern river crangonyctid) after exposure to a pulse of ammonium chloride [18]. In an in situ experiment at the Rhine River, *G. pulex* showed decreased

activity due to an oil pollution peak [19]. This corresponds well with the narcotic effect of 100 % WAF observed in our exposures with *C. volutator* and might have occurred in the pulse experiment if 100 % WAF had been used.

It seems that *C. volutator* was able to recover from aqueous exposure to WAF approximately 18 h after the exposure period, but for a more detailed interpretation, further experiments with a longer recovery time and perhaps a second exposure period would be necessary. In a pulsed exposure experiment, carbamate insecticides were less toxic to *Chironomus riparius* (a midge larva) larvae if recovery in clean water was permitted, but exposure to organophosphate insecticides proved to be equally toxic even after changes in the conditions [20]. In a pulsed exposures experiment with the freshwater amphipod *Hyalella azteca* using $CuSO_4$ and Na pentachlorophenol, recovery time had a significant effect on the mortality at secondary exposure [21]. If the animals were provided enough time between exposures, the amphipods were able to recover to a state similar to their original condition [21]. No data were available in the literature for pulsed exposure to oil or WAF.

According to Diamond et al. [22], pulsed exposure effects are dependent on the frequency, magnitude, and duration as well as the recovery period between pulses. They suggest that chronic water quality criteria and effluent permit limits may not be sufficient for protecting against such effects [22].

Conclusions

The MFB proved to be suitable for detecting behavioral effects of WAF on *C. volutator*. When comparing aqueous and sediment exposure, the effects of WAF on the locomotor activity of *C. volutator* were more pronounced in aqueous exposures than in sediment exposures. The higher sensitivity of aqueous exposure is partly outweighed by the lower environmental relevance because *C. volutator* spends most of the time in the sediment. This may also indicate that *C. volutator* can seek refuge in the sediment as a shelter from aqueous oil pollution spills and hence minimize toxic short-term effects. Clear differences between behavior in sediment and in water could be observed. The locomotor activity in sediment was lower than in water. *Corophium volutator* seemed to be able to recover from WAF exposure after approximately 18 h of recovery, but further and longer experiments are necessary to prove this conclusion.

Acknowledgements

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Kapitel 7: Biomonitoring with *Gammarus pulex* at the Meuse (NL), Aller (GER) and Rhine (F) rivers with the online Multispecies Freshwater Biomonitor®

Almut Gerhardt,[*a] Cornelia Kienle,[a] Ian J. Allan,[b] Richard Greenwood,[b] Nathalie Guigues,[c] Anne-Marie Fouillac,[c] Graham A. Mills,[d] and Catherine Gonzalez[e]

[a] LimCo International, An der Aa 5, D-49477 Ibbenbüren, Germany, Fax/Phone+495451970390, e-mail: AlmutG@web.de
[b] School of Biological Sciences, University of Portsmouth, King Henry Building, King Henry I Street Portsmouth, PO1 2DY, UK,
[c] Bureau de recherches géologiques et minières, 3 av. Claude Guillemin, BP6009, 45060 Orleans cedex 2, France,
[d] School of Pharmacy and Biomedical Sciences, University of Portsmouth, White Swan Road, PO1 2DT, UK,
[e] École des Mines d'Alès - 6, Avenue de Clavières - 30319 Alès Cedex, France

Abstract

Biological early warning systems represent a set of tools that may be able to respond to certain chemical monitoring requirements of recent European legislation, the Water Framework Directive (WFD2000/60/EC) that aims to improve and protect water quality across Europe. *In situ* biomonitoring was performed along the rivers Meuse (NL), Aller (GER) and Rhine (F) within the frame of the European Union-funded Project SWIFT-WFD. *Gammarus pulex* was used as test organism during the evaluation of the Multispecies Freshwater Biomonitor® (MFB), an online biomonitor to quantitatively record different behaviour patterns of animals. At the river Meuse *G. pulex* reacted to pulse exposure of either a mixture of trace metals or of several organic xenobiotics by showing up to 20 % decreased locomotory activity (already at the 1st pulse) and increased mortality (at 2nd or 3rd pulse only). *G. pulex* deployed within the MFB system were observed to survive well at the monitoring station on the Aller (100 %) and monitoring did not result in the measurement of chemical irregularities. In contrast, deployment at the monitoring station on the Rhine river demonstrated that the test organism was able to detect chemical irregularities by up to 20 % decreased locomotory activity in the animals. The MFB proved to be an alert system for water quality monitoring at sensitive sites and sites with accidental pollution.

