Auswirkungen von Umweltchemikalien auf die Biologie von Chironomiden und Fischen als deren Prädatoren

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Die Neugier steht immer an erster Stelle eines Problems, das gelöst werden will.

(Galileo Galilei)

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Zusammenfassung

1. Promotionsthema

Auswirkungen von Umweltchemikalien auf die Biologie von Chironomiden und Fischen als deren Prädatoren.

2. Einleitung

Weltweit werden über 100 000 Chemikalien kommerziell produziert und eingesetzt. Diese Entwicklung hat ihren Höhepunkt jedoch noch nicht erreicht, da jährlich zwischen 500 und 1000 weitere Chemikalien hinzukommen (Fent 2003). Wirtschaftlich gesehen stellt die Chemikalienproduktion einen der wichtigsten Industriezweige in der Welt dar. Beispielsweise ist die chemische Industrie in Deutschland die größte in Europa, wobei sie einen Anteil von ca. 25% des Gesamtumsatzes im europäischen Chemiesektor besitzt (Statistisches Bundesamt 2008). Von den insgesamt 100 000 Chemikalien gelangen zahlreiche in den Naturhaushalt und einige davon haben aufgrund ihrer physikalisch-chemischen Eigenschaften das Potential, negative Auswirkungen auf die Umwelt zu erzeugen.
Seit den Siebziger Jahren des letzten Jahrhunderts wurde die Umweltverschmutzung durch Chemikalien als ernsthaftes Problem erkannt (Fent 2003). Aus dem wachsenden Bewusstsein, dass Chemikalien negative Auswirkungen auf die Umwelt haben können, hat sich auch das Fachgebiet der Ökotoxikologie entwickelt (Newman 2010). Die Ökotoxikologie untersucht „die Auswirkungen von Chemikalien auf die belebte Umwelt“ (Fent 2003). Hierbei sollen neben der Untersuchung des Verbleibs von Chemikalien in der Umwelt auch deren negative Auswirkungen identifiziert, quantifiziert und bestenfalls abgewendet werden (Calow 1993; Fent 2003). Um dieses Ziel zu erreichen, werden verschiedene Bereiche aus der Toxikologie, der Ökologie und der Umweltchemie integriert (Fent 2003).

Letztlich ist es das regulatorische Ziel, Effekte von Schadstoffen auf Populationen zu vermeiden (Daniel et al. 2007). Hierfür müssen jedoch Auswirkungen auf niedrigeren Organisationsebenen bei Molekülen, Zellen und Organismen, bis hin zu höheren Organisationsebenen wie Populationen, und auch darüber hinaus auf Lebensgemeinschaften oder ganzen Ökosystemen berücksichtigt werden (Calow 1993). Die Erfassung der Effekte auf den niedrigeren biologischen Ebenen vom Molekül bis hin zum Organismus ist dabei von besonderer Bedeutung, da sich hier bereits direkte Wirkungen manifestieren. Diese können sich im Folgenden auch auf die höheren Ebenen wie Lebensgemeinschaften auch durch indirekte Aktionen auswirken (Fent 2003). Das Ziel der Regulatorischen Ökotoxikologie ist es, den Mensch und die Umwelt vor schädlichen Einwirkungen gefährlicher Stoffe zu schützen, diese erkennbar zu machen und sie somit abzuwenden oder ihrer Entstehung vorzubeugen (Fomin et al. 2003). Dafür müssen derartig Stoffe, wenn nötig, reguliert werden. Eine solche Regulierung setzt ein standardisiertes Vorgehen voraus, bei dem ermittelt wird, ob ein Risiko für den Naturhaushalt durch die jeweilige Substanz besteht. Die Risikoanalyse ist hierbei durch gesetzliche Regelungen abgedeckt. Regulatorisch gesehen werden Chemikalien in Pflanzenschutzmittel, Industriechemikalien und Human- und Veterinär-Pharmazeutika eingeteilt.

Pflanzenschutzmittel werden dabei direkt in die Umwelt eingebracht (Walker et al. 2006) - mit dem Ziel, dort negative Auswirkungen auf bestimmte Lebewesen zu verursachen. Deshalb sind sie aufgrund ihres Gefährdungspotentials die am besten untersuchte Chemikaliengruppe. Reguliert werden sie international durch die Richtlinie 91/414/EWG der EU über das Inverkehrbringen von Pflanzenschutzmitteln (European Commission 1991) sowie deren Erneuerung, die 2011 in Kraft getreten ist. National ist dieses Recht in Deutschland durch das Pflanzenschutzgesetz (PflSchG 2011) abgedeckt. Pflanzenschutzmittel können durch verschiedene Eintragspfade in Nichtzielflächen wie zum Beispiel Gewässer eingetragen werden. Als Nichtzielflächen werden allgemein diejenigen Flächen bezeichnet, in die kein

beabsichtigter Eintrag stattfindet und bei denen keine Auswirkungen der Pflanzenschutzmittel erwünscht ist. Als wichtigste Eintragspfade sind die Verfrachtung von Pflanzenschutzmitteln bei der Applikation durch Wind (als *Drift* bezeichnet), die Abschwemmung durch Regen (*run-off*), aber auch die Deposition nach Aerosolbildung und durch Drainage zu nennen (Daniel et al. 2007; Walker et al. 2006, Kreuger 1998).

Industriechemikalien stellen zwar mengenmäßig die größte Gruppe der anthropogen produzierten Chemikalien dar, aber von ihnen werden nicht *per se* negative Auswirkungen auf die Umwelt erwartet. Reguliert wird die Gruppe der Industriechemikalien seit 2007 durch die EU-Chemikalienverordnung REACH (REACH 2007). Darin werden abhängig von den intrinsischen chemischen Eigenschaften und der Herstellungstonnage verschiedenen Tests verlangt (REACH 2007).

Pharmazeutika werden nach der Tierarzneimittelrichtlinie 2001/82/EG (2001) und der Humanarzneimittelrichtlinie 2001/83/EG (2001) bewertet. Hier geht es jedoch vor allem um den ökotoxikologischen Erkenntnisgewinn, da nur begrenzt Empfehlungen ausgesprochen werden, bestimmte Pharmazeutika durch weniger umweltschädliche Substanzen zu ersetzten oder die Vernichtung zu verändern. Bei Tierarzneimitteln ist eine Regulation überhaupt möglich, wohingegen bei Humanarzneimitteln Umweltrisiken zwar definiert werden, ein Versagen der Zulassung aus Umweltgründen jedoch nicht vorgesehen ist. Im Allgemeinen gelangen Pharmazeutika, neben unsachgemäßer Entsorgung, erst nach der Therapie durch Ausscheidung oder durch die Entsorgung in die Umweltkompartimente (Hickmann 2007).

Die Risikobewertung für die drei Chemikalialiengruppen basiert beinahe auf den gleichen Grundprinzipien: Zunächst wird durch standardisierte modellhafte Annahmen zusammen mit den physikalisch-chemischen Eigenschaften der jeweiligen Substanz eine Umweltkonzentration, der PEC (*predicted environmental*

concentration), errechnet. Mit Hilfe von Modellierungen soll hierbei ein „worst case", also die höchstmögliche Umweltkonzentration ermittelt werden.

In einem nächsten Schritt werden die Effektkonzentrationen der Chemikalien bestimmt. Allgemein können Schadstoffe die verschiedensten Auswirkungen auf Organismen haben. Sie können zum einen direkt toxisch auf den Organismus wirken, zum Beispiel, indem sie negative Auswirkungen auf Gene, Proteine oder ganze Zellverbände verursachen. Aber sie können auch den Stoffwechsel, die (Embryonal-) Entwicklung und letztlich die Überlebensrate oder Fortpflanzungsrate beeinflussen (Fent 2003). Es ist ebenfalls möglich, dass sie indirekte negative Auswirkungen für einen Organismus verursachen können (z.B. durch die Verringerung des Futterangebotes). Um einige dieser Effekte abschätzen zu können, werden standardisierte ökotoxikologische Tests zum Beispiel nach der *International Organization for Standardization* (ISO) oder *Organisation for Economic Co-operation and Development* (*OECD) Guideline* verwendet. Es ist ausschlaggebend, dass diese Tests standardisiert, nachvollziehbar, verlässlich, relevant und kostengünstig sind (Calow 1993). Allgemein werden für diese Tests Organismen eingesetzt, die bestimmte trophische Ebenen des Ökosystems oder, allgemeiner gesagt, ganze Klassen oder Stämme des Tier- bzw. Pflanzenreichs repräsentieren und die typisch für die Kompartimente Wasser und Boden sind (Fomin et al. 2003). Die am häufigsten getesteten Organismen sind Fische, Algen und Daphnien. Neben Tests mit diesen kann je nach Tonnage, Applikationsort oder Wirkmechanismen von Chemikalien zudem noch die Testung mit weiteren Organismen gefordert werden. Mithilfe der Tests wird nun die jeweilige NOEC (*No observed effect concentration*), die LOEC (*Lowest observed effect concentration*) oder EC_{50} Werte bestimmt (Walker et al. 2006). Als EC_{50}-Wert wird die Konzentration einer Chemikalie bezeichnet, bei der 50 % der getesteten Individuen einen Effekt zeigen. Da die Bestimmung der Effektkonzentration nur mit ausgewählten Organismen als Stellvertreter für ganze Ökosysteme durchgeführt wird, wird versucht, anhand von zugerechneten Sicherheitsfaktoren Unsicherheiten die z.B. bei der Übertragung von akuten zu

chronischen Wirkungen oder von Labor- zu Freilanduntersuchungen auftreten, abzudecken (Walker et al. 2006).

Aus der Umweltkonzentration und der Effektkonzentration wird anschließend ein Risikoquotient berechnet, anhand dessen eine Risikobewertung durchgeführt wird. Ab diesem Zeitpunkt unterscheiden sich die Vollzüge je nach Substanzklasse wieder voneinander.

Das vorgestellte standardisierte Testen von Chemikalien bei der Zulassung hat das Ziel, Mensch und Umwelt vor negativen Auswirkungen zu schützen. In diesem Rahmen scheint eine starke Standardisierung im Zusammenhang mit der Auswahl einzelner Standardorganismen und bestimmter Endpunkte die einzige pragmatische Möglichkeit zu sein, ein realistisches Kosten-Nutzen Verhältnis zu erreichen. Dies ist, neben der dadurch gewährleisteten regulatorischen Sicherheit für die Hersteller der Chemikalien, der wichtigste Grund für ein solches Vorgehen. Aber auch die Nachvollziehbarkeit und Vergleichbarkeit der erzielten Resultate sind von Vorteil (Calow 1993). Selbst wenn es praktisch möglich wäre, alle Chemikalien mit allen existierenden Organismen zu testen, haben wir eine ethische und moralische Verantwortung, dies nicht zu tun. Jedoch verbleiben durch einen solchen vereinfachenden Ansatz Unsicherheiten, welche auch durch die Anwendung von Sicherheitsfaktoren nicht ganz abgedeckt werden können. Es sollte deshalb weiterhin kritisch diskutiert werden ob z.B. von

- akuter auf chronische Toxizität,
- Ergebnissen aus dem Labor aufs Freiland,
- Einzelindividuen auf ganze Populationen bzw. sogar Ökosysteme,
- Einzelsubstanzeffekten auf Effekten von Substanzkombinationen

geschlossen werden darf. Zusätzlich stellen sich die Fragen, ob die jeweilig getesteten Endpunkte wirklich umweltrelevant sind und ob durch ihre Auswahl auch neue Gefährdungen wie z.B. endokrines Potential erkannt werden können. Insgesamt hinterlässt das standardisierte Vorgehen bekannte, aber geduldete Lücken. Erkenntnisse zum Füllen dieser Lücken zu erzeugen ist eine der Aufgaben der

universitären Forschung. In diesem Rahmen ist es essentiell, dass auch nichtstandartisierte Tests durchgeführt werden, um hierbei weitergehende Informationen über das normalen Zulassungsverfahren hinaus zu erhalten.

Ein für diese Arbeit bedeutender Aspekt ist, dass Schadstoffe das Potential beinhalten, auch das Verhalten von Organismen beeinträchtigen zu können. Allgemein kann man Verhalten als die Reaktion eines Organismus auf interne (physiologische) und externe (umwelt-, soziale-) Faktoren bezeichnen, weshalb es eine Vielzahl von biotischen und abiotischen Interaktionen integriert (Dell`Omo, 2002). Generell ist das Verhalten auch die Schnittstelle, über die ein Organismus mit anderen in Beziehung tritt. Da Verhalten relativ leicht adaptiert werden kann, stellt es einen der Hauptmechanismen dar, durch welchen sich Organismen an Änderungen in ihrer Umwelt anpassen (Evans 1994). Verhaltensweisen sind grundsätzlich derart gestaltet, dass sie es einem Organismus ermöglichen, bestmöglich in seiner Umgebung zu bestehen (Begon et al. 2006). Wenn nun z.B. eine neurotoxische Substanz Verhaltensweisen beeinträchtigt, kann dadurch die Leistung in der jeweiligen Umwelt beeinträchtigt werden. Beispielsweise kann das Fraßverhalten, die Räuber-Vermeidung, allgemeine soziale Interaktionen und somit auch der Fortpflanzungserfolg durch Schadstoffe beeinträchtigt werden, was zum einem das Überleben des Individuums und andererseits auch das Schicksal ganzer Populationen beeinflussen kann (Dell`Omo, 2002).

Bisher fokussiert sich der Großteil aller ökotoxikologischen Studien auf die direkten Auswirkungen von Schadstoffen auf Organismen (Langer-Jaesrich et al. 2010). Jedoch ist es ökologisch gesehen genauso relevant, ob Schadstoffe auch Auswirkungen auf die inter- oder intraspezifische Interaktionen zwischen Organismen haben. Beispielsweise ist es äußert bedeutsam ob bzw. wie Räuber-Beute-Beziehungen durch Schadstoffe beeinträchtigt werden. Bei Räubern können viele verschiedene Aspekte wie Mustererkennung, Lernverhalten, Strategien zum Aufspüren der Beute oder Auswahl und Handling der Beute beeinflusst werden, was sich schließlich auf die Effizienz der Nahrungsaufnahme auswirkt (Walker et al.

2006). Aber auch von Seiten potentieller Beuteorganismen ist es eine absolute Notwendigkeit, Prädation zu vermeiden. Bei der Schadstoffzulassung haben bisher solche Fragestellungen, mit Ausnahme von Mesokosmenstudien, kaum Beachtung erhalten.

Für die Untersuchung von Effekten spielen sowohl Bioindikatoren als auch Biomarker eine besondere Rolle. Anhand beider kann bestimmt werden, wie stark Organismen einer Belastung ausgesetzt ist. Zur Bioindikation kann eine Reaktion eines Individuums, einer Population oder einer Lebensgemeinschaft zum Beispiel auf einen Schadstoff verwendet werden (Walker et al. 2006). Zum Beispiel wird schon seit Jahrzehnten diskutiert, ob sich die Mundwerkzeug-Deformationen von Chironomiden zur Bioindikation von Kontaminationen in Gewässern und deren Sedimenten eignen (Bird 1994; Gerhardt et al. 2006; Groenendijk et al. 1998; Janssens De Bisthoven et al. 1998; Meregalli et al. 2000; Warwick 1990).

Als Biomarker hingegen werden messbare molekulare, biochemische, zelluläre und physiologische Reaktionen innerhalb eines Organismus bezeichnet, die als Indikatoren für z.B. Schadstoffbelastungen herangezogen werden (van Gestel und van Brummelen, 1996). Sie zeigen Veränderungen im Metabolismus des untersuchten Organismus an. Hierbei wird jedoch zwischen unspezifischen Effektbiomarkern und spezifischen Expositionsbiomarkern unterschieden (Peakall 1992). Expositionsbiomarker zeigen die Qualität oder Quantität der Exposition eines Organismus gegenüber einem Stressor an, geben jedoch per Definition keine Information darüber, ob der Organismus durch den Stressor negativ beeinträchtigt ist. Ein Effektbiomarker gibt Informationen über den Gesundheitszustand eines Organismus, jedoch ohne Rückschlüsse auf den verursachenden Stressor zu liefern. Ein Beispiel für einen Effektbiomarker sind die Hitzeschockproteine der Größe 70 kD (hsp70). Diese Familie der Hitzeschockproteine stellt eine phylogenetische stark konservierte Art von Chaperonen dar, welche andere Proteine bei der Faltung in die richtige Tertiär- und Quartärstruktur unterstützt (Berg et al. 2003). Werden Teile von Proteinen in ihrer ‚korrekten' Form gestört, zum Beispiel durch Hitzestress oder die

Anwesenheit von Schwermetallen, kann dieser Effekt durch eine Erhöhung des hsp70 Levels, der durch einen Rückkoppelungsmechanismus induziert wird, kompensiert werden (Beckmann et al. 1992, Gething & Sambrook 1992, Morimoto 1993). Deshalb werden Veränderungen des hsp70 Levels als genereller Biomarker für Proteotoxizität bei Umweltuntersuchungen angesehen (Yoshimi et al. 2002).

In der Ökotoxikologie werden vorwiegend Einzelsubstanzen gestestet (Walker et al. 2006). Dies entspricht natürlich nicht einem realistischen Umweltszenario, da es beispielsweise in der Landwirtschaft üblich ist, Kombinationspräparate, Tankmischungen oder verschiedene Spritzfolgen einzusetzen (PAN 2005). Auch beinhalten die Ausflüsse von häuslichen oder industriellen Kläranlagen häufig äußert komplexe Mischungen an Schadstoffen (Walker et al 2006). Deshalb kann festgehalten werden, dass in der Natur Kontaminationen von in der Regel mehr als nur einer Substanz auftreten. Es ist allgemein anerkannt, dass Schadstoffmischungen im Gegensatz zu den Einzelsubstanzen ein deutlich abweichendes toxisches Potential aufweisen können (Faust et al. 1996, Junghans et al. 2006). Es gibt verschiedene Möglichkeiten, wie die Toxizität von Mischungen von der Toxizität der Einzelsubstanzen abweichen kann. Von Bedeutung sind hierbei additive, antagonistische oder synergistische Effekte. Aber auch dosis- oder konzentrationsabhängige Interaktionen der Toxizitätsmischungen sind zu beachten (Jonker et al 2005). Besonders für Sedimente ist es realistisch anzunehmen, dass sie Schadstoffmischungen enthalten, da sie nicht nur aktuell eingetragene, sondern auch in früheren Zeiträumen absorbierte Chemikalien enthalten.

