Interpretation and extrapolation of ecological responses in model ecosystems stressed with non-persistent insecticides

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Interpretation and extrapolation of ecological responses in model ecosystems stressed with non-persistent insecticides

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Interpretation and extrapolation of ecological responses in model ecosystems

Samenvatting

In de aquatische ecotoxicologie worden modelecosystemen, ook wel micro- of mesocosms genoemd, regelmatig gebruikt als onderzoeksinstrument. Vergeleken met natuurlijke ecosystemen worden deze testsystemen gekarakteriseerd door een reductie in grootte en ecologische complexiteit. Ondanks die beperkingen bevatten ze – eigenlijk net zo als in een goed functionerend aquarium - wel een levensgemeenschap die de verschillende niveaus in de voedselketen vertegenwoordigen (zoals predatoren, prooien en waterplanten en/of algen). Die levensgemeenschap is bovendien min of meer in evenwicht met haar omgeving.

Toelatingsprocedures voor bestrijdingsmiddelen (o.a. in de EU) vereisen dat toelatingshouders de potentiële ecologische risico's van hun producten aangeven. Hiervoor wordt vaak een getrapt risico-evaluatiesysteem gebruikt. In het geval van aquatische ecosystemen worden voor de eerste trap gestandariseerde acute en chronische laboratorium-toxiciteitstoetsen uitgevoerd met vissen, ongewervelden (o.a. watervlooien) en planten (o.a. algen en kroos). Vervolgens wordt door middel van veiligheidsfactoren een kritische drempelwaarde vastgesteld en gekeken of verwachte concentraties van het middel al dan niet boven deze ecologisch veilig beschouwde concentratie liggen. Als uit deze eerste evaluatie blijkt dat er mogelijk risico's voor het aquatisch milieu zijn, dan kan men die risico's verder evalueren met vervolgonderzoek (hogere trap onderzoek). Kenmerkend voor hogere trap risico-evaluaties is dat er meer gegevens worden aangeleverd met betrekking tot de risico's en dat potentiële effecten onder meer realistische omstandigheden worden onderzocht. Deze nieuwe informatie kan dan gebruikt worden in de verdere afwikkeling van de toelatingsprocedure.

Binnen de getrapte benadering kunnen micro- en mesocosmstudies tot de complexere mogelijkheden van vervolgonderzoek gerekend worden. Het voordeel van dit type studies is de mogelijkheid om realistische blootstellingsregimes van het te onderzoeken middel te integreren met de analyse van effecten op hogere biologische organisatieniveaus en om interacties tussen soorten en indirecte effecten te bestuderen. Verder is het ook mogelijk om herstel van populaties en levensgemeenschappen te onderzoeken.

Ondanks dat modelecosysteemstudies een belangrijke rol kunnen spelen in de toelatingsprocedure van bestrijdingsmiddelen blijft de vraag of de uitkomsten van deze studies representatief zijn voor andere locaties en tijdstippen. Met dit proefschrift wil ik daarom ingaan op de vraag of de resultaten van micro- en mesocosmstudies reproduceerbaar zijn en kunnen bijdragen aan een degelijke risicoevaluatie van bestrijdingsmiddelen. Om die reden worden de resultaten van verschillende micro- en mesocosm studies die uitgevoerd zijn met de insecticiden chloorpyrifos (organofosfaat) en lambda-cyhalothrin (pyrethroïde), en die tevens verschillen in

experimentele condities, gepresenteerd en vergeleken. Deze studies worden vervolgens in het kader geplaatst van andere experimenten die uitgevoerd zijn met vergelijkbare insecticiden om daarmee inzicht te krijgen in de consistentie van de uitkomsten van micro- en mesocosmstudies.