Journal of Environmental Monitoring, 2007, 9(9), 979-985

Introduction

The objectives of the Water Framework Directive (WFD2000/60/EC) are to improve, protect and prevent further deterioration of water quality across Europe. Different types of monitoring are demanded: surveillance monitoring to assess long-term water quality changes and for the generation of baseline data on river basins, operational monitoring for additional and essential data on water bodies at risk or those failing environmental objectives of the WFD and investigative monitoring to determine causes of such failures. For each type, monitoring of a number of quality elements such as biology, hydromorphology, physico-chemistry and chemistry (in/organic priority substances) and adequate techniques are required.[1] Within the EU-funded project SWIFT-WFD (**S**creening methods for **W**ater data **I**n**F**orma**T**ion in support of the implementation of the Water Framework Directive) one important focus was on the evaluation and validation of existing and emerging tools and technologies for water quality monitoring in various case studies[2] and to establish a link between information provided by chemical sensors and by biological methods. Biological techniques that were evaluated included biological early warning systems that enable real-time monitoring of changes in water quality.[2]

Since *Gammarus pulex* is a detritus feeder and commonly-found or frequent inhabitant of European streams,[3] it was chosen as test organism for the present work. Until now, this species has never been used within another online biomonitor system; the use of the Multispecies Freshwater Biomonitor® (MFB) (LimCo International, Ibbenbueren, Germany) with Gammarids has been the focus of many studies, both in the laboratory and *in situ* (*e.g.* ref. 3-6). Importantly, this species has repeatedly been proposed as a standard test organism for ecotoxicological tests worldwide.[3]

The Multispecies Freshwater Biomonitor® (MFB) allows the quantitative behavioural recording of a variety of animal species in water, soil and sediment in a fully automated manner.[5] The MFB has been at the centre of numerous laboratory- or field-based ecotoxicological studies (*e.g.* ref. 2, 3, 7-9) such as on the river Rhine[3] or at a sewage treatment plant,[10] however *in situ* applications are currently limited. In the present study the MFB was evaluated in three European river basins and more specifically in the rivers Meuse (Eijsden, NL), Aller (GER) and Rhine (F).

The MFB presents a number of advantages when compared with other *in situ* online biomonitoring systems:

(1) No requirements for filtration or pre-treatment of the water under evaluation. This allows the realistic validation of the effects of both dissolved and bioavailable but particle-bound pollutants on the test organism. This is especially important since detritus serves on the one hand as food for numerous stream organisms such as *Gammarus pulex* and on the other hand contributes to the sediment dynamics (deposition, remobilisation); thus the "sediment" component is included towards an integrated approach in online biomonitoring.

(2) The MFB also allows the measurement of sediment inhabitants directly in their substrate. Organisms are exposed in flow-through measurement chambers that may be filled with sediment since sediments do not interfere with the non-optical measurement principle.[11]

In order to validate the MFB for *in situ* application with *G. pulex* as new indicator species, three different field sites were chosen with different characteristics in order to answer the following research questions:

(1) Is *G. pulex* able to survive in clean unfiltered surface water with detritus as food source in the MFB?

The Aller (D), a small, relatively unpolluted stream, allowed the operation of the MFB test system without alarms or disturbances, *i.e.* the undisturbed base operation. The Aller and its adjacent land areas are announced as Fauna Flora Habitat (FFH) areas, parts are Special Protection Areas (SPA) under the Birds Directive.[12]

(2) Is *G. pulex* able to react to a cocktail of metals or organic xenobiotics applied as pulse pollution in concentrations relevant to those occurring under accidental circumstances?

The Meuse River (Eijsden, NL) represents natural river conditions with average water quality conditions, such as those found in many river basins across Europe. A manned monitoring station is present on the Meuse River between Liège and Maastricht.[13] There, many chemical and biological parameters are recorded online, and in the past were proved to be able to detect pollution accidents. However, biomonitors used there are based on video technique and require filtering of the original water as well as changes in water velocity. Accidental Cd pollution levels in the Meuse have been reported before.[14]

(3) After demonstrating that *G. pulex* is a pollution-sensitive, robust and easy to handle indicator species, the final question is to test the MFB in combination with *G. pulex* during a long-term evaluation at a location with frequent pulse pollution and changes in water quality.

The Rhine river flowing through Huningue and Basel is a waterway heavily used for navigation, and with many nearby chemical industries. Therefore this portion of the Rhine river is at risk and monitoring is required. Water quality of the Rhine is monitored through 29 national and international monitoring stations.[15] The monitoring station at Huningue, located at the border of Switzerland, France and Germany, has been working since 1986, year of the Sandoz incident that resulted in the contamination of the Rhine with organophosphorus pesticides and mercury compounds.

Materials and Methods

Maintenance of test organisms

G. pulex was sampled from an unpolluted reference stream flowing through agricultural and forested areas a few days before the tests (Eijsden: Aa, Germany, 11.04.05; Langlingen: Aa, Germany, 15.11.05; Huningue: Kander, Germany, 14.05.06) and brought to the respective measurement stations in Eijsden (Meuse), Langlingen (Aller) and Huningue (Rhine) and kept in aerated stream water with detritus as food source until use.