Allgemein werden Vertreter aus der Familie der Chironomiden (Zuckmücken) häufig für die ökotoxikologische Testung herangezogen. Diese Insekten werden systematisch zur Ordnung der Diptera (Zweiflügler) und Unterordnung der Nematocera (Mücken) zugeteilt (Pinder 1986). Sie sind als Testorganismus besonders interessant, da sie als Vertreter der Insekten zu der artenreichsten und ökologisch äußerst wichtigen Gruppe der Invertebraten gehören (Taenzler et al. 2007). Die Familie der Chironomiden ist weltweit verbreitet, sie sind häufig die

individuenreichsten Insekten im Süßgewässer (Pinder 1986) und stellen mit ihren Larven dort auch die artenreichste Gruppe des Makrozoobenthos dar. Besonders in Weichbodensubstraten können sie in sehr hohen Dichten vorkommen (bis zu 100.000 Ind./m^2). Die hohe Biomasse sowie ihre wichtige Rolle als Detritusfresser im Nährstoffkreislauf bekräftigen ihre ökologische Bedeutung (Gerhardt et al. 2006). In vielen fließenden und stehenden Süßwasserhabitaten fungieren sie als dominante Primärkonsumenten und erreichen extrem hohe Biomasseumsatzraten (Benke 1998; Calow 1993). Nicht zuletzt macht sie ihre Funktion als Futtertiere für andere Organismen ökologisch äußert relevant (Pinder 1986). Da Chironomiden sowohl für Invertebraten als auch Vertebraten wichtige Beuteobjekte darstellen, können ihre Umsatzraten die Struktur der (Lebens)Gemeinschaft stark beeinflussen (Hooper et al. 2003).

Für ökotoxikologische Tests werden vorwiegend die Arten *Chironomus riparius* oder *Chironomus tentans* verwendet (OECD 2004a,b). Das hat neben ihrer Relevanz im Ökosystem vor allem etwas mit ihren Ansprüchen an eine Laborkultur und der Dauer des Lebenszyklus zu tun. *C. riparius* weist zum Beispiel einen relativ kurzen Lebenszyklus von ca. 28 Tagen auf und ist äußert unkompliziert unter Standardbedingungen im Labor zu halten (OECD 2004a,b).

Auch im Feld gehört *C. riparius* zu den relativ anspruchslosen Arten. Die Art bevorzugt eutrophe Gewässer und weist dabei eine hohe Toleranz gegenüber geringen pH-Werten und Sauerstoffgehalten auf. Aufgrund des multivoltinen Lebenszyklus, der kurzen Generationszeiten und der hohen Reproduktionsrate bevölkert sie deshalb auch schnell neu entstandene Habitate. Ein vollständiger Lebenszyklus von *C. riparius* gliedert sich in ein erstes kurzes pelagisches Stadium (2-3 Tage), gefolgt von drei benthischen Stadien (röhrenbildende Formen in den oberen Sedimentschichten) und einem Puppenstadium vor dem Schlupf der Imagines (Fig.1).

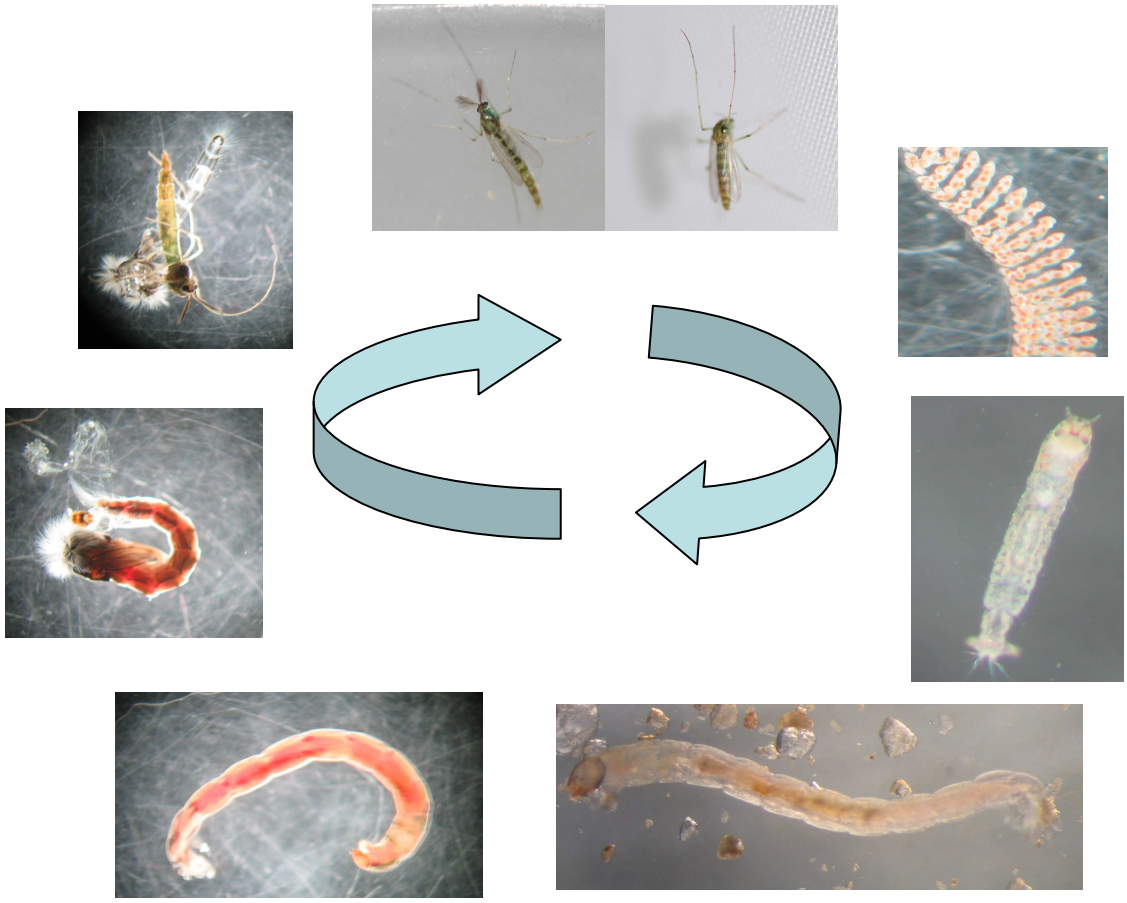

Fig.1: Lebenszyklus von *Chironomus riparius* (Fotos: Miriam Langer-Jaesrich)

Durch die insgesamt recht kurze Lebensdauer ist es möglich, auch in Standardtests die Auswirkungen von Schadstoffen über eine gesamte Generation, bei dem alle Entwicklungsstadien auf Effekte getestet werden, durchzuführen (Gerhardt et al. 2006). Zusätzlich existieren bereits viele wissenschaftliche Publikationen über die Biologie und die Sensitivität dieser Art gegenüber Schadstoffen (unter anderem Forbes and Cold 2005; Gerhardt et al. 2006; Hooper et al. 2003; Läppänen et al. 2006; Meregalli et al. 2001; Pinder 1986). Dies ermöglicht den Vergleich und die Einordnung von neu erhobenen Daten in den Gesamtkontext und hierdurch eine angemessene Bewertung derselben (Gerhardt et al. 2006).

Eine weitere Besonderheit, die *C. riparius* als Testorganismus interessant macht, ist, dass sie zwar im aquatischen System lebt, sich aber als benthischer Organismus vorwiegend im Sediment aufhält. Für Sedimenttoxizitätstests sind diese Mückenlarven deshalb die idealen Testorganismen, da sie im engen Kontakt mit dem

Sediment leben bzw. sogar Sedimentpatikel als Nahrung aufnehmen (Calow 1993). Zusätzlich stellen sie einen wichtigen Stellvertreterorganismus für eine Vielzahl von Sedimentbewohnern dar (Gerhardt et al. 2006). Mit ihnen können deshalb auch Schadstoffe, welche durch ihr hohes Adsorptionspotential an Sedimente gebunden sind, und über deren Bioverfügbarkeit keine Informationen vorliegen, getestet werden (OECD 2004b). Mit Chironomiden ist es somit möglich, wasserlösliche und wasserunlösliche Substanzen gleichermaßen zu untersuchen. Des Weiteren können mit ihnen natürliche Sedimente untersucht und somit die Toxizität von aktuellen wie historischen Verschmutzungen integriert werden.

Aus diesen Gründen wurde im Jahr 2004 ein von der OECD standardisiertes Testsystem 218/219 mit sedimentbewohnenden Chironomiden als Testorganismen (OECD 2004 a,b) entwickelt. Dabei werden zwei bis drei Tage alte Chironomidenlarven gegenüber mit Chemikalien versetztem Wasser oder Sediment exponiert. Als Testparameter dienen die Anzahl der emergierten Tiere, der Zeitraum bis zur Emergenz und die Wachstumsrate.

Aufgrund der hervorragenden Eignung und der guten Resultate wurden seither weitere Testsysteme, darunter ein akuter Immobilisationstest (OECD 235) und ein l*ife-cycle* Test (OECD 233) für Chironomiden entwickelt. Beim akuten Immobilisationstest werden Larven des ersten Larvenstadiums für 48 Stunden gegenüber einer Testlösung exponiert und danach die Überlebensrate, repräsentiert durch die Fähigkeit zur Bewegung, evaluiert (OECD 2011). Der *life cycle* Test stellt eine Erweiterung von OECD 218/219 dar, da hier neben den Effekten der Schadstoffe auf die erste Generation auch die Auswirkungen auf die zweite Generation (F1) untersucht werden (OECD 2010). Als Endpunkte werden die Anzahl der emergierten Chironomiden und das Geschlechterverhältnis (1. und 2. Generation) untersucht. Zusätzlich wird die Anzahl der Eigelege pro Weibchen und die Fertilität der Eigelege der ersten Generation evaluiert. Anhand dieses Test können neben der chronischen Toxizität von Schadstoffen auch Hinweise auf potentiell endokrine Effekte gewonnen werden (Taenzler et al. 2007).

3. Zielsetzung

In dieser Doktorarbeit wurden in mehreren Teilprojekten die Auswirkungen von Umweltchemikalien auf Chironomiden als Schlüsselorganismen in aquatisch-benthischen Lebensräumen untersucht. Dabei lag der Fokus besonders auf der Erfassung von nicht-standardisierten Endpunkten und Bioindikatoren sowie auf der Bearbeitung einer weiterführenden ökologisch relevanten Fragestellung zum Räuber-Beute Verhältnis unter Schadstoffeinfluss.

Im ersten Teil der Dissertation wurde das herkömmliche Standardtestsystem mit *Chironomus riparius* (OECD 2004 a,b) erweitert, um die Informationslage von Thiacloprid als Vertreter der neuen Insektizidklasse der Neonikotinoide im subletalen und letalen Bereich auf Nicht-Zielorganismen zu erweitern.

Die etablierten Testparameter aus den anerkannten OECD Testrichtlinien wurden dabei mit einer Batterie von weiteren Testparametern kombiniert, um den Test sensitiver und aussagekräftiger zu gestalten. Somit wurde eine große Bandbreite an zusätzlichen Parametern, nämlich biochemische (Induktion von Stressproteinen), entwicklungsbiologische (z.B. Larvalentwicklung) und organismische (Verhalten) als Endpunkte integriert, quantifiziert und deren Sensitivität miteinander verglichen. Mit diesem erweiterten Testsystem wurden exemplarisch die Auswirkungen des Insektizids Thiacloprid auf die verschiedenen biologischen Ebenen des Nicht-Zielinsekts *C. riparius* untersucht. Allgemein ist es mit diesem Testsystem möglich, die Auswirkungen von Einzelsubstanzen, Substanzmischungen und natürlichen Proben zu testen.

Im zweiten Teil der Dissertation wurde untersucht, ob sich Deformationen der Mundwerkzeuge von Chironomiden als Bioindikator bei Schadstoffbelastungen eignen. Es sollte dabei geklärt werden, ob Schadstoffe mit verschiedenen Wirkweisen Deformationen der Mundwerkzeuge bei *C. riparius* induzieren können und ob die Anzahl bzw. die Intensität der Deformationen mit der Schadstoffmenge korreliert. Zusätzlich wurde getestet, ob Einzelsubstanzen die gleiche Reaktion induzieren wie

Substanzgemische. Dies wurde mit dem gleichen Testdesign anhand von vier verschiedenen Schadstoffen (Imidacloprid, Thiacloprid, Nickelchlorid und Chlorpyrifos) und deren Mischungen getestet. Darüber hinaus wurden die Ergebnisse in den Kontext der aktuellen, zum Teil widersprüchlichen Literatur gestellt.

Im dritten Teil der Dissertation wurden die Auswirkungen des Insektizids Chlorpyrifos auf Räuber-Beute-Beziehungen anhand eines Modellsystems mit Zebrabärblingen als Räuber und Chironomidenlarven als Beute untersucht. Ziel war es, auf einem höheren biologischen Niveau die eventuell indirekten Auswirkungen von einer kurzzeitigen umweltrelevanten Schadstoffexposition zu untersuchen. Dabei wurden in verschiedenen Ansätzen die Räuber, die Beute oder beide kurzzeitig dem Schadstoff gegenüber exponiert. Es wurde als Hypothese formuliert, dass Prädatoren das Räubervermeidungsverhalten (Vergraben) bei Chironomiden stimulieren, unabhängig davon ob die Beute vorexponiert war oder nicht. Dabei wurde jedoch angenommen, dass vorexponierte Chironomiden ein vermindertes Räubervermeidungsverhalten aufweisen, weshalb sie anfälliger für Prädation sind. Bei gleichzeitiger Vorexposition von Räuber und Beuteorganismen sollte 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. Allgemein sind Räuber-Beute-Beziehungen sehr komplex, weshalb als Versuchsaufbau bewusst ein einfaches und unmissverständlich zu interpretierendes System entwickelt wurde.

4. Material und Methoden

Chironomus riparius

Die in Kapitel 1 bis 3 beschriebenen Versuche wurden mit der Zuckmückenart *Chironomus riparius* durchgeführt. Die Versuche selbst erfolgten in den Laboren der Abteilung Physiologische Ökologie der Tiere, Institut für Evolution und Ökologie an der Universität Tübingen. Die Tiere für die Stammkultur stammten aus unterschiedlichen Quellen (LimCo International, Ibbenbüren, Deutschland, University of Joensuu, Finnland und Universidade de Coimbra, Portugal), um einer genetischen Verarmung vorzubeugen. Die Organismen wurden in einer Klimakammer bei 21 ± 0,5 °C bei mit einem Lichtzyklus von 16:8 Stunden (hell:dunkel) gehalten. Die Hälterung erfolgte in großen Becken (50x55 cm) mit Quarzsand und entchloriniertem Wasser bei ständiger Belüftung. Täglich wurden die Chironomiden mit einer Suspension aus fein gemahlenen Fischfutterflocken und Wasser gefüttert (50% TetraMin: 50% TetraPhyll, Tetra Germany). Sobald *C. riparius* zu emergieren begann, wurde ein Schwarmkäfig über den Becken installiert, in dem die adulten Tiere schwärmen und sich paaren konnten. Die Eigelege wurden täglich gesammelt und entweder für neue Kulturen oder für Experimente verwendet.

Danio rerio

Vier bis sechs Monate alte *Danio rerio* der Wildtyp-Stämme *Danio rerio* ZFIN ID: ZDB-FISH-010531-2 und *Danio rerio* Tue.G14 wurden als Räuber für die Räuber-Beute-Versuche verwendet. Diese wurden in einem belüfteten Aquarium bei 25 ± 0,5 °C und einem 12:12 hell:dunkel-Rhythmus gehalten, wobei pro Fisch mindestens 1L Wasser zur Verfügung stand. Zweimal täglich wurden die Fische mit getrockneten Fischflocken oder mit gefrorenen Crustaceen, Tubifex und Chironomidenlarven gefüttert. Um *D. rerio* mit den Beuteobjekten vertraut zu machen, wurden die Fische

während einer einmonatigen Akklimatisationsphase mehrmals mit lebenden *C. riparius* gefüttert.

Whole life cycle Test

Für die in dieser Dissertation bearbeiteten Fragestellungen (Kapitel 1 und 2) wurde das gängige OECD Testprotokoll 218/219 erweitert (OECD 2004a,b).
Dafür wurden mehrere Eigelege aus der Kultur gesammelt, aufgeteilt und gegenüber den jeweiligen Testlösungen exponiert. Nach drei Tagen begannen die Larven zu schlüpfen. Pro Replikat wurden 30 bzw. 33 Larven des Larvenstadiums 1 (L1) in 250 ml-Testgefäße mit der entsprechenden Testlösung und mit Chemikalien versetzten Quarzsediments überführt. Gefüttert wurde täglich mit ≥ 0,36 mg Fischflocken/Tag/Larve und dabei die Belüftung kontrolliert. Die Testlösung wurde jeden dritten Tag gewechselt. Ein Mal pro Woche wurden die Larven in neue Testgefäße mit frischer Testlösung und mit Chemikalien dotiertem Sediment überführt.
Während der Experimente wurden folgende Endpunkte untersucht:

- Schlupfrate aus dem Ei
- Larvalmortalität
- Lokomotorisches und ventilatorisches Verhalten in verschiedenen Larvenstadien
- Wachstumsrate (Trockengewicht)
- Stressproteinlevel bei L4-Larven
- Emergenzrate
- benötigte Entwicklungsdauer
- Geschlechterverhältnis
- Deformationen des Mentums der L4-Larven

Die Überlebensraten der L3- und L4-Larvenstadien wurden bei der Umsetzung in neue Testgefäße bestimmt. Durch die Untersuchung der Überlebensrate zu verschiedenen Zeitpunkten konnte im Folgenden zwischen Mortalität und Verzögerungen des Emergenzprozesses unterschieden werden.

Zusätzlich wurde die Bewegungs- und Ventilationsaktivität von zwölf L3- bzw. L4-Larven pro Testkonzentration für zwei Stunden mit dem *Multispecies Freshwater Biomonitor* (MFB, LimCo International) untersucht. Nachdem Emergenz auftrat wurde täglich die Anzahl der emergierten *C. riparius* bestimmt. Durch die Erfassung des Emergenzzeitpunktes konnte die Entwicklungsrate errechnet werden (OECD 2004a,b). Zusätzlich wurde das Geschlechterverhältnis bestimmt. Zur Bestimmung des Trockengewichtes der Larven wurde das Experiment wie oben beschrieben wiederholt. Nach 10 Tagen wurde das Trockengewicht der überlebenden *C. riparius* bestimmt und daraus das durchschnittliche Trockengewicht der Larven in jedem einzelnen Replikat errechnet. Zusätzlich wurden die Mundwerkzeuge auf Deformationen und der Hsp70 Gehalt von L4 Larven untersucht.

Mundwerkzeuguntersuchung

Zur morphologischen Untersuchung der Mundwerkzeuge (Kapitel 1 und 2) wurden nach Emergenz von *C. riparius* deren Exuvien gesammelt und in 100% Ethanol konserviert. Ein Tag vor der Weiterbearbeitung wurden die Kopfkapseln mechanisch vom Rest der Exuvien getrennt und anschließend für zwölf Stunden in Rotihistol (Carl Roth GmbH, Germany) gelagert. Am darauf folgenden Tag wurden die Kopfkapseln mit der ventralen Seite nach oben in Rotihistokit (Carl Roth GmbH, Germany) auf einem Objektträger eingedeckelt und anschließend getrocknet. Die stark sklerotisierten Mandibeln und das Mentum wurden mit einem Mikroskop (Zeiss Axiostar Plus) bei 40 facher Vergrößerung ausgewertet.