Multispecies Freshwater Biomonitor® (MFB)

The Multispecies Freshwater Biomonitor® (MFB) allows fully automatic, online, real-time based quantitative recordings of the whole behavioural pattern of all aquatic in/vertebrates. It consists of test chambers with usually one animal in each, that can be placed *in situ* or in a tank (recirculation or flow-through), a recorder (impedance converter instrument) and a PC-unit (Laptop or PC). Organisms are exposed continuously in the test water. A quantitative recording of the behaviour (swimming (0.5-2 Hz), ventilation (2.5-8 Hz), inactivity (zero line)) is conducted automatically every 10 min and lasts for 4 min. Data recording and analyses (*via* a time series model) are fully automated.[7,16] Since the MFB is based on a non-optical recording principle—the tetrapole impedance conversion—it is suitable for applications both in laboratory and *in situ* with unfiltered raw water. It is a modular test system (8-96 channels) which allows

a high replication, as well as the monitoring of several species at the same time. The control of the system is done by the monitoring of an empty chamber with leaves (without *G. pulex*) for detection of signal disturbances due to physico-chemical changes in the water or man-made disturbances. Alarm calculation was based on a moving average time series models as well as a series of jump detectors, such as the Hinkley detector, the double sigma detector and the slope detector.[15]

Experimental setup and design

***In situ* monitoring in the Aller.** Several chemical and physico-chemical parameters (e.g. temperature, pH, conductivity, oxygen content) are continuously monitored at the Langlingen monitoring station at the Aller River. *In situ* monitoring with the MFB was performed from the 17th of November to the 1st of December 2005.

Experimental tank tests with Meuse River water. While the manned monitoring station in Eijsden allows near continuous monitoring of a wide range of physico-chemical characteristics, it is difficult to predict changes in water quality or levels of trace pollutants under field conditions or to incorporate these into field studies. Therefore, a series of 5 day-long tank tests were performed to enable the evaluation of a number of water quality monitoring tools under more controlled trace pollutant concentrations that, otherwise, would be impossible in field studies.[13,17-19] The experiments were performed in spring, 14th to 26th of April 2005. In this time pulsed spiking with metals and organics was performed.

Tank set-up for dosing experiments. Two tank tests were undertaken for a period of five days each by using 200 L tanks filled with fresh natural river water from the Meuse. Re-circulating (plastic tank) and flow-through (stainless steel tank) systems were used for the simulation of fluctuating trace metals and organic pollutants, respectively (Fig. 1). River water from the Meuse was used in the system and spiking with a mixture of metals or organic pollutants allowed to evaluate a wide range of tools under controlled conditions. Mixing was by a carousel housing a number of *in situ* monitoring devices such as passive sampling devices and the flow-through systems that enable control over variations in pollutant concentrations with time.

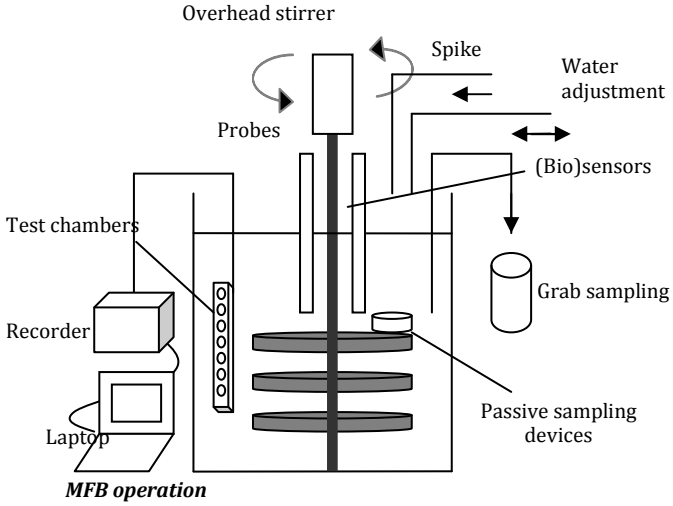

Fig. 1 General diagram of the set-up for the tank tests conducted with Meuse river water for the evaluation of a wide range of water quality monitoring tools (off, on-line sensors, biosensors, passive sampling devices, chemical test kits and biological early warning systems).

In short, spiking solutions were prepared in nitric acid and methanol for metals and organics, respectively, and were added by direct addition for metals or with the use of a peristaltic pump for organic pollutants and volumes were optimised in order to ensure minimal influence of spiking media on the performance of the tools evaluated. Fortification with trace metals included Cd, Cu, Ni, Pb and Zn and while additions of polycyclic aromatic hydrocarbons, polychlorinated biphenyls, and a range of polar and hydrophobic pesticides was undertaken (for concentrations see Results and discussion section). Water samples were collected at regular intervals in order to determine total organic pollutant concentrations or total, filtered (0.45 µm) and ultrafiltered (5 kDa) metal concentrations.[17] A total of 16 triplicate and 20 replicate samples were collected during the tank test, with fortification with metals and organic pollutants, respectively. Sample analysis was undertaken by an accredited commercial laboratory.

***In situ* monitoring at the Rhine.** Several parameters (physico-chemical, trace metals, organic matter and toxicity) were continuously monitored at the water monitoring station of Huningue. This site is particularly important since it allows the monitoring of episodic pollution events and the control of a lock to isolate a canal downstream the river in order to protect groundwater from contamination. At this point, canal water infiltrates into the groundwater, that is mainly used as drinking water or for agricultural activities in the area. The test systems were located inside the monitoring station in Huningue, at the border of Switzerland, France and Germany. The measurements of toxicity were carried out by two BEWS systems (Multispecies Freshwater Biomonitor® and Mosselmonitor® (www.mosselmonitor.nl). The measurement of total organic carbon (TOC) was carried out by an on-line UV spectrophotometer (STAC-Secomam). General toxicity was measured at the station by a biosensor, the Fluotox device that exploits the photosynthetic activity of algae cells. In principle, the presence of pollutants (such as pesticides) inhibits photosynthesis and thus induces an increase in fluorescence emission.[2] Monitoring with the MFB and *G. pulex* was conducted from the 18th of May to 29th of June 2006. Fig. 2 shows the experimental setup for the measurements at Aller and Rhine.

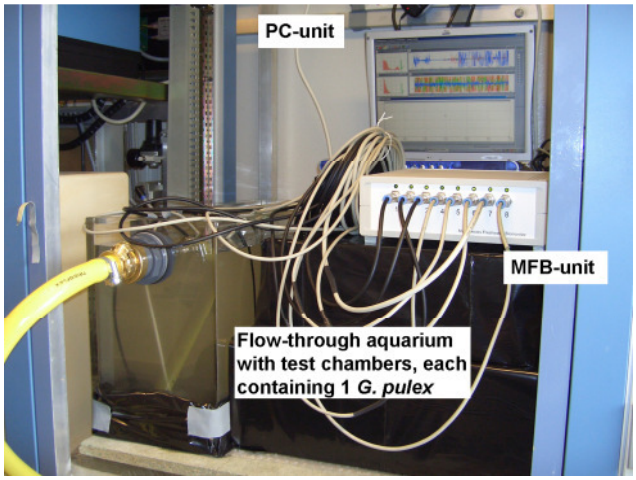

Fig. 2 Experimental setup for the Aller and the Rhine experiments.

Handling of the test organisms and behaviour measurements. The animals (size: *ca.* 5-8 mm) were carefully and separately placed in the measurement chambers (length: 4 cm, diameter: 2 cm, mesh width: 1 mm). Additionally, a piece of conditioned leaf (size: 1 cm) was introduced into the chamber as food. The chambers were arranged in a flow through aquarium (flow velocity: 40 mL s^{-1}., Aller test; >10 mL s^{-1}, Meuse test; approx. 200 mL s^{-1}, Rhine Test) parallel to the direction of flow and fixed. For the test a measuring device with 8 (Aller, Rhine) or 9 (Meuse) measurement channels was used with 7-8 Gammarids and 1 empty chamber as a control. The lead time including

assembly of the measurement device and insertion of the animals lasted 1-2 h. The MFB was then left to operate without disturbances for two weeks (Aller) without maintenance and for 1.5 months at the Rhine (Fig. 2).
The survival of the animals was checked at the Aller and the online data analysed after the 2 week exposure. During the Rhine test, survival was checked after 2 weeks and dead animals were replaced and new food provided. Three weeks later, the constitution of the animals was checked once more and the online data was analysed.

Data analysis. Data analysis consisted of the calculation of the percentage of time the animal spent on different behaviours, characterised by their signal frequencies, such as locomotion (≤2 Hz) and ventilation (>2 Hz). If the measured value deviated by more than 20 % from the predicted value (mean of five last records) on three subsequent recording occasions, a warning was given (light grey bars, see Fig. 4) and if more than 50 % of the animals were immobile, an alarm for mortality was given (dark grey bar, see Fig. 5).

Results and discussion

In situ monitoring at the Aller

Chemical analysis. Continuous chemical measurements revealed a constant decrease of the water temperature over time especially in the second week of exposure (from 6.7 to 3.8 °C), while oxygen, pH and conductivity levels remained mainly constant (Oxygen saturation: 91-101 %; pH: 7.46-7.59; Conductivity: 810-830 µS cm^{-1}). Chemical analysis of the Aller water (01.12.05) showed detectable concentrations of isoproturon (<1 ng L^{-1}) as well as metals (Al, Cr, Mn, Co, Ni, Cu, Zn, As, Cd, Pb) in the µg L^{-1} range. The water contained 90 mg Cl L^{-1} and 146 mg SO_4 L^{-1}. The TOC concentration was 6.8 mg L^{-1} while dissolved organic carbon (DOC) ranged around 5 mg L^{-1}. No chemical irregularities concerning organic compounds (pesticides, PAHs, PCBs) were detected.

Behaviour measurements. No alarms based on behaviour were detected by the MFB during the entire two week measurement period and the Gammarids were observed to have survived well in these conditions (100 % survival after 2 weeks exposure).

Behaviour pattern over time. The animals showed constant activity, consisting of swimming movements and crawling in the measurement chambers. However, in the second week a slight decrease in activity occurred. This could most easily be explained by the decrease of water temperature (from 6.7 °C in the beginning to 3.8 °C). The empty chamber demonstrated that no outside disturbances occurred during the exposure, which would have led to the measurement of a signal in that chamber (constant zero line and dark grey mark means "no signals").

The double sigma detector did not reveal any alarms, but exhibited some non-significant variations, particularly during the second week. This may be attributed to the short inactivity phases of the animals.

Survival. All 7 animals were observed to survive in the measurement chambers and to eat the food they were given (leaf). Additionally, they received detritus as food and substrate *via* sedimentation of detritus in the measurement chambers since approximately 1/4 of each measurement chamber was filled with mud. This is desirable since it reflects natural condition without disturbing the measurement signals.