Als Deformationen wurden fehlende `Zähne´, zusätzliche `Zähne´, gespaltene Mittel`zähne´ und *Köhn Gaps* beurteilt (Bird 1994, Gerhardt und Janssens De Bisthoven 1995, Servia et al. 1998). Bei der Auswertung wurde auf die sorgfältige Unterscheidung von mechanischer Abnutzung und entwicklungsbiologischer Deformation geachtet. Die Anzahl der Larven mit deformierten Mundwerkzeugen wurden im Verhältnis zur Anzahl der untersuchten Larven gesetzt (Hämäläinen 1999). Dabei wurden zwei verschiedene Ansätze für die Interpretation dieser

Verhältnisse verwendet: Im ersten wurde die Gesamtsumme der Deformationen ins Verhältnis zu den untersuchten Kopfkapseln gesetzt. Im zweiten Ansatz wurden die verschiedenen Deformationstypen ins Verhältnis zu den untersuchten Kopfkapseln gesetzt.

Hsp70

Für die Analyse des Hsp70-Gehaltes (Kapitel 1) wurden von jedem Versuchsansatz zehn Larven des L4-Stadiums (17 Tage nach Oviposition) zufällig entnommen, in flüssigem Stickstoff schockgeforen und bei -20°C gelagert.
Zuvor wurde dieses Larvenstadium auf einen ausreichenden Gesamtporteingehalt zur Analyse des Hsp70-Gehaltes getestet. Alle Proben wurden mit einer auf die Chironomiden angepassten immunologischen Methode nach Köhler et al. (1992, 2007) bearbeitet und ausgewertet. Die gefrorenen Larven wurden einzeln mit Phosphatpuffer homogenisiert und anschließend zentrifugiert. Der Überstand wurde für die weiteren Analyse verwendet. Zunächst wurde der Gesamtproteingehalt des Überstandes mit der Methode nach Bradford (1976) quantifiziert. Im Folgenden wurde von jeder Probe eine konstante Proteinmenge (40 µg) auf Minigele aufgetragen und eine modifizierte SDS-PAGE nach Laemmli (1970) durchgeführt. Dann wurden die Proteine im Western-Blotting-Verfahren auf eine Nitrozellulosemembran übertragen und dort mit einer Peroxidasefarbreaktion (erster Antikörper: Mouse anti-human Hsp70 IgG, zweiter Antikörper: Peroxidase-konjugierter goat anti-mouse IgG) angefärbt. Die Auswertung der Farbintensität der Proteinbanden erfolgte densitometrisch-planimetrisch. Im Folgenden wurde das optische Volumen (durchschnittliche Farbintensität x Fläche) der Banden mit einem parallel analysierten Standard in Relation gesetzt.

Räuber-Beute-Versuche

Um das Räuber-Beute-Verhalten (Kapitel 3) nach vorhergehender Schadstoffbelastung zu untersuchen, wurden L4-Larven von *C. riparius* als Beute und adulte *D. rerio* als Räuber für zwei Stunden gegenüber jeweils einer niedrigen (1µg/L) und einer hohen (6µg/L) Chlorpyrifoskonzentration exponiert. Dabei wurden neben einem Kontrollszenario drei verschiedenen Szenarien getestet: Im ersten Szenario waren nur die Chironomiden vorexponiert (*C. riparius* contaminated, Cc), im zweiten nur die Zebrabärblinge (*D. rerio* contaminated, Dc), im dritten beide, *C. riparius* und *D. rerio* exponiert (both contaminated, Bc). Jedes Szenario wurde drei Mal repliziert.
Damit die Fische bereits vor Versuchsbeginn mit den neuen Beuteobjekten vertraut waren wurden diese während der Akklimatisierungsphase regelmäßig mit lebenden *C. riparius* Larven gefüttert. Ebenfalls wurde in dieser Zeit das (Ver)-Grabeverhalten der L4-Larven von *C. riparius* beobachtet. Dabei wurde die benötigte Zeit zum Eingraben, die ursprüngliche Fraßrate durch *D. rerio* und die Wiederfindungsrate von *C. riparius* in verschiedenen Vortests bestimmt.
In jedes Versuchsreplikat wurden insgesamt 5 *D. rerio* und 100 L4-Larven von *C. riparius* eingesetzt. Dafür wurden *C. riparius*-Larven zufällig aus der Präkultur gesammelt und in der entsprechenden Testlösung (Wasser oder Chlorpyrifos-Lösung) und Quarzsediment für zwei Stunden exponiert. Im Anschluss wurden die Mückenlarven in ein Zehn-Liter-Aquarium mit einer 2 cm dicken Quarzsedimentschicht und acht Liter Wasser überführt. In den folgenden zwei Stunden hatten die Larven Gelegenheit sich in das Sediment einzugraben. Währenddessen wurden fünf *D. rerio* je Replikat in Vier-Liter-Aquarien gegenüber der entsprechenden Wasser- oder Chlorpyrifoslösung für zwei Stunden exponiert. Bevor die Zebrabärblinge in die Zehn-Liter-Aquarien mit den Chironomiden gesetzt wurden, wurde die Anzahl von *C. riparius*-Larven, die vollständig oder teilweise an der Oberfläche sichtbar waren, gezählt. Nach dem Transfer hatten die Fische zwei Stunden Zeit, nach den Chironomiden zu suchen und diese zu fressen. Danach wurde erneut die Anzahl der Chironomiden, die sich vollständig oder teilweise an

der Oberfläche befanden, erneut gezählt. Die Fische wurden entfernt, anästhesiert und deren Länge bestimmt. Die Anzahl der überlebenden *C. riparius* im Sediment wurde bestimmt.

Schadstoffe

In der vorliegenden Arbeit wurden in verschiedenen Fragestellungen die Auswirkungen von unterschiedlichen Schadstoffen auf *C. riparius* untersucht. Davon waren drei Substanzen Insektizide, wobei die beiden Neonicotinoide Thiacloprid und Imidacloprid als Agonist an den nikotinergen Acetylcholinrezeptoren des Nervensystems (von Insekten) fungieren (Abbink 1991, Liu & Casida, 1993). Hierdurch wird eine ständige Übererregung verursacht, die zum Tod der Tiere führen kann. Chlorpyrifos, ebenfalls ein Insektizid, weist einen anderen Wirkmechanismus auf. Als Breitband-Organophosphat wirkt es als Inhibitor der Acetylcholinesterase und löst dadurch eine Übererregung aus (Richardson 1995). Das Schwermetall Nickelchlorid weist eine eher unspezifische Wirkweise auf, da es allgemein die Integrität von Proteinen und dadurch deren Funktion beeinträchtigt (Scheil et al. 2009). Die Schadstoffe wurden, vor allem im Hinblick auf Versuche zur Kombinationsauswirkung, so ausgewählt, dass zum einen zwei Schadstoffe gleiche Wirkweise und gleichen Wirkort aufwiesen, wohingegen die anderen beiden an unterschiedlichen Orten mit unterschiedlichen Mechanismen wirken.

5. Ergebnisse und Diskussion

Kapitel 1: Miriam Langer-Jaesrich, Heinz-R. Köhler, Almut Gerhardt (2010): Assessing Toxicity of the Insecticide Thiacloprid on *Chironomus riparius* (Insecta: Diptera) Using Multiple End Points. Archives of Environmental Contamination and Toxicology 58:963-972

Die Auswirkungen von Thiacloprid auf die Zuckmücke *Chironomus riparius* wurden in einem Konzentrationsbereich von 0,1 bis 1000 µg/L untersucht. Um den größtmöglichen Informationsgehalt zu erzielen, wurde eine Batterie von verschiedenen Endpunkten z.B. die Larvenmortalität, das Verhalten, die

Wachstumsrate, die Emergenzrate, die Entwicklungsdauer, das Geschlechterverhältnis, der Hsp70-Stressproteinlevel und Deformationen der Mundwerkzeuge untersucht.

In dieser Studie konnte ermittelt werden, dass die Schlupfrate von *C. riparius* aus dem Ei bei Exposition gegenüber 1000 µg/L Thiacloprid signifikant reduziert war. Zusätzlich starben die Larven bei dieser Konzentration direkt nach dem Schlupf. Die Überlebensrate der Larven war ab einer Konzentration von 0,5 µg/L im Vergleich zur Kontrolle verringert (LC_{50}: 5,18 µg/L 10 d und 1,50 µg/L 17 d). Dabei nahm mit fortschreitender Expositionszeit die Anzahl der überlebenden Larven ab. In den höheren Konzentrationen starben die Larven vor dem Erreichen des Pupalstadiums. Aufgrund der hohen Mortalität der Larvenstadien verringerte sich auch die Emergenzrate ab einer Konzentration von 0,5 µg/L Thiacloprid signifikant (EC_{50}: 0,54 µg/L). Thiacloprid scheint damit nicht direkt mit dem Emergenzprozess zu interferieren, beeinträchtigt diesen aber durch die verursachte Mortalität der Larven indirekt.

Darüber hinaus konnte jedoch sowohl bei der Entwicklungsdauer als auch beim Geschlechterverhältnis zwischen weiblichen und männlichen Chironomiden kein Unterschied zwischen der Kontrolle und den mit Thiacloprid exponierten *C. riparius* nachgewiesen werden.

Überlebende L3 Larven von *C. riparius* Larven (Alter: 10 d) zeigten bei Konzentrationen ≥5 µg/L Thiacloprid eine signifikante Reduktion der Lokomotions- und Ventilationsaktivität. Nach 17 Tagen konnte bei 1 µg/L Thiacloprid eine signifikante Verringerung der Lokomotionsaktivität nachgewiesen werden. Zu diesem Zeitpunkt war auch der relative Hsp70-Stressproteinlevel in dieser Konzentration signifikant im Vergleich zur Kontrolle erhöht. In allen Ansätzen wiesen einige Individuen Deformationen der Mundwerkzeuge insbesondere der Mandibeln und am Mentum auf. Allerdings konnte weder in der Deformationsart noch in der Deformationsrate ein Unterschied zwischen der Kontrolle und den gegenüber Thiacloprid exponierten Chironomiden nachgewiesen werden. Es konnte

gezeigt werden, dass Thiacloprid in den Konzentrationen, in denen ein Überleben der Chironomiden möglich war, keinen negativen Einfluss auf die Integrität der Mundwerkzeuge hatte.

Durch eine Erweiterung des in der OECD Guideline vorgegeben Ablaufes des Tests konnte auch die Embryonalentwicklung in die Beobachtungen integriert werden. Allerdings lag die Konzentration von Thiacloprid, bei der die Embryonalentwicklung signifikant beeinträchtigt wurde, deutlich über den Effektkonzentrationen der anderen untersuchten Endpunkte. Hingegen eigneten sich die neu integrierten Verhaltensparameter gut als frühzeitig reagierende, subletale Endpunkte, um die neurotoxische Aktivität von Thiacloprid abzuschätzen. Die jeweilige Konzentration, bei der Verhaltensänderungen der Larven nach Tag 10 bzw. 17 auftraten, führte innerhalb der nächsten sieben Tage zu einer hohen Mortalitätsrate.

Ein direkter mechanistischer Zusammenhang zwischen der signifikanten Erhöhung des Hsp70-Stressporteinlevels und Thiacloprid kann, begründet auf der speziellen neurotoxische Wirkweise von Thiacloprid, als unwahrscheinlich angesehen werden. Allerdings kann die Erhöhung des Hsp70-Levels als Indikator einer allgemeinen Stressreaktion des Tieres ausgelegt werden.

In dieser Studie stellte der in den meisten Studien betrachtete Parameter „Emergenzrate“ in Verbindung mit Larvalmortalität den sensitivsten der untersuchten Endpunkte dar. Zusammenfassend erbrachte die Studie weitere Hinweise, dass Thiacloprid für Nicht-Zielinsekten (Beketov and Liess 2008a,b; Beketov et al 2008), in diesem Fall den Modellorganismus *C. riparius*, hochtoxisch ist. Der LOEC von 0,5 µg/L kann dabei als umweltrelevant eingestuft werden, da einzelne Thiaclopridmessungen in der Umwelt Konzentrationen von bis zu 4.5 µg/L im Oberflächenwasser neben Apfelplantagen im `Alten Land´ bei Hamburg nachweisen konnten (Süß et al. 2006). Zur abschließenden Bewertung der Chemikalie sollten, vor allem bei der Beurteilung chronischer Effekte, Faktoren wie die

Lebensdauer von Insekten oder die Verbreitung der Nicht-Zielinsekten im aquatischen Bereich berücksichtigt werden (Beketov et al. 2008).

Kapitel 2: Miriam Langer-Jaesrich, Heinz-R. Köhler, Almut Gerhardt (2010): Can mouth part deformities of *Chironomus riparius* serve as indicators for water and sediment pollution? A laboratory approach. Journal of Soils and Sediments 10:414–422

Um zu untersuchen, ob sich Deformationen der Mundwerkzeuge von Chironomiden als zuverlässige Indikatoren für Wasser- und Sedimentverschmutzung eignen, wurden in dieser Studie die Auswirkungen verschiedener Schadstoffe mit unterschiedlichen Wirkmechanismen als Einzelstoffe und Mischungen auf diesen Endpunkt bei Chironomiden untersucht. Testsubstanzen waren das Schwermetall Nickelchlorid, das Acetylcholinesterase hemmende Organophosphat Chlorpyrifos, und die beiden als Agonisten des Acetylcholinrezeptors wirkenden Insektizide Imidacloprid und Thiacloprid.

Allgemein konnten in den Kontrollen Deformationsraten bis zu 17% nachgewiesen werden, wobei dieser Prozentsatz jedoch mit Kontrolldeformationsraten anderer Studien vergleichbar ist (Janssens de Bisthoven et al 1998a,b; Meregalli and Ollevier 2001; Meregalli et al. 2001; Vermeulen et al. 2000a). In Expositionen mit höheren Konzentrationen von Imidacloprid, Thiacloprid, Chlorpyrifos und deren Mischungen starben die Chironomiden vor dem Erreichen des Pupalstadiums, weshalb keine Kopfkapseln zur Analyse vorhanden waren. Nach Applikation derjenigen Konzentrationen, bei denen Chironomiden dieses Stadium erreichten, konnte für alle Einzelsubstanzen kein signifikanter Unterschied zwischen den Kontrollen und den exponierten Chironomiden weder in Bezug auf die Deformationsart noch in Bezug auf die Deformationsrate der Mundwerkzeuge nachgewiesen werden. Dies galt auch für die Exposition gegenüber Mischungen von Nickelchlorid und Chlorpyrifos. Dementsprechend konnte bei diesen Ansätzen auch keine Konzentrations-Effekt Beziehung zwischen den jeweiligen Substanzkonzentrationen und dem Auftreten von Deformationen ermittelt werden.

Eine der grundlegenden Fragestellungen in dieser Studie bestand darin, zu klären ob Einzelsubstanzen im gleichen Maß Deformationen hervorrufen wie Substanzmischungen.

Obwohl Imidacloprid und Thiacloprid einen ähnlichen Wirkmodus aufweisen (Jeschke et al. 2001; Liu und Casida 1993, Maienfisch et al. 2003) und als Einzelsubstanzen keine erhöhte Deformationsrate verursachten, konnte bei der Mischungsexposition aus Imidacloprid und Thiacloprid eine steigende Deformationsrate vor allem in Bezug auf die Deformationen des Mittelzahns nachgewiesen werden. Dies weist darauf hin, dass die Effekte von Einzelsubstanzen und Substanzmischungen sich in Bezug auf die Mundwerkzeugdeformationen deutlich unterschieden können.

Die Verwendung von Exuvien für die Analyse der Mundwerkzeuge hat den Vorteil, dass es den Chironomiden ermöglicht, ihren Lebenszyklus zu vollenden. Hierdurch können Vergleiche zu anderen Endpunkten gezogen werden. In dem in Kapitel 1 dargestellten Versuch zeigten sich die ebenfalls untersuchten Parameter, wie beispielsweise das Verhalten oder die Emergenzrate, deutlich sensitiver. Darüber hinaus konnten bei diesen konsistente Konzentrations-Wirkungsbeziehungen nachgewiesen werden. Da, wie bereits erwähnt, die Deformationsrate der Mundwerkzeuge nicht beeinträchtigt war, scheinen diese als Testparameter jedoch eher ungeeignet zu sein.

Infolge dessen muss kritisch hinterfragt werden, ob die Deformationen der Mundwerkzeuge von Chironomiden deren Gesundheitszustand adäquat widerspiegeln und ob die Anwendung als zuverlässiger Indikator im Bezug auf Süßwasser- und Sedimentkontaminationen möglich ist.

Für die Anwendung im Freiland sollte zusätzlich beachtet werden dass weitaus komplexere Situationen durch mannigfaltige Einflüsse auftreten können. Zum Beispiel kann es bei einer über mehrere Generationen andauernden Exposition zu einer Adaption an einen bestimmten Schadstoff kommen. Auch kann die

Interpretation von Freilandstudien, beeinflusst durch saisonale Schwankung der Deformationsrate (Jeyasingham und Ling 2000), erschwert werden.

Kapitel 3: Miriam Langer-Jaesrich*, Cornelia Kienle*, Heinz-R. Köhler, Almut Gerhardt (2010): Impairment of trophic interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) by chlorpyrifos. Ecotoxicology 19:1294-1301

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Da bisher die Effekte von Chemikalien auf biotische Interaktionen wie z.B. Konkurrenz oder Räuber-Beute-Beziehungen in der aquatischen Ökotoxikologie nur selten untersucht wurden, wurde bei der folgenden Studie das Räuber-Beute Verhältnis zwischen Zebrabärblingen (*Danio rerio*) als Räuber und Zuckmückenlarven (*Chironomus riparius*) als Beute nach einer vorausgegangenen Pulsexposition gegenüber zwei verschiedenen Chlorpyrifoskonzentrationen (1 und 6 µg/L) analysiert. Dabei wurden vier verschiedene Ansätze betrachtet: Neben einem Kontrollansatz wurde in einem zweiten Ansatz nur die Beute, im dritten nur der Räuber und im vierten Ansatz sowohl Räuber als auch Beute prä-exponiert. Als Endpunkte wurde zum einen das Verhalten der Chironomidenlarven, sich im Sediment einzugraben, was als Vermeidungsstrategie gegenüber potentiellen Räubern interpretiert wurde, und die Fraßrate durch *Danio rerio* visuell erfasst.

Durch 1 µg/L Chlorpyrifos wurden weder das Grabverhalten der Chironomiden noch die Futtersuche der Zebrafische beeinflusst. Bei 6 µg/L wurde das Grabverhalten der exponierten Mückenlarven im Vergleich zu Kontrolltieren bereits vor dem Zusammentreffen mit *D. rerio* signifikant verändert. Der Anteil der Larven, die sich nicht vergruben, sondern an der Sedimentoberfläche blieben, war bei diesen Ansätzen signifikant erhöht.