Experimental tank test with Meuse river water

Main physico-chemical parameters. For the tank experiments with metals, most parameters remained stable over time. The water temperature ranged between 13 and 16 °C, the pH was ~8, conductivity lay between 490 and 500 µS cm^{-1} and dissolved oxygen ranged between 9 and 10 mg L^{-1}. The turbidity showed a constant decrease after stirring at the beginning of the measurements (14.04.05) and after adding fresh Meuse water (16.04.05).

Concerning the tank experiments with organic pollutants, most parameters were also relatively stable, with a temperature between 15 and 17.5 °C, a conductivity around 500 µS cm^{-1} and a pH of ~5. However, there was a significant drop of dissolved oxygen (from ~5.5 to 0 mg L^{-1}) and an increase in the redox potential (from ~40 to ~150 mV).

Temporal variations in metal and organic pollutant concentrations. Fluctuations in metal concentrations were generated during the course of the experiment and an example of these variations can be observed in Fig. 3a.

Peaks of metal concentrations were successfully obtained and reached a maximum of 69, 85, 58, 69 and 140 µg L^{-1} for Cd, Cu, Ni, Pb and Zn, respectively. While the filtered

fraction of Cd, Cu, Ni and Zn varied between 80 and 90 % of total concentrations, for Pb this was close to 45 %. Ultrafiltration at 5 kDa resulted in 90 % of Cd and Ni in the filtrate and 40, 20 and 60 % of Cu, Pb and Zn in the filtrate. During this tank test with metals, metal concentrations varied over time following the spiking scheme (Fig. 3a).[17]

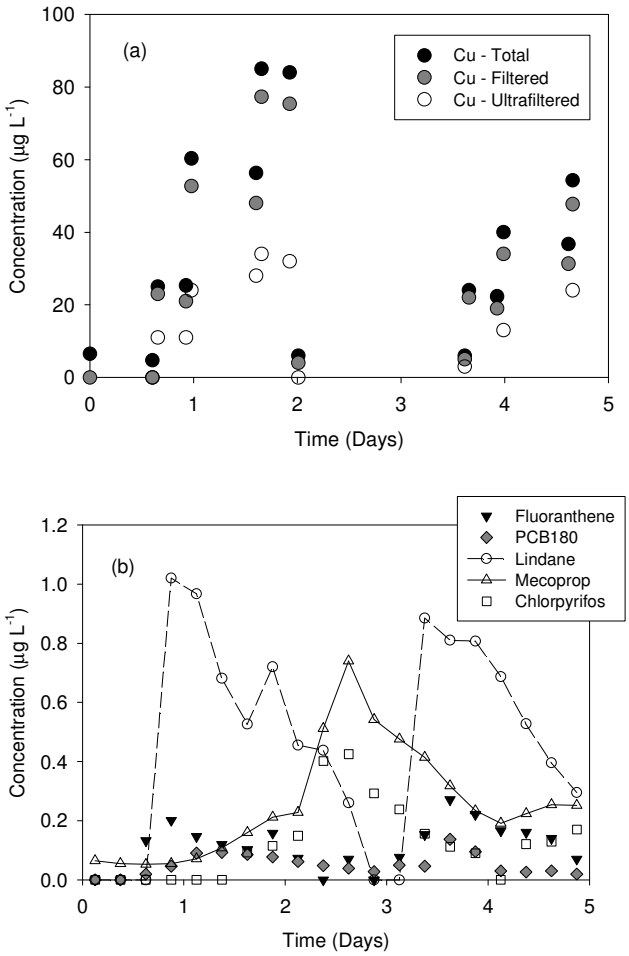

Fig. 3 Examples of changes in (a) total, filtered (0.45 µm) and ultrafiltered (5 kDa) Cu concentrations and (b) fluoranthene, PCB 180, lindane, mecoprop and chlorpyrifos concentrations (µg L^{-1}) with time, during the two tank tests.

Examples of scenarios of temporal variations in concentrations in PAHs, PCBs, organochlorine pesticides, carbamates or other phenoxy acetic acid herbicides may be found on Fig. 3b. Two peaks of concentration may be observed for lindane, and at a lower concentration, for fluoranthene and PCB 180. The total sum of concentrations of pesticide varied widely and approached 13 µg L^{-1} for the maximum peak concentration. While this value is highly unrealistic, concentrations for the various pesticides are generally relevant and this test was designed to evaluate a number of different tools and

techniques. The sums of 16 PAHs and seven PCB congeners approached a maximum of 3 and 1 µg L^{-1}, respectively.

Behaviour responses. During the tank test with metals, the first pulse (Fig. 4) was detected soon after the second dosing by 30 % of the animals, while the third and fourth metal additions were detected by 90 % of the animals. 30 % of the animals died during the second pulse. The first pulse certainly weakened the animals that subsequently became more sensitive towards the second pulse.