Bei nicht exponierten *C. riparius*-Larven konnte nach dem Zusammentreffen mit den Fischen ein nahezu vollständiges Vergraben beobachtet werden. Hieraus ist zu folgern, dass Chironomiden in der Lage sind, Räuber aktiv wahrzunehmen und sich diesen durch eine erhöhte Eingrabrate zu entziehen versuchen (Hölker und Stief 2005). Im Vergleich hierzu befand sich bei den vorexponierten Chironomiden ein

deutlich größerer Teil, auch nach dem Zusammentreffen mit dem Räuber weiterhin an der Oberfläche des Sediments. Hierdurch waren die Chironomidenlarven offensichtlich besser für die Räuber zu detektieren. Es konnte nachgewiesen werden, dass nach Exposition gegenüber 6 µg/L Chlorpyrifos Chironomidenlarven signifikant häufiger gefressen wurden als im Kontrollansatz. Interessanterweise wurde dieser Effekt in demjenigen Versuchsansatz, bei dem beide Organismen, Räuber und Beute prä-exponiert wurden, wieder aufgehoben. Zwar befanden sich mehr Chironomiden an der Oberfläche, jedoch die Fraßrate lag auf dem des Kontrollniveaus. Dies legt die Annahme nahe, dass die Beuteerkennung oder der Trieb zur Nahrungsaufnahme bei exponierten Zebrabärblingen beeinträchtigt war.
Durch die zweistündige Pulsexposition sollte ein natürliches Expositionsszenario, wie es durch Regenauswaschungen oder Drift nach Pestizidapplikation auftreten kann, dargestellt werden. Von der höheren getesteten Konzentration von 6 µg/L wird jedoch angenommen, dass sie nur in Ausnahmefällen potentiell in Gewässern auftritt z.B. bei starkem Regen direkt nach Pestizidapplikation (Schulz 2001).
Es ist bekannt, dass Räuber-Beute-Interaktionen auch Effekte auf die Ökosystemfunktionen haben können (Stief und Hölker 2006; Townsend et al. 2003). Deshalb legt unsere Studie nahe, dass ökotoxikologische Tests mit Einzelspezies nicht adäquat alle potentiellen Effekte von Toxinen auf die Struktur und Funktion von Ökosystemen repräsentieren, da hierdurch Effekte auf organismischer Interaktionen zwischen Arten nicht detektierbar werden. Die vorgelegte Studie zeigt jedoch die Relevanz dieses Themas und stellt eine einfache Methodik vor, die es erlaubt, solche Effekte zu quantifizieren.

6. Schlussfolgerungen

Es konnte gezeigt werden, dass die Zuckmücke *Chironomus riparius* höchst geeignet ist, um verschiedenste ökotoxikologische Fragestellungen zu adressieren.
Durch die Erweiterung des OECD Tests mit zusätzlichen Endpunkten (Bioindikatoren und Biomarkern) konnten weiterführende Informationen über die

Toxizität von Thiacloprid gewonnen werden. Es konnte gezeigt werden, dass Thiacloprid in umweltrelevanten Konzentrationen toxisch für das untersuchte Nicht-Zielinsekt ist. Neben den Verhaltensreaktionen zählt die Emergenzrate zu den sensitivsten Endpunkten. Das Testsystem ist gut geeignet, um neben weiteren Substanzen auch Mischungen oder natürlich kontaminierte Sedimente zu untersuchen.

Im Gegenzug hierzu wurde ermittelt, dass Deformationen der Mundwerkzeuge von *C. riparius* sich weniger als zuverlässige Bioindikatoren für Schadstoffe eignen. Neben der Tatsache, dass die Deformationsrate auch bei Kontrolltieren bereits sehr hoch ist, induziert nicht jeder Schadstoff eine Reaktion. Da es nicht möglich war, Dosis-Wirkungsbeziehungen zu ermitteln und auf Grund veränderter Reaktionen bei Substanzmischungen im Vergleich zu Einzelsubstanzexpositionen sollte der Einsatz von Mundwerkzeug-Deformationen als geeigneter Bioindikator überdacht werden. Es besteht weiterer Forschungsbedarf für dieses Thema, insbesondere wenn die Mundwerkzeug-Deformationen für Feldversuche verwendet werden sollten, da bei diesen weit komplexere Umwelteinflüsse eine Rolle spielen können.

Des Weiteren konnte gezeigt werden, dass Räuber-Beute-Beziehungen, am Beispiel von Zebrabärblingen und Chironomidenlarven durch Schadstoffe beeinflussbar sind, auch wenn es sich hierbei nur um kurzeitige Expositionen handelt. Es sollten bei solchen Fragestellungen unbedingt beide Organismen, also Räuber und Beute gleichermaßen beachtet werden. Es ist dabei entscheidend welche trophische Ebene exponiert ist, um zu ermitteln, auf welche Weise sich die organismische Interaktion verändert. Es wäre erstrebenswert bei Risikoabschätzungen von Chemikalien zukünftig auch interspezifische Reaktionen zu beachten, insbesondere da gezeigt werden konnte, dass relativ einfache experimentelle Systeme hierfür ausreichend sind.

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen

7.1 Kapitel 1: Miriam Langer-Jaesrich, Heinz-R. Köhler, Almut Gerhardt (2010): Assessing Toxicity of the Insecticide Thiacloprid on *Chironomus riparius* (Insecta: Diptera) Using Multiple End Points. Archives of Environmental Contamination and Toxicology 58:963-972

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

7.2 Kapitel 2: Miriam Langer-Jaesrich, Heinz-R. Köhler, Almut Gerhardt (2010): Can mouth part deformities of *Chironomus riparius* serve as indicators for water and sediment pollution? A laboratory approach. Journal of Soils and Sediments 10:414–422

Versuchsdurchführung der Chironomidentests mit Chlorpyrifos, Nickelchlorid und deren Mischungen durch LimCo International (Dr. Almut Gerhardt und Mitarbeiter). Vollständiger Eigenanteil an der Versuchsplanung, Durchführung und Auswertung der Chironomidentests mit Imidacloprid, Thiacloprid und deren Mischungen. Kompletter Eigenanteil an der Analyse der Mundwerkzeugdeformationen und deren Auswertung. Die fachliche Betreuung erfolgte durch Dr. Almut Gerhardt (LimCo International) und Prof. Dr. H.-R. Köhler (Universität Tübingen).

7.3 Kapitel 3: Miriam Langer-Jaesrich*, Cornelia Kienle*, Heinz-R. Köhler, Almut Gerhardt (2010): Impairment of trophic interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) by chlorpyrifos. Ecotoxicology 19:1294-1301
**beide Autoren sind gleichberechtigt als Erstautoren zu betrachten.*

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Kapitel 1: Assessing Toxicity of the Insecticide Thiacloprid on *Chironomus riparius* (Insecta: Diptera) using multiple endpoints

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Abstract

Since data documentation on neonicotinic toxicity to nontarget organisms should be enhanced, we investigate the effects of thiacloprid, a novel neonicotinoid insecticide, on the sediment-dwelling non target insect *Chironomus riparius*. Further we wanted to validate the sensitivity of end points on different biological levels and obtain the greatest amount of information regarding the effects of this compound by using a battery of several end points such as larval mortality, behavior, body weight gain, emergence rate, time of development, gender ratio, the Hsp70 stress protein level and larval mouth part deformities after exposure at a concentration range of 0.1 to 1000 µg/L thiacloprid. *C. riparius* was impacted starting at concentrations of 0.5 µg/L, a concentration which can be considered as environmentally relevant. Larval mortality, behavior, emergence, and Hsp70 protein level were sensitive indicators for the toxic effect of thiacloprid, whereas gender ratio and mouthpart morphology were not affected. In our case life cycle endpoints like the survival rate (LC_{50}: 1.57 µg/L) or emergence rate (EC_{50}: 0.54 µg/L) proved to be more sensitive than tested physiological endpoints for the neurotoxic insecticide.

Archives of Environmental Contamination and Toxicology, 2010, 58:963-972

Introduction

The use of pesticides can affect non target species of organisms, since they can be at risk from pesticide leaching, spray drift, or surface runoff into aquatic ecosystems (Kreuger 1998; Palma et al. 2004). To evaluate this risk for aquatic organisms, a number of test systems have been developed. However, these methods focus primarily on pelagic organisms, such as fish and daphnids. Communities of benthic organisms play a key role in energy, nutrient and contaminant fluxes and they play a key role in transferring environmental contaminants to higher trophic levels (Burton 1991; Reynoldson 1987). For this reason, the consequences of toxic substance exposure to benthic communities should be studied more closely.

Thiacloprid, a neurotoxic insecticide, belongs to the new and commercially very successful family of the neonicotinoids. Both its structure and mode of action is related to imidacloprid – one of the highest selling insecticides worldwide (Jeschke et al. 2001). In 2007 it was registered in more than 50 countries (Bayer Crop Science 2008). Thiacloprid, as all neonicotinoids, acts on the insect nervous system as an agonist of the nicotinic acetylcholine receptor (nAChR) (Jeschke et al. 2001). It exhibits high water solubility (184-186 mg/L) and a relatively low log K_{ow} (1.26) at 20°C. This insecticide has a half life (DT_{50}) in water of 6-11 days and a DT_{50} in water sediment systems, lasting between 11-27 days (European Commission 2004). Thiacloprid may therefore contaminate surface waters following rainstorm events (Beketov and Liess 2008b). The currently predicted worst case environmental concentration for thiacloprid via spray drift in surface water has been predicted to be 1.99 (ornamentals) and 17.52 µg/L (orchards) respectively (Schmuck 2001). For runoff events this scenario is not yet available. Studies on measured thiacloprid concentrations in the environment are rare due to its brief time of availability on the market. A single study conducted in apple orchards in the surrounding area of Hamburg, Germany, detected thiacloprid concentrations of 4.5 µg/L in a nearby water system (Süß et al. 2006).

Information about the toxicity of thiacloprid to non target freshwater invertebrates and about its potential effects on freshwater ecosystems is limited (Beketov and Liess 2008b). However, in previous experiments insects showed a higher sensitivity to this insecticide than other freshwater arthropods (Beketov and Liess 2008a; Beketov and Liess 2008b; Beketov et al. 2008). This strongly suggests the inclusion of insects in further additional ecotoxicological testing.

The worldwide distributed family of *Chironomidae* are suitable test species due to their aquatic and sediment bound larval stages. Chironomids are frequently the most abundant group of insects in freshwater environments and their function as prey for other species makes them environmentally relevant (Armitage et al. 1995; Pinder 1986). The non biting midge, *Chironomus riparius,* is widely used for toxicity testing (OECD 2004a; OECD 2004b; US-EPA 2000) due to its easy cultivation, its short generation time, and its relative sensitivity to pollutants.

Beketov and Liess (2008b) pointed out that the knowledge about sublethal effects of thiacloprid on life cycle traits of insect species is limited. The aim of this study was to evaluate the effects of thiacloprid on the insect *C. riparius* using established endpoints such as emergence rate, the time needed for development, and the larval body weight gain (OECD 2004a; OECD 2004b). In addition, these main parameters were complemented with other endpoints including hatching rate, behaviour changes, larval mortality (L3 and L4), stress protein response (70 kD heat shock protein family, Hsp70), mouth part deformation, and sex ratio.

Material and Methods

Maintenance of Parent animals

Stock cultures of *Chironomus riparius,* from different genetic sources, in order to avoid genetic impoverishment, (LimCo International, Germany; University of Joensuu, Finland and Universidade de Coimbra, Portugal), were kept as larvae in fine quartz sand and dechlorinated tap water under constant aeration. Every day the chironomid

larvae were fed with finely grounded fish flakes (50% Tetramin, 50% Tetraphyll, Tetra, Germany). Declorinated tap water was exchanged one or two times per week. Before emergence occurred, a breeding cage (55 x 65 x 120 cm) was installed over the stock containers, in which the adults were allowed to fly, swarm and breed. The egg masses which were attached to the vessel wall were collected every morning and used subsequently for experiments. The stock breeding and all experiments were conducted in a climatized chamber at 21.0 ± 0.5°C, with a light-dark cycle of 16:8 h artificial daylight (Philips standard daylight 54765, 2500 lumen, Germany).

Preparation of Insecticide Stock Solutions

At room temperature (RT) a 5 mg/L thiacloprid (Riedel-de Haën, CAS 111988499 analytical standard, Germany) stock solution was prepared, every third day with declorinated tap water (pH 7.8 ± 0.2) and stirred for 14 h in the dark. The following nominal test concentrations were directly prepared before use with aerated declorinated tap water: 0.1, 0.5, 1, 5, 10 and 1000 µg/L thiacloprid – as well as one negative control containing only pure dechlorinated tap water. Concentrations of insecticide were chosen on the basis of a preliminary range of tests with L3 larvae and also from measured environmental concentrations (Süß et al. 2006). Each treatment was replicated four times.

Spiking of Sediment

To simulate natural conditions we first spiked the sediments with an aqueous solution assuming that the partitioning between sediments and the water phase takes 24 h to reach equilibrium. The day before being used in the experiment 50 g of quartz sediment (particle size 0.1-0.3 mm, burned for 3 h at 500°C to remove organic matter; Dehner, Germany) was filled into a 250 ml glass beaker. For spiking, the sediment was covered with 200 ml of the respective test solution and subsequently shaken for 24 h under exclusion of light. Subsequently we removed the water phase and added fresh test solution representing the environmental conditions in which thiacloprid is

repeatedly introduced. The aim of this was to stabilize the concentration of insecticide both in the water and the sediment.

Egg Preparation and Exposure

Different egg clutches from the breeding stock were collected at 8 a.m., separated into smaller clusters of visually the same size and mixed randomly. A preliminary experiment was designed to test whether egg clutch disassembly with and without thiacloprid treatment had an effect on hatching rate.

After counting the eggs, between 100 and 120 eggs per replicate were exposed to the appropriate thiacloprid concentration. The number of hatched larvae was counted under a stereomicroscope daily at the same time of day for a period of six days after oviposition. Since larvae exposed to the highest thiacloprid concentration of 1000 µg/L did not hatch, but died immediately after hatching (see results), only up to 10 µg/L thiacloprid were tested in the procedures that follow.

Larval Exposure and Maintenance

After 3 days 33 first-instar larvae from each replicate were transferred with a glass pipette to glass beakers (providing a density of 1.34 individuals/cm^2). The beakers were covered with parafilm to reduce evaporation (Parafilm 'M', American national can, Chicago). The larvae were fed daily with 12 ± 1 mg fine ground fish food (50% Tetramin, 50% Tetraphyll, Tetra, Germany), corresponding to ≥ 0.36 mg/day/larvae. From the second day after transfer the beakers were aerated through a glass pasteur pipette. Water was exchanged every third day with new insecticide spiked water. Temperature, pH, conductivity, dissolved oxygen saturation, and nitrite content were regularly measured in the newly spiked and old exchanged water. Every week the larvae were transferred into new beakers with freshly spiked sand and test solution. The beakers were gently swayed until the larvae came to the sediment surface. The larvae were then aspired gently with a cut plastic pipette, transferred and counted.

Larval Survival

The survival rate of third-instar (L3) and fourth-instar (L4) larvae was monitored 10 and 17 days after oviposition. This made it possible to distinguish between early and latter larval mortality; and furthermore, to differentiate between mortality and disturbances of the emergence process, both resulting in reduced emergence rate.

Behavior Measurement

During the survival monitoring the behaviour of 12 larvae per test insecticide concentration (3 randomly selected animals from each of the 4 replicates) was measured for 2 h with the Multispecies Freshwater Biomonitor® (LimCo International, Germany) in dechlorinated tap water. The Multispecies Freshwater Biomonitor (MFB) is an online biomonitor which continuously and quantitatively records the behaviour pattern of animals (Gerhardt et al. 1994). Behaviour signals of the chironomids were analysed with a Fast Fourier Transformation resulting in a histogram of different signal frequencies, hence enabling distinction between different types of behaviour such as locomotion and ventilation (Gerhardt et al. 1998). For each individual, mean locomotor (0.5–2.5 Hz, band 1) and ventilatory activity (3-8 Hz, band 2) (% time spent on locomotion and ventilation, respectively) was calculated for a time period of 2 h. The MFB chambers, which differed for the different larvae stages (L3, 4 cm long and 1 cm diameter; L4, 4.5cm long and 2 cm diameter), allowed free movements of the chironomids larvae and were sealed with a porus lid (mesh size 0.25 mm). Larvae were put back into their respective beakers after the behaviour measurements.

Emergence

When emergence started, the number and the gender of emerged midges were determined daily. To detect changes in development time, for each concentration the developmental rate was calculated using the formula given in the OECD Guideline (OECD 2004a; OECD 2004b). The gender ratio of the emerged chironomids was calculated in each replicate by the number of male to female organisms.

Mouthpart Deformities

The remaining head capsules exuviae of the emerged midges were collected and stored in 100% alcohol. One day before morphological preparation, the head capsules were separated from the body or exuviae rests and stored overnight in Rotihistol (Carl Roth GmbH, Germany). The next day, the head capsules were placed on a glass slide with the ventral side facing upwards, then covered with Roti-Histokit (Carl Roth GmbH, Germany) and squeezed gently with a cover slip. The strongly scleroticised mentum and mandibles were evaluated with a microscope at 40x magnification. Missing teeth, extra teeth and mentum split medial teeth were counted as deformities (Bird 1994; Gerhardt and Bisthoven 1995; Servia et al. 1998). The ratio of individuals with deformed mouthparts to the number of examined individuals was calculated (Hämäläinen 1999), using two different approaches. First, the total deformity rate was calculated using the number of individuals with deformed mouthparts to the total of examined individuals. Secondly, the different deformity types to examined number of individuals were calculated.

Body Weight Gain

To investigate the body weight gain, the same experiments were conducted as described above. But as recommend in the OECD Guideline 218/219, 10 days after oviposition the surviving animals were collected and dried at 100°C for 24 h in aluminum foil and weighed (Sartorius LE 324S, Germany). Subsequently, the average dry weight of the larvae in each replicate was calculated.

Hsp70 Protein Analysis

Ten fourth-instar larvae (17 days after oviposition) per treatment were shock frozen in liquid nitrogen and stored at – 20°C for later Hsp70 analysis. The Hsp70 content was investigated at day 17, because at this stage (L4) the chironomid larvae had a 'total protein content' that was sufficient to analyse the Hsp70 in individual larvae. For standard Hsp70 analysis see also Köhler et al. (1992) and Köhler et al. (2007). The

frozen larvae were homogenized individually with a plastic pestel in 45 µl extraction buffer (80 mM potassium acetate, 4 mM magnesium acetate, 20 mM Hepes, 2% protease inhibitor Sigma P8340, pH 7.5) and centrifuged for 10 minutes at 20.000 g and 4°C. The supernatant was used for further analysis. The total protein concentration was determined according to the method of Bradford 1976. The supernatant was mixed with 12.5 µL sodium dodecyl sulphate (SDS) and cooked for 5 minutes at 95-100°C. Constant amounts of total protein (40 µg) from each sample were subjected to SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) (12% acrylamid-bisacrylamid) for 15 minutes at 80 V and then 90 minutes at 120 V. The protein was transferred to a nitrocellulose membrane in a semi-dry blotting chamber (360mA, 2h; Panther™Hep-1; Owl). Subsequently, the nitrocellulose membranes were blocked for 2 h in Tris-buffered saline (TBS) solution containing horse serum. After washing for 5 minutes in TBS, the membranes were incubated overnight with a monoclonal antibody at 21°C (mouse anti-human Hsp 70, Dianova, Hamburg, Germany, dilution 1:5000 in 10% horse serum/TBS). After washing again for 5 minutes with TBS, the filters were incubated with a secondary antibody for 2 h at 21°C (peroxidase-conjugated goat anti-mouse IgG, Dianova, Hamburg, Germany, dilution 1:1000 in 10% horse serum/TBS). Following a repeated wash with TBS for 5 minutes, the antibody complex was detected by adding 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 protein bands were quantified using a densiometric image analysis system (Herolab E.A.S.Y., Germany) and set in relation to an internal snail hsp 70 standard (*Xeropicta derbentina*) run in parallel on each gel.

Statistics

Because some data were not normally distributed, non-parametric tests were used. All data were analysed using Friedmann´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 Excel macro REGTOX

(based on the Marquardt algorithm, http://eric.vindimian.9online.fr) was used to compute lethal concentration (LC) and effect concentrations (EC), and to estimate the confidence intervals on the parameters by a bootstrap non-parametric simulation.

Results

Abiotic parameters

Water quality parameters measured during the experiment were comparable among the treatments (pH 7.8-8.2, temperature 21.2-21.5°C; conductivity 400-500 µS/cm, dissolved oxygen level above 60% of saturation, nitrite content 0-0.5 mg/L).

Hatching Rate

In the preliminary experiment no difference was found between disassembled egg clutches and nonmanipulated egg clutches in the two additional treatments investigated, the control and in the treatment with 1000 µg/L thiacloprid (Wilcoxon test n.s.).

Hatching of the first-instar larvae started 2 days after oviposition, but peaked at 3 and 4 days after oviposition. No impact on hatching rate could be observed in the presence of 10 µg/L thiacloprid compared to the control treatment (overall mean hatching rate 92.44 ± SD 6.77 after oviposition) (Wilcoxon n.s.). Only at 1000 µg/L the hatching rate was significantly reduced (mean hatching rate 5.57 ± SD 8.47) (Wilcoxon $p<0.0209$) compared to control and other treatments with lower insecticide concentrations. Even the small number of hatched larvae in the 1000 µg/L treatment died immediately after hatching; for this reason only up to 10 µg/L thiacloprid was used in further tests.

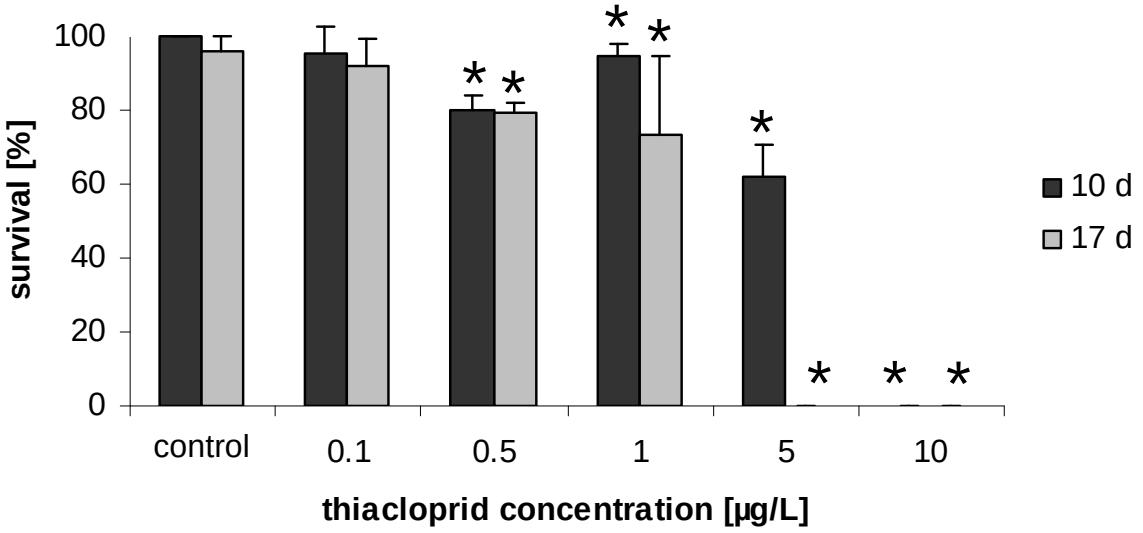
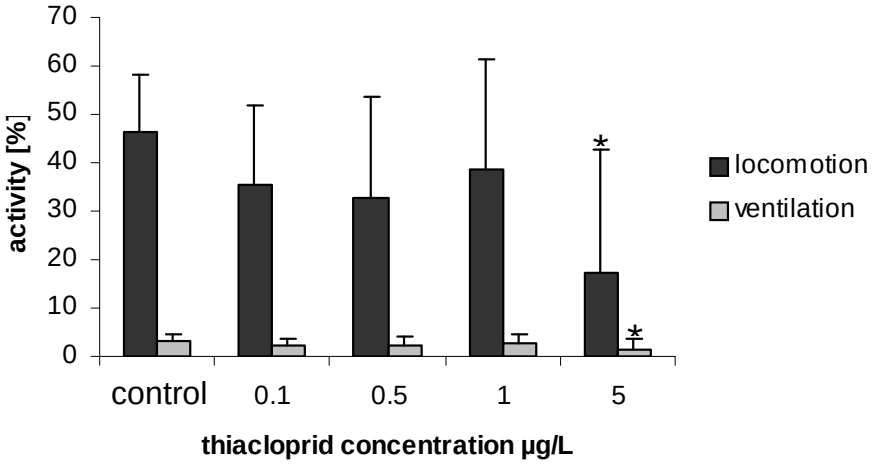


Fig 1: Mean survival rate of *Chironomus riparius* larvae exposed to different thiacloprid treatments at the age of 10 and 17 days after oviposition. Mean ± SD (four replicates per treatment with 33 larvae each). Significant difference to the control at * $p \leq 0.05$ (Wilcoxon test).

Larval survival

Ten and seventeen days after oviposition the larval survival rate was significantly decreased at 0.5 µg/L and higher concentrations of insecticide compared to the control treatment. With increasing exposure time fewer larvae survived at the respective exposure concentrations (Fig. 1). The calculated LC_{50} was 5.18 µg/L (10 d) and 1.5 µg/L (17 d).

A:



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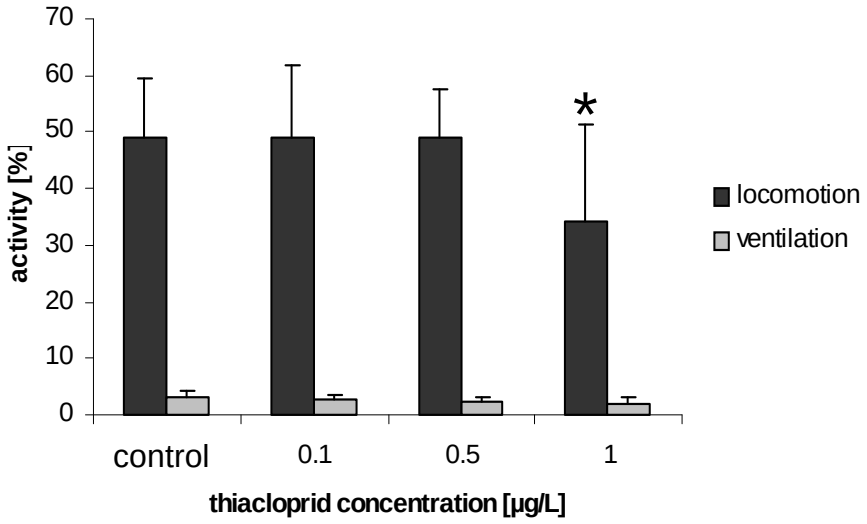

Fig. 2: Mean activity [%] of *Chironomus riparius* larvae exposed to different thiacloprid concentrations at an age of 10 days (A) and 17 days (B) after oviposition. Mean ± SD, n = 12. Significant differences to the respective control treatment at * $p \leq 0.05$ (Wilcoxon test).

Emergence

The first emergence of the organisms was observed at day 19 after oviposition. At the highest test concentrations (5 and 10 µg/L of thiacloprid) the larvae died before achieving the pupae stage, therefore no emergence could be observed (Figs. 1, 5). At 1 µg/L only two animals emerged. At 0.5 µg/L the total emergence was significantly reduced compared to the control. At 0.1 µg/L the number of emerged animals did not differ significantly from the control, although the emergence rate was higher. The calculated EC_{50} for the endpoint 'total emergence' was 0.54 µg/L thiacloprid.

The developmental rate in the treatments with emerging *C. riparius* (0.1, 0.5 and 1 µg/L) did not differ significantly between the different thiacloprid treatments (Friedmann's ANOVA n.s.). The sex ratio of emerged chironomids varied, but did not differ significantly (Friedmann's ANOVA n.s.).

Behavior

In both larval stages (L3 and L4) the locomotor activity was significantly reduced at the highest insecticide concentrations of 5 µg/L after 10 days and 1 µg/L at 17 days (Figs. 2a,b), respectively, which still allowed survival as shown in Fig. 1. L3 larvae also showed reduced ventilatory activity at 5 µg/L of thiacloprid (Fig. 2a).

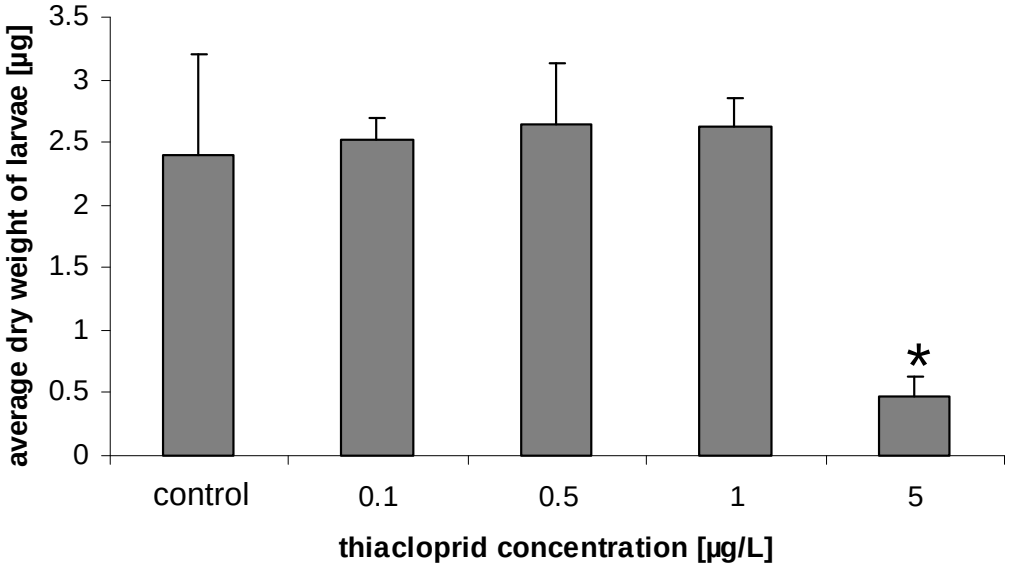

Fig. 3: Average dry weight per larva (mean ± SD) of *Chironomus riparius* exposed to thiacloprid 10 days after oviposition (n = 4). Significant difference to the respective control treatment at * $p \leq 0.05$ (Wilcoxon test).

Body weight gain

The average mean dry weight of *Chironomus riparius* larvae exposed to 5 µg/L of thiacloprid for 10 days was significantly reduced compared to the control (Fig. 3).

Hsp 70

The relative level of the stress protein Hsp70 significantly increased at 1 µg/L thiacloprid compared to the control (Fig. 4). Higher concentrations of insecticide exposure could not be investigated because the animals did not survive until day 17.

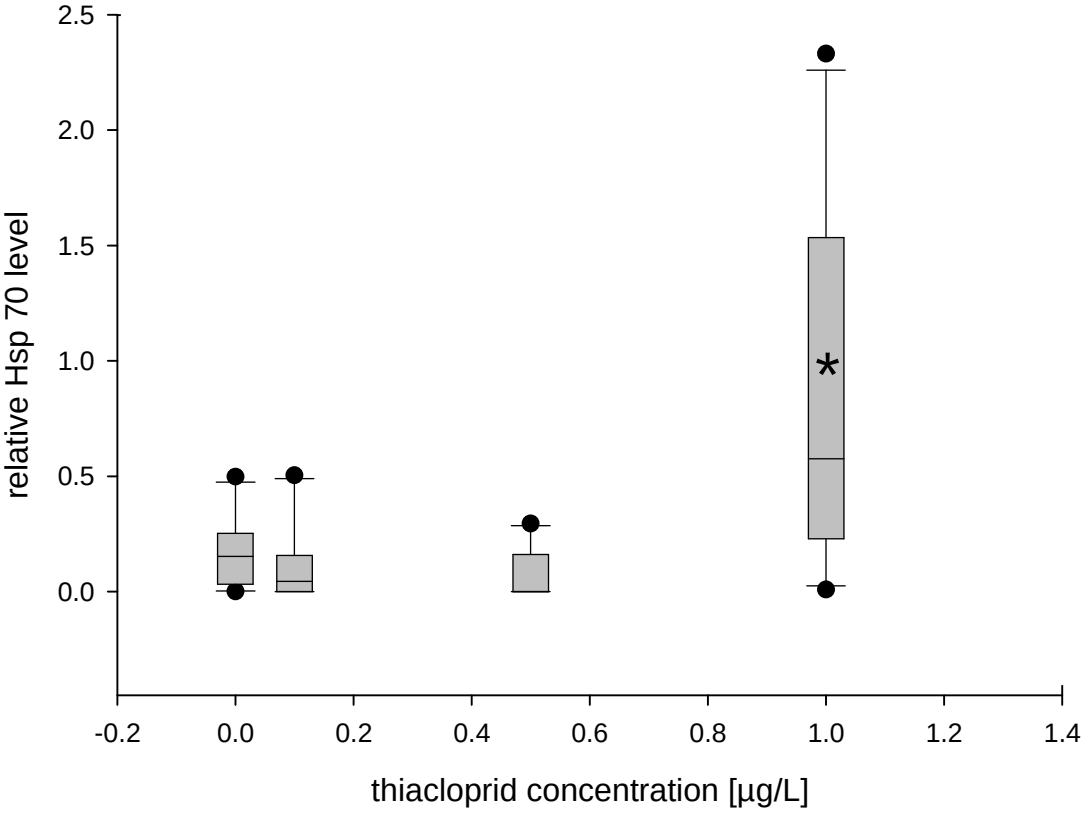

Fig. 4: Hsp70 levels (relative to standard) of L4 larvae of *Chironomus riparius* exposed to different thiacloprid concentrations for 17 days (n = 10). Box plots display the median, lower (25%) and the higher percentile (75%) and outliers. Significant difference to the control at * $p \leq 0.05$ (Wilcoxon test).

Mouth part deformities

In all treatments some chironomids showed deformities in the mentum and the mandibles. Slight deformities were observed such as missing teeth, extra teeth, and mentum split medial teeth. No severe deformities such as the Köhn gap were found (Köhn and Frank 1980). The control treatment showed a mean total deformity rate of 17%. No significant differences in the deformity rates between the thiacloprid treatments and the control were observed.

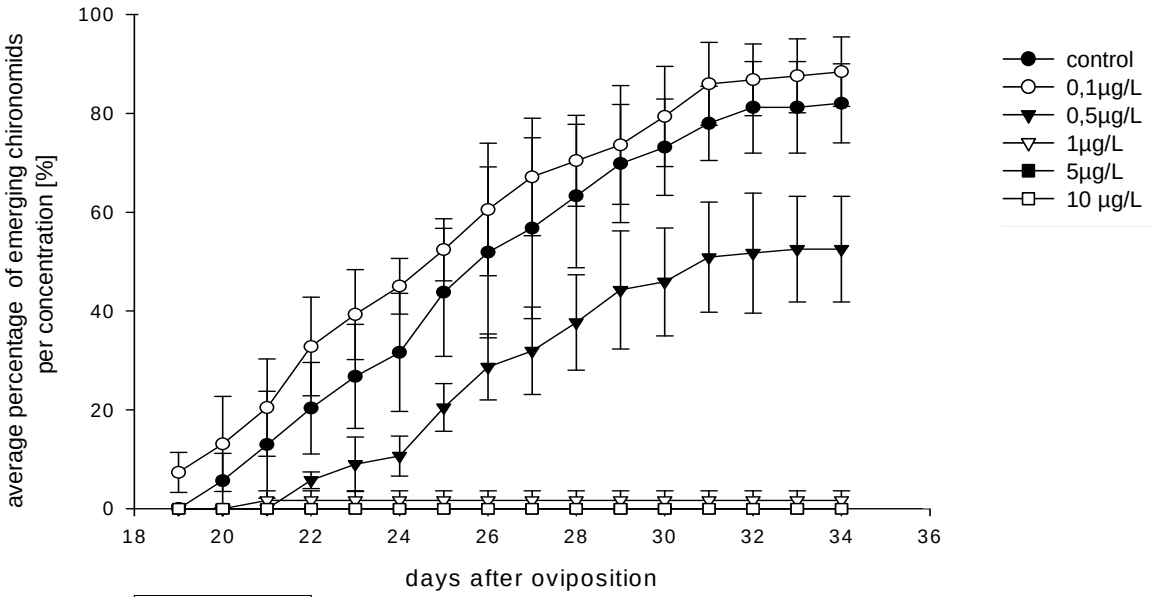

Fig. 5: Mean ± SD cumulative numbers of emerged *Chironomus riparius* imagos exposed to different thiacloprid treatments ± SD, n = 4 (30 or 31 animals per replicate). The number of emerged *C. riparius* was significantly lower in the 0.5, 1, 5 and 10µg/L treatment compared to the control, * $p \leq 0.05$ (Wilcoxon test). Data obtained for 5 and 10 µg/L are identical.

Discussion

The aim of the present study was to quantify the effects of thiacloprid on different life-cycle traits of *Chironomus riparius*, as well as to compare the sensitivity of those end points from different biological levels which have rarely been addressed in this combination in previous studies. To achieve these goals, a number of modifications to the OECD Guideline (OECD 2004a, b) were made.

In the present study, chironomid eggs were exposed to thiacloprid directly after oviposition, and therefore, effects on embryonic development were likely to be detected. The hatching rate was affected above 10 µg/L, and thus it was less sensitive compared to other tested end points, which reacted at considerably lower concentrations. It was shown that dissection of the egg clutches, along with partial removal of the gelatinous matrix, had no negative effects on hatching rate. Therefore, in our study, dissection of egg clutches was found to be a suitable tool to determine

the exact number of exposed eggs, allowing reliable calculation of the hatching rate. The aim of this study was to test the toxicity of the hydrophilic insecticide thiacloprid ($logK_{ow}$, 1.26) on *C. riparius* and not to focus on its binding potential to natural sediments. Therefore pure inert quartz sand was used, in accordance with other chironomids studies (Hahn et al. 2001; Nowak et al. 2008; Vogt et al. 2007a, b). Since thiacloprid is stable to hydrolysis at pH values of 5–9 at 25°C, and has a reported half-life in aerobic natural water sediment systems varying between 12 and 20 days (Public Release Summary 2001), and since water was exchanged twice a week and replaced by fresh solutions, nominal thiacloprid concentrations can be assumed to closely represent actual concentrations.

In this study, an intermediate larval density of 1.34 larvae/cm^2, ranging between low densities of 0.2 larvae/cm^2 (Forbes and Cold 2005) and high densities of 4 larvae/ cm^2 (Hooper et al. 2003), was used. However, this larval density value was lower than the recorded field densities of *C. riparius* of 10 individuals/cm^2 (Groenendijk et al. 1998). At thiacloprid concentrations [1 μg/L all larvae died within 17 days of exposure. Reduced survival (LC50:1.57 μg/L after 17 days) and total emergence rate (EC50:0.54 μg/L) were the most sensitive end points.