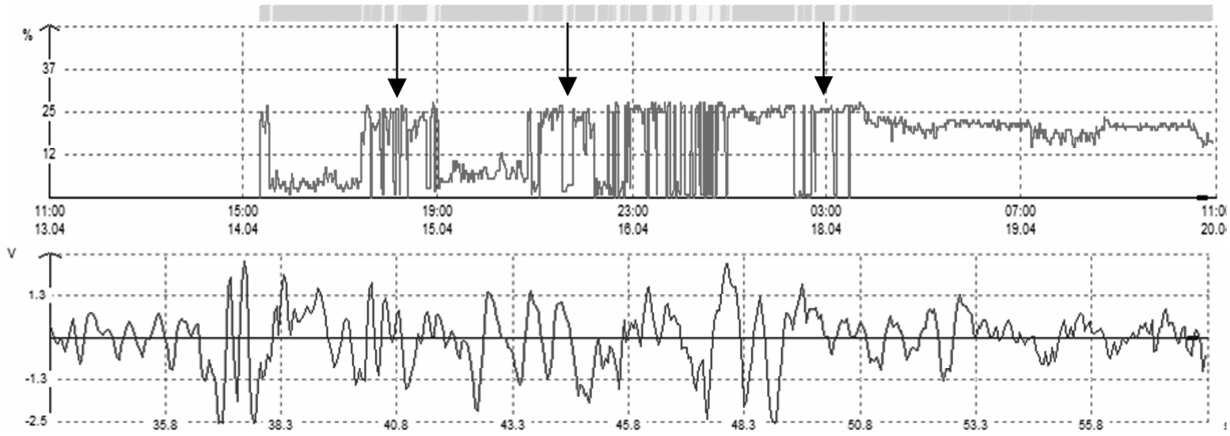

Fig. 4 Experiment 1. Top: locomotory activity of *G. pulex* (n = 7) in response to the subsequent metal pulses (percentage activity time [%] versus time [hh:mm]). Light grey marks in grey bar: Behavioural warning responses (decreased locomotion). Black arrows: metal pulses. Bottom: typical movement pattern of *G. pulex* consisting mostly of locomotion signals (Amplitude [V] versus time [sec].

When spiking with organic pollutants, the first dosing was detected by 10 % of the animals (Fig. 5), while the second dosing was detected by 40 % of the animals. After the third dosing 20 % of the animals were observed to die, and 50 % of the animals were dead after the fourth dosing.

In summary, *G. pulex* reacted very rapidly to the trace metal pulses. The behavioural warnings were detected within a few hours at sublethal levels, whereas mortality alarms occured towards the end of the exposure and only after repeated pulses and stress. The response of *G. pulex* to episodic exposure to organic pollutants occurred in a slower way than for trace metals; however behavioural warnings and mortality alarms were also recorded by the MFB, showing that the MFB is a reliable system for detection of rapid changes both in metals and organic pollutant concentrations.

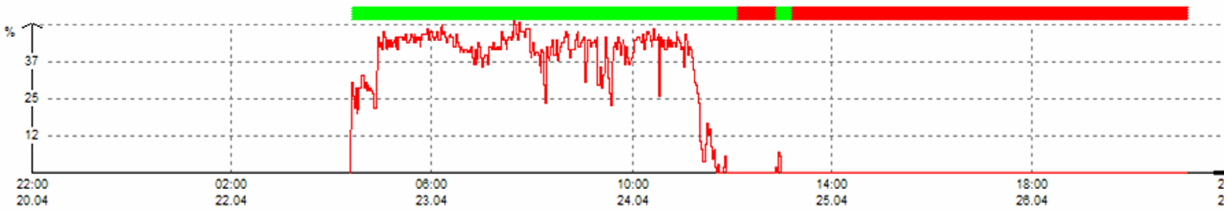

Fig. 5 Experiment 2. Locomotory activity of *G. pulex* in response to the subsequent organic xenobiotic pulses (percentage activity time [%] *versus* time [hh:mm]). Dark grey bar on top of graph: increasing mortality *versus* grey bar: alive animals (mean response of 7-8 animals).

In situ monitoring at the Rhine

Chemical analysis. At the Rhine monitoring station (Station d´Alerte de Huningue) several chemical irregularities could be detected (Fig. 7, see black arrows).

Behaviour measurements. No disturbances were observed with good zero baselines over the whole exposure period. Small sporadic signals (at background noise level) were observed but may be due to variations in flow conditions. Fig. 6 presents the behaviour of *G. pulex* from 18th of May to 29th of June and Fig. 7 presents the data available and provided by the station during the same period.