As the second test end point behavioral changes were used, as behavior integrates biochemical and physiological processes (Dell'Omo 2002). Consequently, it is sensible to use this end point, especially when testing substances like the neurotoxic insecticide thiacloprid. The detected behavioral changes occurred regularly at 5 μg/L (L3 larvae) and 1 μg/L (L4 larvae) thiacloprid and caused high mortality rates after a period of 7 days. In the *C. riparius* life cycle, behavioral parameters can therefore be regarded as early-response sublethal end points. Thiacloprid is also known to induce behavioral changes in the aquatic insect *Simulium latigonium* at concentrations several times lower than the LC_{50} as soon as 2 h after contamination (Beketov and Liess 2008a).

To investigate general stress, the expression of Hsp70 was included as an end point. This protein family is ubiquitous, and its isoforms can act as chaperones for correct

protein folding (Berg et al. 2003). When proteins start to unfold, for example, due to heat stress, the presence of toxic metals, or other proteotoxic conditions, the Hsp70 level is increased to compensate for these effects via feedback coupling (Beckmann et al. 1992; Gething and Sambrook 1992; Morimoto 1993). Therefore, a change in the Hsp70 level has been proposed as a general biomarker for proteotoxicity in environmental monitoring (Yoshimi et al. 2002). In our study, the Hsp70 level was significantly higher in L4 *C. riparius* larvae at 1 µg/L thiacoprid exposure compared to the control. Considering the neurotoxic mode of action, a direct proteotoxic effect of thiacloprid is unlikely, although the upregulation of Hsp70 might indicate a reaction of the animal to the harmful stress situation. Up to now only a few studies have used Hsp70 as a biomarker for toxic stress in chironomids. An increase in hsp70 gene expression was found in *C. riparius* exposed to 10 mM cadmium (Martinez-Guitarte et al. 2007) or to 1 µg/L nonylphenol, a potential endocrine disruptor (Lee and Choi 2006). In addition, an increase in hsp70 expression was reported for *Chironomus yoshimatsui* exposed to 0.4 µg/L fenitrothion, an organophosphorus insecticide, and to 1.1 µg/L ethofenprox, a synthetic pyrethrioid (Yoshimi et al. 2002). Karouna-Renier and Zehr (2003) were able to demonstrate an increase in the Hsp70 protein level in *C. riparius* exposed to 0.25 mg/L copper for 24 h. Considering the results presented in this study and those found in the literature, the Hsp70 response seems to be a quite sensitive sublethal indicator for the toxicity of substances with different modes of action or other unfavorable conditions in *C. riparius*.

The end point body weight gain was only significantly reduced when the survival was also considerably reduced. Consequently, upon thiacloprid exposure, the end point 'body weight gain' is less sensitive than the others. Although the developmental rate of the surviving chironomids was not impaired as evidenced by the simultaneously occurring emergence peaks (Fig. 5), the total number of emerged chironomids was significantly reduced at ≥0.5 µg/L thiacloprid (EC_{50}: 0.54 µg/L). It is reasonable to assume that thiacloprid did not affect the emergence process itself, but the survival of larvae, due to the high young larval mortality. The emergence period

(between day 19 and day 34) of control *C. riparius* in this study is rather lagged compared to the results of some other chironomid studies (Paumen et al. 2008; Vogt et al. 2007c), but our results are comparable to those of Forbes and Cold (2005) and Watts and Pascoe (2000). Additionally, in contrast to OECD guidelines 218 and 219, days were counted from oviposition, which might explain the seemingly later start of emergence.

A positive spatial relationship between sediment contamination level and larval deformities of midges in the field has been documented (Hämaäläinen 1999). Therefore, the use of Chironomidae morphological deformities in bioassessment and biomonitoring of contamination stress in lakes and streams has been suggested. However, few experimental studies have demonstrated an exposure–response relationship between contaminants and deformities (Hämäläinen 1999; Janssens De Bisthoven et al. 1998a, b; Martinez et al. 2001). Also, in the present study, no dose–response dependence was found between the total mouthpart deformity rate or single deformities and increasing thiacloprid concentration. It is still unknown how pollutants may induce deformities. However, there is an ongoing discussion that mouthpart deformities develop at the endocrine-regulated molting stage, and a disruption of this complex process is likely at the base of their ontogeny (Meregalli and Ollevier 2001). DDT, 4-nonylphenol, and heavy metals are known to induce mouthpart deformities. The organics, DDT and 4-nonylphenol, disrupt endocrine processes directly and heavy metals have been described to act indirectly on the endocrine system (Meregalli et al. 2001). It seems that thiacloprid at concentrations which allowed for the survival of some individuals (at 0.1 and 0.5 µg/L) does not interfere with these processes. It was also not possible to find a relation between the deformity rate and any of the other affected end points. Therefore, it must be questioned whether mouthpart deformities really mirror the health conditions of *C. riparius*. A high percentage (17%) of mouthpart deformities in the control seems not to be unusual for *C. riparius*, a chironomid genus which naturally shows a high percentage of deformities (Servia et al. 1998). Similarly, in other studies the deformity

rate ranged between 7 and 19% (Janssens de Bisthoven et al. 1998b; Meregalli and Ollevier 2001; Meregalli et al. 2001).

The use of a complete set of end points at different developmental stages gave detailed insight into the lethal and sublethal effects of thiacloprid on *C. riparius*. Hatching rate, body weight gain, larval mortality, behavior, Hsp70 level, and emergence rate were affected in the tested concentration range, whereas developmental rate, gender ratio, and mouthpart deformations seemed not to be impaired in surviving chironomids. The widely accepted end point ´total emergence rate´, which is established in the OECD guidelines (OECD 2004a, b), seems to be the most sensitive end point in connection with larval survival. Considering the neurotoxic potential of thiacloprid, it was expected that, first, behavior, then survival, and, finally, emergence rate were affected at lower concentrations than growth rate or hatching rate. Additionally, this mode of action suggests that the gender ratio and the deformity rate of the surviving chironomids were not affected by chronic exposure to thiacloprid.

The results of this study provided further evidence that thiacloprid is highly toxic to the nontarget insect *C. riparius*. The obtained LOEC level was 0.5 μg/L for larval survival and emergence rate; the calculated EC_{50} ranged between 5.18 μg/L for survival (10 days) and 0.54 μg/L for total emergence rate. In a previous *C. riparius* study (28 days) with thiacloprid an EC_{15} of 1.75 μg/L (range, 1.54–1.99 μg/L) was obtained (Schmuck 2001), however, the measured end point was not clarified. Although not directly comparable, recently published acute studies with thiacloprid also indicated a high toxicity to aquatic nontarget insects. Beketov and Liess (2008b) were able to demonstrate that thiacloprid is toxic to several freshwater arthropods after a 24-h exposure. Especially *Notidobia ciliares* (LC_{50}, 5.47 μg/L) and *Simulium latigonium* (LC_{50}, 5.76 μg/L) were very sensitive to thiacloprid. In another study, *Baetis rhodani* exhibited an LC_{50} of 4.6 μg/L after 96 h of exposure (Beketov and Liess 2008a). In a stream mesocosm experiment a single thiacloprid pulse contamination (0.1, 3.2, and 100 μg/L) resulted in long-term (7-month) alteration of the overall invertebrate

community structure, with an LOEC of 3.2 µg/L (Beketov et al. 2008). This value is slightly below the acute LC_{50} for sensitive invertebrates relevant in this mesocosm study. Therefore this indicates that concentrations of pesticides at which the majority of species are affected can be predicted by acute organism-level toxicity tests with sensitive species (Beketov et al. 2008).

The total insect abundance was recovered in the mesocosm study after 10 weeks, whereas no recovery was observed for insect taxon richness at 3.2 and 100 µg/L during 7 months of observation time. In addition, the monitored abundance and taxon richness of emerged insects were suppressed but had fully recovered 4 and 8 weeks after thiacloprid contamination. As in the present experiment, Chironomidae were severely affected by thiacloprid contamination, but in the mesocosm study they had fully recovered 10 weeks following the exposure. The fast recovery was attributed to the multivoltine lifestyle of the Chironomidae because, in contrast, uni- or semivoltine species did not recover during the same observation time (Beketov et al. 2008). Based on this fact it can be concluded that life-cycle characteristics are an important factor for recovery dynamics of single species after stressor occurrences (Beketov et al. 2008).

The present study, in accordance with the other mentioned results, demonstrates that *Chironomus riparius* can be regarded as a model organism that is very sensitive to thiacloprid. But for evaluation of further long-term risks on the macroinvertebrate community in the field, other information, such as life-cycle characteristics, spatial variation, and additional stressors, must be considered as well (Beketov and Liess 2008c; Beketov et al. 2008).

Since the lowest ecologically acceptable concentration of 1.54 µg/L thiacloprid found in a mesocosm study (Schmuck 2001) suggested a high environmental risk of thiacloprid to aquatic nontarget organisms, appropriate risk mitigation procedures concerning spray drift, also suggested by the manufacturer (Schmuck 2001), might be applied to protect nontarget species in aquatic systems.

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Kapitel 2: Can mouth part deformities of *Chironomus riparius* serve as indicators for water and sediment pollution? A laboratory approach

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Abstract

The significance of chironomids mouth part deformities as suitable indicators for pollutant contamination of natural waters and sediments has been investigated and discussed for several decades. Uncertainties still exist as further laboratory studies, with different pollutants and with the same experimental design are required. In this study, the effects of four substances (i.e. nickelchloride, chlorpyrifos, imidacloprid, and thiacloprid) were tested on the mouth part deformity rates and patterns in *Chironomus riparius*. These compounds were investigated either individually or in mixtures. No significant increase in the frequency of mouth part deformities was found using different single substance treatments when compared to the controls. Consequently no concentration-effect relationships between substance concentration and deformity frequency were detected. In mixture experiments an increase in mouth part deformities of *C. riparius* exposed to imidacloprid-thiacloprid mixtures was detected. This indicated that the effects of single substances and mixtures on mouth part deformity frequency may differ considerably. The findings in this study from different laboratory approaches in combination with the published literature questions the reliability of chironomids mouth part deformities as indicators of freshwater and sediment contamination by toxic substances.

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Keywords Chironomids, Mouth part deformities, Mixture effects, Pollutant indicator

Introduction

Mouthpart deformities of chironomids have been discussed as potentially suitable indicators for in situ bioassessment and biomonitoring of contamination stress in streams and lakes for decades (Bird 1994; Gerhardt et al. 2006; Groenendijk et al. 1998; Janssens De Bisthoven et al. 1998a; Meregalli et al. 2000; Warwick 1990). In numerous field studies a higher rate of chironomids with deformed mouthparts could be detected in polluted water bodies (Dermott 1991; Groenendijk et al. 1998; Janssens De Bisthoven et al. 1998a; Warwick 1990; Wiederholm 1984), possible reasons for these include contamination with radionuclides, oil compounds, Polycyclic aromatic hydrocarbons (PAH's), organochlorine pesticides, Polychlorinated biphenyls (PCBs) and especially heavy metals (Janssens de Bisthoven et al. 1998b). Although often the stressors and the increased frequency of mouth part deformities coincide spatially, a number of criticisms need to be made, as follows: (1) When an increased frequency of mouthpart deformities occurs, some studies fail to find a correlation between toxicants and the deformities (Bird 1994; Dermott 1991; Jeyasingham and Ling 1997; Jeyasingham and Ling 2000; Nazarova et al. 2004; Reynolds and Ferrington 2001).

(2) Due to the fact that many of the mentioned compounds have been found simultaneously in situ, no direct relationships could be established in most cases.

(3) In some unpolluted areas an increased rate of chironomids with deformed mouth parts could be found which varied seasonally (Jeyasingham and Ling 2000). Several studies suggest that the season does have a significant impact on the frequency of mouth part deformities (Jeyasingham and Ling 1997; Reynolds and Ferrington 2001; Servia et al. 2000; Urk et al. 1992). These results complicate, even after the long time of discussion, the usage of chironomid deformities as pollutant indicators. Therefore, several questions remain to be addressed in further laboratory studies.

The mechanisms by which the deformities are induced by pollutants are not yet well understood. To date the issue whether mouthpart deformities develop in the

endocrine-regulated moulting phase and if the disruption of this complex process is likely to occur at the basis of their ontogeny (Meregalli and Ollevier 2001) has not been resolved. Several studies have been conducted with endocrine disruptors which might induce deformities via interrupting the endocrine regulated moulting process. With β-sitosterol (5–500 µg/L) and 17α-ethynylestradiol (1–10 µg/L) no significant induction of deformation frequency was found, whereas in the presence of 4-*n*-nonylphenol (10–100 µg/L), lower 17α-ethynylestradiol concentrations (10 ng/L) and bisphenol A (10 ng/L) a significant increase in deformation frequency was induced (Meregalli and Ollevier 2001; Meregalli et al. 2001; Vermeulen et al. 2000a; Watts et al. 2003). Nonlinear concentration induction pattern has been observed before with endocrine disruptors and invertebrates (Matthiessen 2008; Oehlmann et al. 2000; Welshons et al. 2003), but more information is needed to understand the mechanism of the induction of mouthpart deformations in chironomids.

Additionally, field observations of polluted sites and the increased frequency of severe deformities could not easily be reproduced in laboratory experiments. Even in the cases were the examined pollutants were able to induce an elevated deformity rate, in many studies a linear exposure-response relationship between contaminants and deformities has yet to be found (Dias et al. 2008; Martinez et al. 2001; Vermeulen et al. 2000a). These observations are not reliable to support the rational application of chironomids deformities as pollutant indicators, especially when other stressors in the field might also generate deformities in chironomids.

In most published laboratory studies just a single stressor, in most cases a heavy metal, was used to induce mouth part deformities in chironomids (Bird et al. 1995; Janssens de Bisthoven et al. 2001; Janssens de Bisthoven et al. 1998b; Martinez et al. 2001; Vermeulen et al. 2000a). However, polluted areas are usually contaminated by more than just a single substance. Reviews on this subject emphasize the need to provide more consistent background data on the casual agents of chironomid deformation (Vermeulen et al. 2000a). Because no standardised protocol exists,

comparison of published results in terms of causal agents of deformation, is limited (Vermeulen et al. 2000a).

Therefore, the aim of this study was to investigate *Chironomus riparius* deformities after lifetime exposure to different kinds of pollutants using the same experimental design. Larvae of *C. riparius* were exposed to stressors with different modes of action. The organisms were exposed to single substances as well as binary mixtures. In the first set of experiments, the toxic metal nickel with its unspecific mode of action (affects protein integrity and function) and the widely used neurotoxic insecticide chlorpyrifos, which is acting as an acetylcholinesterase inhibitor, were chosen. In the second set of experiments, the pesticides imidacloprid and thiacloprid, both exhibiting the same mode of action as agonists of the nicotinic acetylcholine receptor, were tested in single and mixed exposures. The rationale for the choice of the selected mixture combinations is that effect information from different mixture scenarios is needed. In the first combination (nickel and chlorpyrifos) the substances aim at different target sides and show different mode of actions, whereas in the second combination (imidacloprid and thiacloprid) the two substances have the same target side and mode of action.

The specific questions addressed in this study are:

1) Can substances with different modes of action induce mouth part deformities in *C. riparius* at environmentally relevant concentrations?
2) Is there a concentration-response relationship between the substance concentration and the incidence of deformities?
3) Do single substances induce the same reactions, as when applied in mixtures?

Material and Methods

Maintenance of parent animals

Stock cultures of *C. riparius,* from different genetic sources, in order to avoid genetic impoverishment, (LimCo International, Germany; University of Joensuu, Finland and Universidade de Coimbra, Portugal), were kept as larvae in fine quartz sand and dechlorinated tap water under constant aeration. Every day the chironomid larvae were fed with finely grounded fish flakes (50% Tetramin, 50% Tetraphyll, Tetra, Germany). Declorinated tap water was exchanged one or two times per week. Before emergence occurred, a breeding cage (55 x 65 x 120 cm) was installed over the stock containers, in which the adults were allowed to fly, swarm and breed. The egg masses which were attached to the vessel wall were collected every morning and used subsequently for experiments. The stock breeding and all experiments were conducted in a climatized chamber at 21.0 ± 0.5°C, with a light-dark cycle of 16:8 h artificial daylight (Philips standard daylight 54765, 2500 lumen, Germany).

Exposure

After oviposition, eggs of *C. riparius* were exposed to different nominal concentrations of the toxic metal Nickel(II)chloride hexahydrate ($NiCl_2*6H_2O$, CAS 7791-20-0, Roth, 98,0%), and the insecticides chlorpyrifos (CAS 2921-88-2, Riedel de Haën , 99,2%), imidacloprid (CAS 138261-41-3,Riedel-de Haën, 99,9%), thiacloprid (CAS 111988-49-9, Riedel de Haën, 99,9%) and also to their combination (for detailed list of tested concentrations see Tables 1 and Table 2). Control treatments were conducted with declorinated tap water. Mixtures were prepared using the toxic unit concept with a two-ratio design. In the first ratio one the toxic unit level was dominated by chemical 1 (TU-level 2/3 chemical 2, 1/3 chemical 2), whereas the second ratio the TU-Level was dominated by chemical 2 (TU-level 1/3 chemical 1, 2/3 chemical 2).

Experimental setup

Three or four replicate experiments were performed for each concentration level. After three days, 30 larvae in the first larval stage (L1) were transferred to 250 ml glass beakers containing declorinated tap water spiked with the respective toxic substance and spiked quartz sediments (particle size 0.1–0.3 mm), which were burned for 3 h at 500°C (Dehner, Germany). For spiking, one day before larvae introduction the sediment was covered with 200 ml of the respective test solution and was subsequently shaken for 24 h under exclusion of light. Chironomids were fed every day with powdered ground fish flakes (1:1 Tetraphyll, Tetramin) providing a food supply of≥ 0.36 mg/day/larvae. Surviving larvae were weekly transferred to new beakers containing freshly spiked sand and test solution. Glass beakers were gently swayed until the larvae came to the surface and the remaining sand was rinsed in water to find residual larvae. Larvae that did neither move nor respond to stimulation with a pipette were considered dead. The mortality rate and behavioural activity was recorded during the procedure of sand exchange for the third instar larvae (L3) and for the fourth instar larvae (L4), 10 and 17 days respectively, after oviposition. Number of emerged *C. riparius* and 'time till emergence' was recorded.

Analysis of mouthpart deformation

After the chironomids started to emerge, the remaining exuviae of L4 larva with head capsules were collected and stored in 100% ethanol. Since not hundred per cent of the exuviae's could be recovered the number of examined mouth parts cannot be equalised the number of emerged chironomids. One day prior to the morphological preparation, the head capsules were separated mechanically from the rest of the exuviae and stored in Rotihistol overnight (Carl Roth GmbH, Germany). The next day, the head capsules were mounted on a glass slide in Roti-Histokit (Carl Roth GmbH, Germany) with the ventral side up, then covered with a glass cover slip and squeezed gently. The strongly sclerotinised mentum and mandibles were evaluated through observation with a light microscope at 40x magnification (Zeiss Axiostar

plus). Missing teeth, extra teeth, mentum split medial teeth and Köhn gaps were counted as deformities (Bird 1994; Gerhardt and Janssens de Bisthoven 1995; Servia et al. 1998). Special care was taken to distinguish deformities from physical wearing. The ratio of individuals with deformed mouthparts to the total number of examined individuals was calculated (Hämäläinen 1999). Two approaches were used for the interpretation of calculated ratios. Firstly, the total deformity rate was calculated using the total number of individuals with deformed mouthparts to the total of examined individuals. Secondly, the different deformity types were set in relation to the total number of examined individuals.