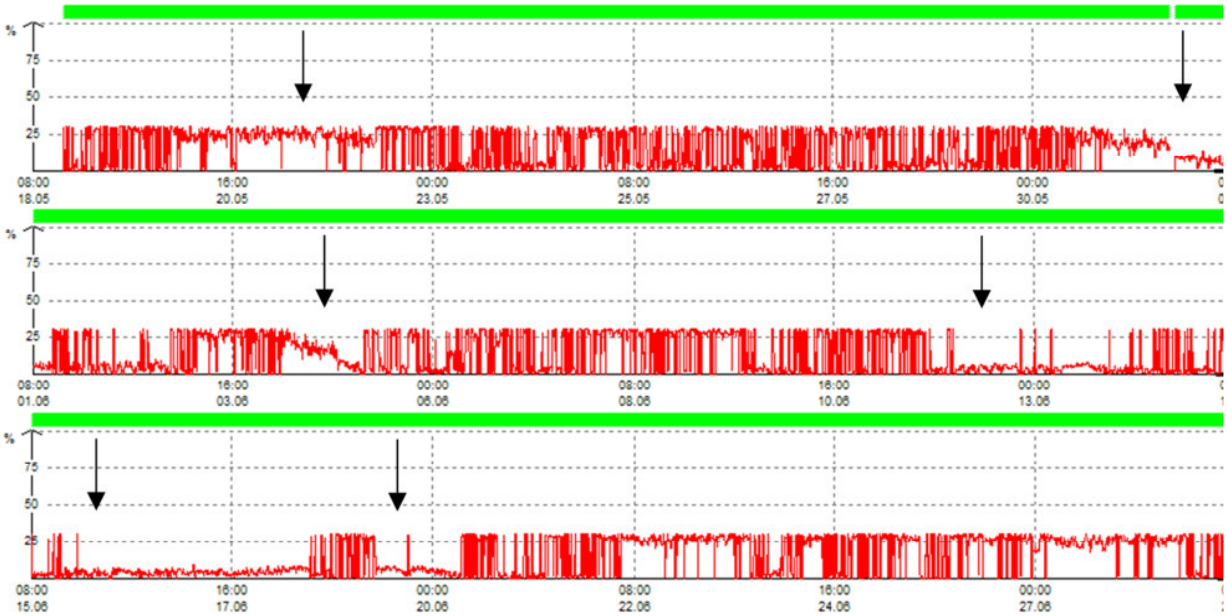

Fig. 6 Locomotory activity of *G. pulex* based on the mean of 8 individual recordings over the measuring period of 6 weeks (percentage activity time [%] *versus* time [hh:mm]). Arrows: chemical irregularities conform with behavioural alterations (decreased locomotory activity).

Locomotion of *G. pulex* showed the typical "up and down signals" (*e.g.* 18 to 20.05.06). However, some phases showed a decrease in activity (*e.g.* 20-21.05.06, 30.05.-01.06.06, 05.06.06., 12-14.06.06 and 15-18.06.06). When the dates of these drops in activity are

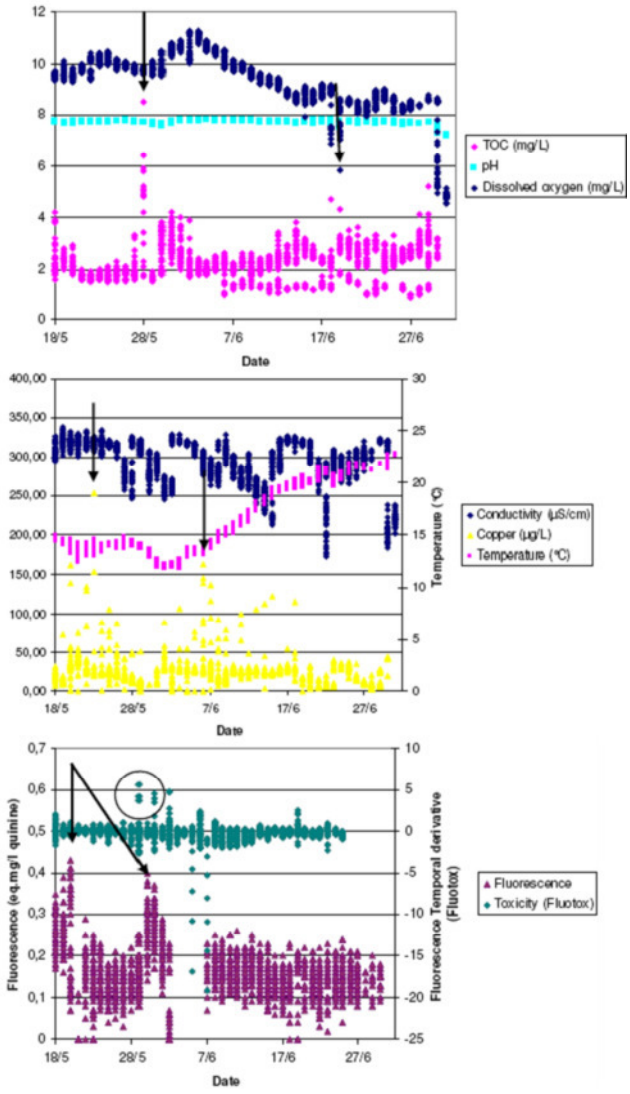

Fig. 7 Evolution of parameters measured by the Huningue Station during the deployment of the MFB. Black arrows: chemical irregularities.

compared with chemical data provided by the station (Fig. 7), the following connections between chemical and behavioural data may be drawn:

20-21.05.06: Slight increases in fluorescence (0.43 eq mg L^{-1} 20.05.06) and in copper concentration from 73 to 163 µg L^{-1} were observed. Hence, the drop in activity shown by the MFB may be linked to the increase in copper concentration in the water and/or oils present in the water.

30.05.-01.06.06: Both an increase in fluorescence (0.4 eq mg L^{-1}, 30.05.06) and a slight increase in toxicity (29.05.06, 31.05.06) were observed. On these two occasions, organic pollutants such as oils and pesticides may be responsible for the decrease in activity of the Gammarids.

05.06.06: no significant modification of the water quality was observed according to the data available.

12-14.06.06: a slight increase in copper concentration and a slight decrease in oxygen were noticed.

15-18.06.06: a slight increase in TOC and a decrease in oxygen (5.84 mg L^{-1} 19.06.06) that often characterises the presence of organic pollutants were observed. The ventilation behaviour, however, did not show any serious irregularities.