Statistics

Due to the non-normal distribution of some data sets, all data were analysed using non- parametric statistics. Significance was tested 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.

Results

In all control and exposure treatments, deformities of the mandibles and the mentum did occur at rates between 0 and 33% (see Tab. 1 and 2). In most cases, minor deformities such as missing or extra teeth occurred. Severe deformities (Köhn gaps) were detected only in two cases (at different treatments). Missing teeth in the mentum or the mandibles were the most abundant types of deformities found.

In the treatment with increasing nickelchloride concentrations (e.g. 100, 1000, 2500 and 5000 µg NiCl/L) no significant differences were found between the control and the different nickel treatments, neither in the total deformity rates nor in the rates of the specific deformations (see Tab. 1). Consequently, no concentration-response relationship could be detected. No significant difference was observed in the total deformity or specific deformity rates between the control treatments and the lowest chlorpyrifos concentration (1 µg/L). At higher chlorpyrifos concentrations, no *C.*

riparius individuals survived into the pupal stage (following L4 larvae stage), therefore no L4 exuvia were available for mouth deformity analysis. For this reason, in the mixture experiments low chlorpyrifos concentrations were combined with those of nickelchloride (see Tab. 1).

Tab. 1: Data from exposure experiments with nickel and/or chlorpyrifos. The numbers of examined *C. riparius* mouthparts, the frequency of total deformities as well as the percentage of the different deformity types are displayed. The exposure lasted over the entire span of larval live (28 d).

Substance 1: Nickel [µg/L]	Substance 2: chlorpyrifos [µg/L]	No. of replicates	No. of examined individuals	Total deformities [%]	Missing teeth [%]	Extra teeth [%]	Mentum split medial teeth [%]	Köhn gap [%]
0		3	56	7.14	7.14	0	0	0
100.00		3	38	15.79	13.16	0	0	2.63
1000.00		3	66	7.58	7.58	0	0	0
2500.00		3	13	0	0	0	0	0
5000.00		3	5	0	0	0	0	0
10 000.00		3	0	-	-	-	-	-
	0	3	51	7.84	5.88	0	1.96	0
	1.00	3	15	6.67	6.67	0	0	0
	5.00	3	0	-	-	-	-	-
	10.00	3	0	-	-	-	-	-
	50.00	3	0	-	-	-	-	-
0	0	6	143	9.79	9.09	0.70	0	0
130.00	0.033	3	24	12.50	8.33	4.17	0	0
130.00	0.13	3	33	3.03	0	0	0	0
270.00	0.066	3	13	15.38	0	0	15.38	0
330.00	0.33	3	46	8.70	0	0	0	0
670.00	0.165	3	51	0	0	0	0	0
1340.00	0.33	3	20	10.00	0	0	0	0
2000.00	10	3	23	-	-	-	-	-

In these mixtures, no significant differences in the total or specific deformity rates between the control treatments and the different mixture levels could be detected. Thus, from these experiments no evidence for a dose response relationship between the stressors and the deformity rate was found.

Also in the thiacloprid treatment (0.1, 0.5 and 1 µg/L) no significant difference in the total or a specific deformity rate between the control and the different thiacloprid treatments was found (see Tab. 2). At higher thiacloprid concentrations *C. riparius* larvae died before reaching the pupal stage. Therefore no mouth parts were available

for analysis at high toxic substance concentrations. Also, in the imidacloprid treatments with 0.1, 0.5 and 1 µg/L, no significant difference in the total or specific deformity rates between the control treatments and the different thiacloprid levels was detected (see Tab. 2). At higher imidacloprid concentrations *C riparius* larvae died again before reaching the pupal stage.

In binary mixtures of thiacloprid and imidacloprid a significant increase of larvae with mentum split medial teeth was found in three experimental conditions: (1) 0.83 µg/L thiacloprid –0.83 µg/L imidacloprid, (2) 0.416 µg/L thiacloprid –1.66 µg/L imidacloprid (3) 0.166 µg/L thiacloprid -0.66µg/L imidacloprid (see Tab. 2 and Fig. 1). The pattern of mentum split and medial teeth formation could be best explained by assuming a model of concentration addition (Jonker et al. 2005). It was noted that in all thiacloprid-imidacloprid mixtures high percentages of deformities occurred, whereas this was not observed when organisms were exposed to single pesticide conditions at sublethal concentrations.

Discussion

In this study, a high total deformity rate (between 7 and 17%, mean ± SD: 11.37% ± 4.14%) was found in the control treatments. This high percentage of mouthpart deformities in the control is not unusual for *C. riparius*, a chironomid species which naturally shows a high percentage of deformities (Reynolds and Ferrington 2001; Servia et al. 2000). In other laboratory studies the deformity rate in the controls ranged between 7 and 34.1% (Janssens de Bisthoven et al. 1998b; Meregalli and Ollevier 2001; Meregalli et al. 2001; Vermeulen et al. 2000a). As a possible explanation they assumed that the high deformity rate in the controls had been due to a high degree of inbreeding in a laboratory culture. In this study, this factor was tried to be avoided, as the founding individuals of our culture originated from three different stocks of *C. riparius*. Additionally, the cultures were refreshed with new genetic material regularly. It has been suspected before that filter tissue used as substrate in some assays (Meregalli et al. 2001) or diatomaceous earth may impact the frequency

of deformity in chironomids. Therefore in this experiment quartz sediment, free of possible organic contaminations, was used.

Tab. 2: Data from exposure experiments with thiacloprid and/or imidacloprid. The numbers of examined mouthparts from *C. riparius*, the frequency of total deformities as well as the percentage of different deformity types are displayed. The exposure lasted over the entire span of larval live. Significant differences to the control treatments are indicated in bold face (Wilcoxon test $p \leq 0.05$).

Substance 1: Thiacloprid [µg/L]	Substance 2: Imidacloprid [µg/L]	rep	No examined N	Total deformities [%]	Missing teeth [%]	Extra teeth [%]	Mentum split medial teeth [%]	Köhn gap [%]
0		4	89	16.85	8.99	6.74	1.12	0
0.10		4	89	19.10	16.85	3.37	0	0
0.50		4	74	9.46	2.70	5.41	1.35	0
1.00		4	65	18.46	6.15	1.54	13.31	0
2.50		4	0	-	-	-	-	-
5.00		4	0	-	-	-	-	-
10.00		4	0	-	-	-	-	-
	0	4	97	12.37	0	9.28	3.09	0
	0.10	4	85	9.41	1.18	3.53	4.71	0
	0.50	4	83	18.07	4.82	9.64	3.61	0
	1.00	4	27	11.11	0	7,41	3.70	0
	2.50	4	0	-	-	-	-	-
	5.00	4	0	-	-	-	-	-
	10.00	4	0	-	-	-	-	-
0	0	8	191	15.70	10.47	4.71	1.05	0.52
0.08	0.33	4	55	20.00	1.82	16.36	1.82	0
0.83	0.83	4	70	27.14	0	4.29	**22.86**	0
0.17	0.66	4	59	22.03	6.78	3.39	**15.56**	0
0.17	0.17	4	36	19.44	11.11	8.33	0	0
0.33	0.33	4	52	11.54	3.85	5.77	1.92	0
0.42	1.66	4	54	**33.00**	0	3.70	**33.33**	0
0.83	3.33	4	0	-	-	-	-	-
1.66	1.66	4	0	-	-	-	-	-
1.66	6.67	4	0	-	-	-	-	-

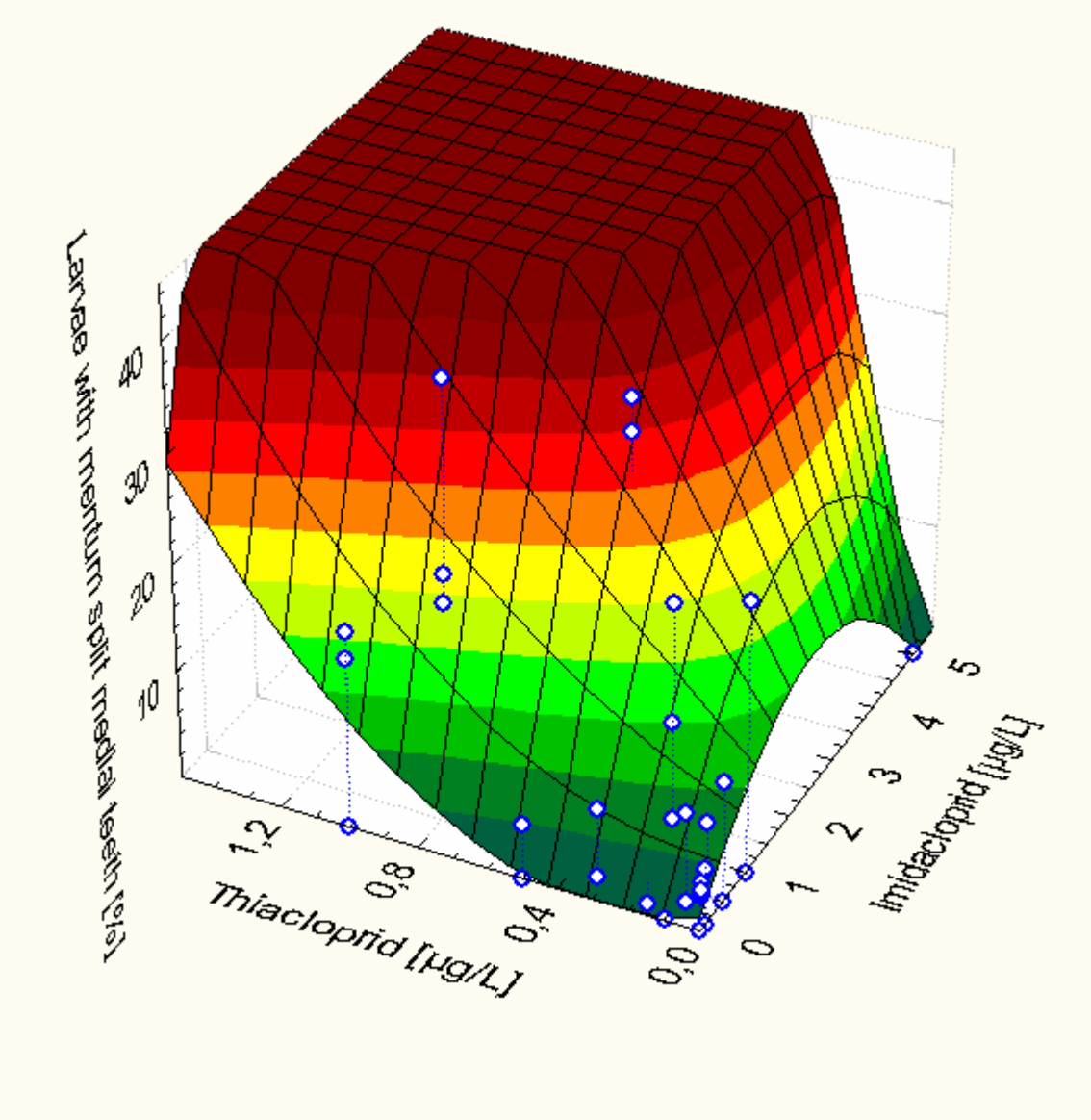

Fig. 1: Three-dimensional diagram (surface plot with isobolic lines calculated on the basis of means) of the number of *C. riparius* larvae with mentum split medial teeth after being exposed to different concentrations of thiacloprid and/or imidacloprid during the whole larval stage.

Therefore, the first approach to induce mouth part deformities in *C. riparius* with substances exhibiting different modes of action was not successful. Hence, it is self-evident that also the second (establishing concentration response relationship between of the substance concentration and deformity incidences) in this study could not be verified. The results of the present study are in good agreement with those of other studies which showed that significant increases in the frequency of mouth part

deformities could not be induced in chironomids by exposure to substances like azaarenes, 17 α ethynylestradiol, lead, mercury, β-sisterol (Bleeker et al. 1999; Meregalli and Ollevier 2001; Vermeulen et al. 2000a). Additionally, a linear concentration response which would simplify the quantification of pollutants in sediment or water, has not yet been found (Dias et al. 2008; Kwak and Lee 2005; Martinez et al. 2001; Martinez et al. 2003; Watts et al. 2003). As well, it would be very desirable to find a connection between a specific deformity pattern and a special pollutant for the further use of deformities as indicators of water contamination. Only two studies have provided evidence for such a correlation (Dickman and Rygiel 1996; Vermeulen et al. 1998). These studies found different mouthparts which seemed to react independently to pollution stress, and which displayed specific deformation frequency profiles (Vermeulen et al. 1998). Even though we tested only a limited number of chemicals we did not observe such a corresponding pattern with specific substance exposure, as well as in other recent studies.

For nickel, the absence of deformities was unexpected due to the fact that this is a widely distributed heavy metal. Heavy metals have been investigated in connection with deformity induction, both in the laboratory and the field (Bird et al. 1995; Janssens de Bisthoven et al. 2001; Janssens de Bisthoven et al. 1998b; Vedamanikam and Shazili 2009), (Bhattacharyay et al. 2005; Bird et al. 1995; Dickman and Rygiel 1996; Gerhardt and Bisthoven 1995; Gerhardt et al. 2006; Groenendijk et al. 1998). Nickel is toxic to chironomids affecting their survival rate (LC_{50} -20 days: 200 µg/L), behavioural activity (lowest observed effect concentration (LOEC) 2500 µg/L), as well as their emergence rate (LOEC 2500 µg/L) (Gerhardt and Janssens de Bisthoven 2006). But although nickel in the tested concentration range is obviously toxic to *C. riparius*, no significant increase in the frequency of mouth part deformation was found, proving mouth part deformities to be a less sensitive parameter than e.g. behaviour or emergence. The higher tested nickel concentrations are above the environmentally relevant levels, with an exception of severely contaminated sited, where nickel concentrations can range between 50 and 2000 µg/L in waters near

industrial sites and 183 000 µg/L near a nickel refinery (Chau and Kulikovsky-Cordeiro 1995; Kasprzak 1987).

The induction of mentum deformities by pesticides was investigated to a lesser extent (Fowlkes et al. 2003; Madden et al. 1992). Only one study (Madden et al. 1992) found a significant concentration-response relationship between DDT and abnormal mentum. In the present study no significant induction or dose response was found in the mouth deformity rate of *C. riparius* exposed to the neurotoxic insecticide chlorpyrifos. Chlorpyrifos is a broad-spectrum organophosphorus insecticide (Richardson 1995) which is one of the most common active compounds in commercial pest control products worldwide (Dow AgroSciences 2008). Environmental chlorpyrifos concentrations of 0.19–0.3 µg/L were detected in urban waterways in California and surface waters in the USA (Bailey et al. 2000; Gilliom et al. 2006). In sediments a maximum concentration of 924 µg/kg chlorpyrifos was detected after a single rainstorm event in the Lourens River, South Africa (Schulz 2001). 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; Lydy et al. 1999; Moore et al. 1998). However, mouth part defomity quantification was not the objective of these works. As it was expected from previous study, chlorpyrifos was highly toxic to *C. riparius* affecting the survival rate (LOEC 5 µg/L; LC_{50}-16 days: 5 µg/L, LC_{50}-29 days: 2.5 µg/L). Only at 1 µg/L chlorpyrifos, the chironomids survived longer than eleven days. In this chase no impairment of behavioural activity, growth, emergence rate, and mouthpart deformity rate could be observed (Gerhardt and Janssens de Bisthoven 2006).

When being exposed to the insecticides thiacloprid or imidacloprid, larvae of *C. riparius* did not show significant changes or concentration-response in mouth deformity rates. Thiacloprid is highly toxic to *C. riparius* affecting the survival rate (LOEC 0.5 µg/L), behavioural activity (LOEC 1 µg/L), the relative Hsp70 level (1 µg/L) and the total emergence rate (L0EC 0.5 µg/L) (Langer-Jaesrich et al. 2010). Imidacloprid is slightly less toxic, affecting the survival rate (LOEC 1 µg/L),

behavioural activity (LOEC 5 µg/L) and the total emergence rate (L0EC 1 µg/L) (Langer-Jaesrich, unpublished data). Both substances are members of a very successful new insecticide group called neonicotinoids (Maienfisch et al. 2003), which have been the fastest-growing class of insecticides in modern crop protection in recent years (Jeschke and Nauen 2008). Information about measured imidacloprid and thiacloprid concentrations in the environment are rare. However, in their pesticide surface water report, Pfeuffer and Matson (2001) - cited in Jemec (2007) - mentioned imidacloprid concentrations between 1 µg/L and 14 µg/L in water systems, USA. In a study by Süß et al. (2006), thiacloprid concentrations of 4.5 µg/L were detected in a surface water system near apple orchards in the surroundings of Hamburg, Germany.

The number of examined mouth parts decreased due to an increasing mortality at higher concentration levels of the toxic substances tested. Because of this, data concerning the induction of mouth deformation by a particular toxic substance condition should be considered with care. This was the reason why Vermeulen et al. (2000a) postulated that a standard protocol should yield an adequate number of larvae to been screened at the end of a toxicology assay. It is of equal importance to observe the induction of deformities over the whole toxic substance concentration range under otherwise identical conditions, in order to quantify concentration-response relationships. Therefore, Vermeulen et al. (2000a) did also postulate that a wide range concentration gradients are needed to obtain significant regression equation parameters. Because of this large concentration range required, a reduced number of surviving larvae at higher concentrations of tested substances had to be accepted in this study.

Using the experimental approach described previously it was not possible to see whether the deformity rate of dead larvae was higher than that of surviving larvae, due to the fact that, even under laboratory conditions, the dead larvae disintegrated within 2 days. Under natural conditions it is likely that dead larvae even disintegrate much faster due to scavengers and microbiological activity. Therefore, it seems to be

likely that under field conditions the most sensitive chironomids die and, consequently, fewer and less sensitive animals remain to show deformities.

The use of exuviae from the L4 stage in this study led to the advantage that the chironomids could complete their life cycle. This made it possible to compare mouth part deformity rates with other accepted endpoints like emergence rates and developmental times (OECD 2004a; OECD 2004b). In this study, it was found inappropriate to include the mouth parts from exuviae of younger larvae mainly because they were difficult to recover in reliable numbers. Additionally Vermeulen et al. (2000b) observed that older larvae displayed a higher frequency of deformities than younger larvae. Most deformities in the Vermeulen study were transferred identically from the L3 to the L4 stadium in an identical shape during moulting. In a very small number of individuals it was shown that a few deformities did not occur again in the L4 stadium. This provides evidence that deformities can be reversible.