In addition, the Alarmdetektor-software (LimCo & Hölle und Hüttner AG) detected several irregularities:

(1) H: Hinkley detector: Locomotion: around 05.06.06, 19.06.06, 29.06.06.

(2) DS: double sigma detector, considering locomotion and ventilation gives even more irregularities, however a strong one around 18-19.06.06.

Survival. The survival of animals in the MFB was verified over the whole exposure period. Since death of 3 animals was observed on the 31.05.06, these were replaced. The high sensitivity of the animals might be due to the fact that they originated from a clean location. It may also be the result of pollution induced by copper and/or organic compounds present in the water. Indeed between 18. to 31.05.06, we can notice an increase in copper concentration (250 µg L^{-1} 23.05.06), in TOC (8.5 mg L^{-1} 28.05.06) and in fluorescence (about 0.4 mg L^{-1}, the 20.05.06 and 30.05.06). At the end of the test (29.06.06), 3 animals were also dead. These deaths, at the end of the exposure may be due to organic pollution (increase in TOC) and a decrease in oxygen that was observed at that time.

In summary the MFB appeared suitable for the real-time detection of changes in water quality, with no occurrence of technical problems and with reasonable baselines during the entire six week exposure period. Deaths of *G. pulex* at the beginning of the test may be explained by an increase in organic compounds/matter and copper concentrations. *G. pulex* responded to sublethal copper concentrations around 100 µg L^{-1}, both in laboratory[3] as well as *in situ*[7], with changes in locomotion and ventilation behaviour in previous experiments, and the response appeared to be dependent on the origin of the test population.[7] Then, *G. pulex* responded to the Rhine water with several irregularities. These irregularities may sometimes be explained by an increase in concentrations of pollutants such as copper and/or organic compounds (oils). At the end of the test, the deaths of animals might be due to organic pollution, pointed out by an increase in TOC and a decrease in dissolved oxygen.

Conclusions

During this evaluation at all three sites, the MFB performed well, without technical problems and with reasonable baselines obtained for all exposure periods in the Meuse, Aller and Rhine. *G. pulex* reacted to the changes in concentrations of metals and the organic pollutants during a series of tank tests undertaken with Meuse river water that resulted in stress behaviour and mortality. At the Aller river site, the undisturbed

baseline operation of the MFB could be established. With regard to the Rhine water *G. pulex* responded with several irregularities, with a particular event where the reaction coincided with a peak of oil pollution. The Alarmdetector software indicated this with both detectors, the Hinkley and the double sigma detector, and it can be verified in the original signals as shown in the MFB longterm graph (less activity).

The following conclusions can be drawn:

(1) *G. pulex* is suitable for water monitoring in the MFB because as detritus feeder, it can show also particle bound effects of pollutants (all bioavailable contaminants).

(2) At the Aller, no alarms were detected; that is, no harmful effects of the water on *G. pulex* were recognized. However *G. pulex* was able to detect several chemical irregularities at the Rhine monitoring station.

(3) The weekly exchange of the test animals (compared with bimonthly replacement in the present study) is standard and should therefore also be performed with the MFB.

(4) It is recommended to operate/run and calibrate the MFB further and for a longer period of time at monitoring stations *e.g.* in order to detect seasonal effects.

Our experiments showed that *G. pulex* were able to survive under the various conditions evaluated at all three monitoring stations and were able to react to changes in concentrations of various different types of pollutants. The MFB operation with a residential and ecologically relevant species such as *G. pulex* proved to be a reliable biomonitor system for surface water quality monitoring.

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Views presented here are those of the authors alone.

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* Beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.

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Lebenslauf

Persönliche Daten

Name	Cornelia Kienle
Geburtsdatum	02.02.1980
Geburtsort	Markt Rettenbach
Familienstand	ledig
Nationalität	deutsch

Schulbildung

1986 – 1990	Grundschule Mindelheim
1990 – 1999	Gymnasium Maristenkolleg Mindelheim

Hochschulstudium

10.2000 – 09.2002	Grundstudium der Biologie an der Universität Konstanz
10.2002 – 11.2005	Hauptstudium der Biologie an der Universität Bremen Diplomarbeit: "Wirkung von unterschiedlichen Schadstoffen und Schadstoffkombinationen auf das Verhalten und die Vitalität von frühen Lebensstadien des Zebrabärblings (*Danio rerio*) mit und ohne zusätzlichen Umweltstress“
02.2006-04.2009	Promotionsstudium an der Eberhard-Karls-Universität Tübingen Promotionsthema: „Toxizität von Umweltchemikalien und deren Mischungen auf ausgewählte aquatische Organismen – Verhalten, Entwicklung und Biochemie“

Stipendien

10.2006 – 09.2008	Landesgraduiertenförderung Baden-Württemberg Promotionsstipendium
2006 – 2008	Reinhold-und-Maria-Teufel-Stiftung, Tuttlingen Finanzielle Unterstützung für den Besuch verschiedener Konferenzen und für einen Forschungsaufenthalt am Helmholtz-Zentrum für Umweltforschung-UFZ, Leipzig im Rahmen der Dissertation (04.2008)