The third goal of this study was to test whether single substances are able to induce the same mouth part deformations as induced by substance mixtures. This is a fundamental question concerning freshwater systems and sediments because many different kinds of pollutants are continuously being introduced into the environment. Several studies in the field have suggested that deformity rates of chironomids can be used as bioindicators for pollutants in aquatic systems (Madden et al. 1992; Meregalli et al. 2000; Servia et al. 1998). However, only single substance tests have been conducted in the laboratory to assess the impact of these substances in nature. In a single field study the concentrations of different toxic substances have been summed up (simulating toxicant additivity) and normalized to the content of organic matter and clay (Meregalli et al. 2000). However this application does not take into account synergistic or antagonistic deviations from the common concepts of concentration addition or independent action (Jonker et al. 2005) where a synergistic deviation would be a more toxic reaction than expected from the concentration addition concept and an antagonistic deviation would correspond to a less toxic reaction than the independent action concept predicts (Escher and Hermens 2002).

In the present study no significant change or dose response in mouth deformities of *C. riparius* exposed to mixtures of nickel and chlorpyrifos was found (see Tab. 1). The survival rate, however, was significantly decreased (Gerhardt and Janssens de Bisthoven 2006). Chironomids exposed to mixtures of imidacloprid and thiacloprid showed a partially increased rate of mouth part deformities (e.g. mentum split medial teeth) when compared to the control treatments (see Tab. 2 and Fig.1). This was unexpected since both substances exhibited the same mode of action and did not induce deformities in single substance exposure experiments, at the same total concentration range. These differences in mouth part deformity induction after exposure to single substances or pollutant mixtures renders unreliable the potential application of mouth part deformities as bioindicators for pollutants.

In summary, the results of the present study show that not all toxicants (including heavy metals and pesticides) are able to induce mouthpart deformities in chironomids, even when these groups have been reported to correlate with these abnormalities (Bhattacharyay et al. 2005; Madden et al. 1992; Vermeulen et al. 2000a). Compared to mouthpart deformity rates other frequently tested endpoints such as activity changes, total emergence rate, and survival rate were more sensitive and consistently showed a concentration-dependence for all of the substances (Gerhardt and Janssens de Bisthoven 2006; Langer-Jaesrich et al. 2010, Langer-Jaesrich, unpublished data). Because of this and the mentioned absence of inducible deformities in most of the experiments in this study, it has to be questioned whether mouth part deformities reflect the health condition of *C. riparius* adequately, and whether these abnormalities can be used as reliable bioindicators of pollution. Furthermore, the often observed nonlinear concentration response and the different response to mixtures and single substances hamper the application of the deformities as bioindicators under laboratory conditions.

In nature a different situation of even higher complexity may occur due to the fact that chironomids are exposed to pollutants over several generations and have the time to adapt (Postma and Davids 1995). Other additional stressors and seasonally

fluctuating stress might complicate the interpretation of deformity data in nature. In the few published multi-generation studies investigating deformity rates (Janssens de Bisthoven et al. 2001; Janssens de Bisthoven et al. 1998b), deformity rates fluctuate and complicate interpretations. Furthermore another challenge will be to extend the existing knowledge on whether mouthpart deformities in a given larval stage could induce similar deformities in the subsequent stage or whether they can be reversed by the moulting process (Vermeulen et al. 2000b).

The findings in this study form different laboratory approaches in combination with the published literature questions the reliability of chironomids mouth part deformities as indicators of freshwater and sediment contamination by toxic substances.

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Kapitel 3 Impairment of trophic interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) by chlorpyrifos

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Abstract

The effects of chemicals on biotic interactions, such as competition and predation, have rarely been investigated in aquatic ecotoxicology. This study presents a new approach for the investigation of predator-prey interactions between zebrafish (*Danio rerio*) and midge larvae (*Chironomus riparius*) impaired by chlorpyrifos (CHP), 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 by visual observation an increase in the feeding rate of zebrafish preying on exposed chironomids after acute (2 h) exposure to 6 µg/L CHP. Previously, 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 therefore propose the use of trophic interactions as parameters in

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higher tier studies for chemical testing and evaluation of ecotoxicological risk assessment.

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

Introduction

Behavioural responses often occur rapidly after exposure to environmental pollutants and represent a sensitive indicator of the influence of pollutants on non target organisms, this being the basis for the rather new branch of behavioural ecotoxicology (Gerhardt 2007). Moreover, pollutant-induced alterations in behaviour affect not only individuals, but also the viability of populations and consequential the structure of ecosystems (Dell`Omo 2002). Up to now, the majority of ecotoxicological studies have focused on the direct effect of pollutants on organisms; although indirect effects, such as an impairment of inter- and intraspecific interactions, are also likely consequences of an exposure event. An ecologically important example of interactions in this context is the predator-prey relationship, as food web interactions influence not only the structure of the populations of both directly involved species but might also affect ecosystem functioning (Townsend et al. 2003). According to the optimal foraging theory (Begon et al. 2006), predators exert specific behaviours to detect (searching behaviour) and hunt (handling behaviour) prey organisms, whereas the prey develop predator avoidance behaviours in co-evolution.

Since the predator prey relationship represents an important biotic interaction it therefore may be susceptible to pollutant exposure. To date, studies of predator-prey interactions in aquatic ecosystems 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). 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).

However, 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. As proposed by Lima (2002), important conclusions about ecological consequences can only be drawn if predators and prey are regarded both equally in the investigation of predator-prey interactions. This approach has been applied in only a few field and laboratory studies with aquatic invertebrates, amphibians and fish (Bridges 1999; Gómez et al. 1997, Rahel and Stein 1988; Taylor et al. 1995; Thorp and Bergey 1981).

In our study we included both, predator (*Danio rerio*) and prey (*Chironomus riparius*), to investigate the indirect effect of a pollution scenario (pulse exposure of chlorpyrifos) on the predator prey relationship. To differentiate between effects on predator or prey we used 3 different exposure scenarios: Predator exposed, prey exposed and both predator and prey exposed. A similar exposure design was conducted by Gomez et al. (1997), using different rotifer species. An important difference to this setup is that in our experiment the prey organisms had the opportunity to avoid the predators via burrowing, providing a higher ecological relevance.

As "model" predator we chose the zebrafish (*Danio rerio*), which naturally occurs in stream habitats rich in macrophytes in South East Asia (Börries 2006). Ecologically, fish represent a very important group of secondary consumers or even top predators.

Our "model" prey organisms were 4th instar larvae of the non-biting midge *Chironomus riparius*. This organism was chosen because of its ecological importance as food item for fish (Pinder 1986). Chironomids have been used as prey objects for *D. rerio* in many studies and husbandry instructions (Béchard et al. 2008; Lawrence 2007; Nyholm et al. 2008). Additionally as sediment-dwelling organisms, they are particularly susceptible to sediment bound pollutants. There is evidence that chironomids are reacting actively to the presence of predators. For example, studies showed that larvae of *C. riparius* burrowed significantly deeper when exposed to fish kairomones, simulating increasing predator density by *Rutilus rutilus* (Hölker and Stief 2005). A predatory damselfly, showing visual orientation like 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, 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).

Studies so far mainly investigated the effects of chlorpyrifos alone and in mixtures on the acute toxicity to *Chironomus 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 both invertebrates and for fish.

In the present study the following hypotheses were tested:

1. Exposed chironomids burrow less than control animals, and are therefore more susceptible to predation by fish.
2. Predation by fish stimulates increased burrowing behaviour in exposed as well as control chironomids.
3. When exposing predator and prey, the decreased success of the predator to prey and of the prey to burrow are outweighed, resulting in insignificant differences in feeding rate compared to the control.

Materials and Methods

In the following experiment zebrafish *Danio rerio* were used as predators, and 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.

Animal culture and maintenance of Chironomus riparius

Egg ropes of *C. riparius* have been collected from a breeding stock at 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; 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) were 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.

Animal culture and maintenance of Danio rerio

The 4-6 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 during that time with living *C. riparius* larvae several times before the start of the experiment.

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. In order to simulate possible pulse pollution concentrations, 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. 2009).

Experimental design of preliminary tests

First, the burrowing behaviour of *C. riparius* in the L4 stage has been observed (unpublished data) as follows: The natural burrowing behaviour of *C. riparius* L4

larvae was assessed in three replicate treatments with 50 chironomids each. The numbers of totally visible and partly visible larvae were counted manually every 20 minutes. Due to those experiments, a 2 hour period was determined as the adequate time for healthy *C. riparius* to dig entirely into the sediment and to show natural behaviour. Second, the recapture rate of 100 *C. riparius* L4 larvae burrowed in quartz sediment was observed (replicated 9 times). The recapture rate was 97.2%. Third, 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. Therefore in the main experiment we chose 100 chironomids as an adequate number for the predator-prey experiments.

Experimental design of main experiments

In this study, three different treatments and one negative control were 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 predators and 100 *C. riparius* as prey were introduced. A total of 100 chironomids per replicate were collected randomly and transferred for exposure into large glass dishes (15 cm diameter, depth 8 cm) containing 50 g of quartz sediment and the corresponding chlorpyrifos solution made from dechlorinated tap water. After 2 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 dechlorinated tap water and distilled water to obtain a conductivity of 400-450 µS/cm, optimal for *D. rerio* cultivation. During the transfer of the test organisms into the feeding aquaria special attention was paid to ensure that neither contaminated sediment nor water

were transferred. In the two hours following the transfer the chironomids had the opportunity to burrow into the sediment.

Meanwhile, 5 *D. rerio* per replicate were transferred into 4 L aquaria containing 3 L of the respective chlorpyrifos solution or control water. All aquaria were 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 h periods, the number of chironomids completely at the surface and those partly visible was re-counted. Then, the fish were removed and anaesthetised with benzocain (3-5 ml of 40 mg Bencocain/ml Aceton). The total length of each fish was measured with a sliding calliper (powerfix EMC, model number Z11155, resolution 0.01 mm). Subsequently, surviving chironomids were searched and counted in the 10 L aquaria and the sediment.

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.

Results

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

Tab. 1. Total length of *D. rerio* (mm) (mean ± SD). No significant difference was found in *D. rerio* size between the different treatments. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated.

	Treatment			
	control	**Cc**	**Dc**	**Bc**
Mean	28.42	27.60	28.34	27.36
SD	3.47	4.13	4.60	3.64

At a nominal 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). 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 vs Dc) or exposed chironomids (Cc vs Bc), respectively. After the introduction of zebrafish, 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).

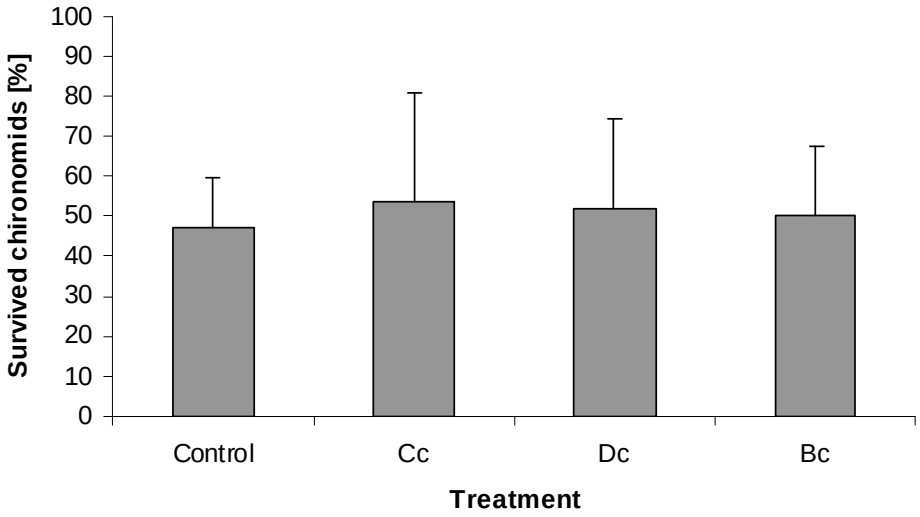

Fig. 1. Feeding rate [%] of *D. rerio* on larval chironomids after two hours. Fish and/or chironomids were exposed to 1 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. n = 3,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

At a nominal 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). 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 vs Dc) or exposed chironomids (Cc vs Bc), respectively. After the introduction of zebrafish, 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).

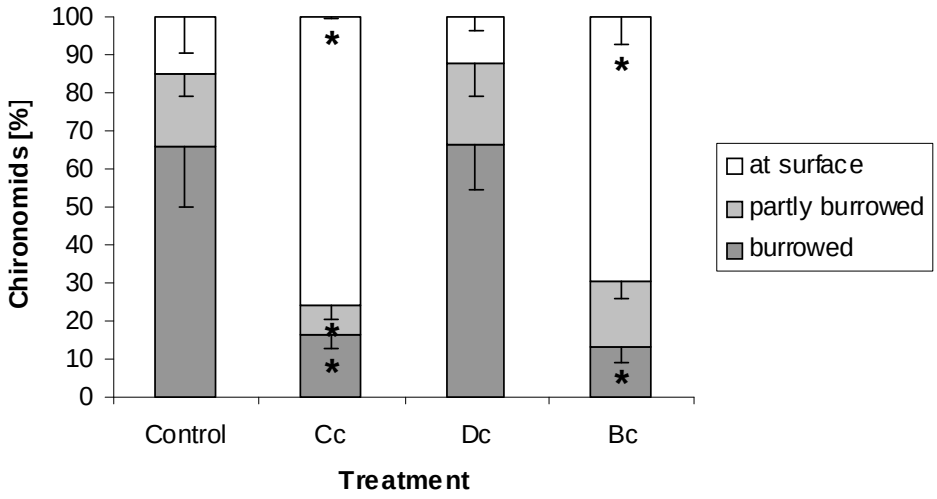

Fig. 2. Percentage of chironomids at the sediment surface, partly burrowed and totally burrowed before introducing the zebrafish. Fish and/or chironomids were exposed to 6 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc**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

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).

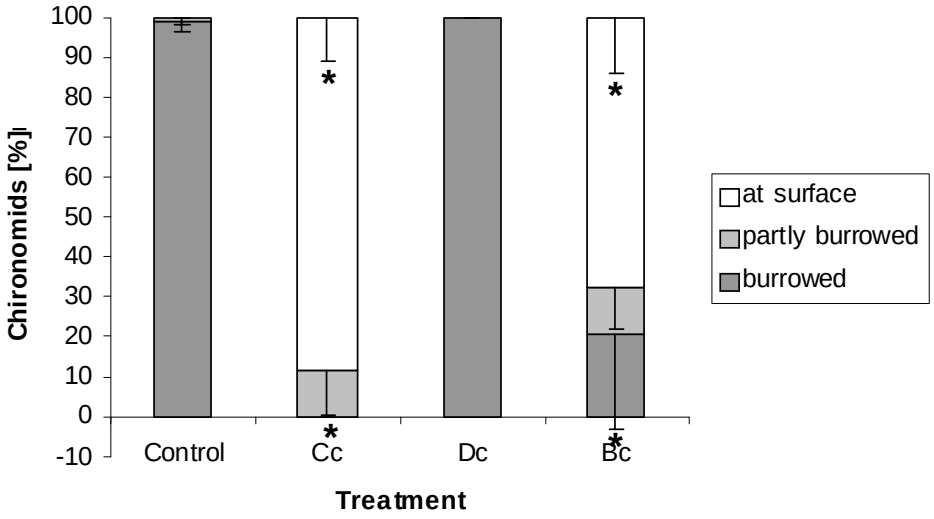

Fig. 3. Percentage of chironomids at the surface, partly burrowed and totally burrowed after introducing zebrafish. Fish and/or chironomids were exposed to 6 µg/L CHP prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids)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

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. For example it is known that 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. 2009; Levin et al. 2003; Levin et al. 2004; Roex et al. 2002; Scheil and Köhler 2009), where effectively imparing concentrations were 10 µg/L for locomotor activity and 250 µg/L for morphological abnormalities (Kienle et al. 2009). For adult freshwater fish, 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).

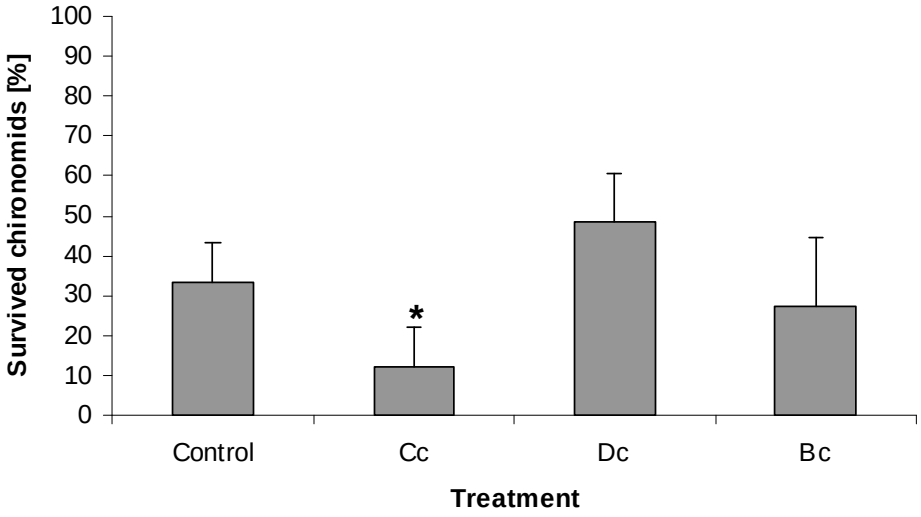

Fig. 4. Feeding rate [%] of *Danio rerio* on larval chironomids after two hours. Fish and/or chironomids were exposed to 6 µg/L CHP for 2 h prior to the feeding trials. Treatments: **Cc** *C. riparius* contaminated, **Dc** *D. rerio* contaminated, **Bc** Both (fish and chironomids) contaminated. * Significantly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

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). In the present study, the observed effects on predator prey relationship between zebrafish and chironomids can presumably be attributed to the neurotoxic mode of action of chlorpyrifos resulting in behavioural changes.

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. The main exposure route for aquatic ecosystems is pesticide 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 pulse exposures both at low and high doses. 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, which indicates, that CHP rapidly binds to sediment and therefore might pose a high risk for sediment inhabiting organisms such as chironomids as well as for organisms feeding on them.

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 in our study. 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 (pulse pollution). 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. 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 escaped from the presence of fish and survived. In the treatment with only zebrafish 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 having been exposed to 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 might be interpreted as a result of a reduced ability to burrow presumably due to increased convulsions caused by the effects of chlorpyrifos on the nervous system. 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 confirmed 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 for Chironomids in our study is an order of magnitude higher compared to 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 (96 h 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 (LOEC after 5 d exposure, Kienle et al. 2009) and chronic effects on response latency and spatial discrimination of adult zebrafish occurred after early life stage exposure to 0.1 µg/L chlorpyrifos for 5 days (Levin et al. 2003). Our effective concentration is again one order of magnitude higher than the one mentioned above, but with a much shorter exposure time, which might explain the deviation of the effect concentrations. Another possible reason might be the differences in the experimental setup and the addressed endpoints (response latency and spatial discrimination vs feeding rate). 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 even on ecosystem function. Our results suggest that simple single species ecotoxicity tests do not reflect adequately potential effects of a toxin on ecosystem structure and function. Up to now the relevance of predator-prey interactions has not been considered in chemical risk assessment, with the exception of sporadically conducted mesocosm studies. Our study shows the relevance of the mentioned problem and also proposes a simple method to quantify the effects of a toxic compound, CHP, on interactions between predator and prey organisms.

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