In Hoofdstuk 2 wordt een experiment gepresenteerd met als doelstelling de voorspellende waarde van (gestandariseerde) laboratoriumtoxiciteitstoetsen voor effecten in het veld te onderzoeken. Hiervoor zijn 12 proefsloten gebruikt (mesocosms met een waterinhoud van ca. 60 000 L) die een levensgemeenschap bevatten die kenmerkend zijn voor ondiepe, door waterplanten gedomineerde zoetwaterecosysemen. Hierin werd een concentratiereeks van enkelvoudige doseringen (concentratieniveau's: 0 – 0,1 – 0,9 – 6 - 44 μg/L) met chloorpyrifos getest onder veldcondities. De concentratie van chloorpyrifos in het water is gevolgd in de tijd. Effecten op macrofauna (met het blote oog waarneembare dieren zoals insecten, kreeftachtigen, slakken, bloedzuigers) en zoöplankton (met de microscoop waarneembare dieren zoals watervlooien, eenoogkreeftjes, raderdiertjes) zijn onderzocht en vergeleken met toxiciteitsgegevens uit het lab. Acute directe effecten zijn alleen waargenomen bij geleedpotigen (bijv. insecten, kreeftachtigen). Dit is in overeenstemming met de informatie uit het laboratorium. De korte-termijn directe effecten in de proefsloten zijn voor zeven soorten gekwantificeerd in de vorm van veld-EC50s. De EC50 is de concentratie waarbij 50% van een blootgestelde populatie een bepaald effect vertoont, zoals, bijvoorbeeld, immobiliteit of sterfte.Voor deze soorten waren de veld-EC50s van dezelfde ordegrootte als hun acute lab-EC50s. Het gevoeligste standaard toetsorganisme uit het lab, de watervlo *Daphnia magna*, blijkt een goede indicator voor de gevoelige soorten in de proefsloten.

Op de korte termijn zijn in de proefsloten bij het 0,1 μg/L-behandelingsniveau geen wezenlijke effecten waargenomen. Dit behandelingsniveau is te beschouwen als de niet-toxische drempelwaarde. Een veiligheidsfactor van 10 toegepast op de 48 uurs-EC50 van *D. magna* zou voldoende zijn geweest om verreweg de meeste soorten (99,5%) in de levensgemeenschap van de proefsloten te beschermen tegen korte-termijn effecten. Deze studie geeft aan dat, wanneer blootstellingsconcentraties die in het veld optreden in het laboratorium min of meer worden nagebootst, toxiciteitsdata uit het lab overeenkomen met de directe toxische effecten die men in het veld kan waarnemen.

Hetzelfde proefslotenexperiment is ook uitgevoerd om de respons van de levensgemeenschap op langere termijn te volgen, en om inzicht te krijgen in factoren die het herstel van aangetaste populaties bepalen (Hoofdstuk 3). Voor de gehele studie is de $NOEC_{proefsloot}$ op 0,1 μg/L chloorpyrifos vastgesteld. De NOEC is de hoogste concentratie waarbij geen effecten waarneembaar zijn. De NOEC van 0,1 μg/L geldt zowel voor het populatie- als voor het levensgemeenschapsniveau. De niet-toxische drempelwaarde van 0,1 μg/L die voor korte-termijn effecten is vastgesteld blijkt dus

ook beschermend voor de levensgemeenschap in de proefsloten op de langere termijn.

De snelheid van herstel van populaties blijkt sterk afhankelijk van de overlevingsstrategie van de betreffende soorten. Belangrijke factoren zijn: het aantal generaties per jaar (voltinisme), de aanwezigheid van ongevoelige levensstadia, en de mogelijkheid om van het ene systeem naar het andere te migreren. In deze studie is dat geïllustreerd door de verschillende responsen van twee eendagsvliegen, watervlooien, en een vlokreeft te vergelijken. De eendagsvliegen *Cloeon dipterum* en *Caenis horaria* zijn even gevoelig voor chloorpyrifos. Herstelpatronen blijken echter wel verschillend. Bij het hoogste behandelingsniveau herstelt de *C. dipterum*-populatie zich binnen 12 weken, terwijl dit voor *C. horaria* 24 weken in beslag neemt. Dit verschil kan verklaard worden door de verschillen in voltinisme. *C. dipterum* heeft meerdere generaties per jaar, en heeft daardoor binnen het seizoen meerdere kansen om de proefsloten te herkoloniseren nadat het water niet-toxische niveaus heeft bereikt. *C. horaria* heeft één generatie, met als gevolg dat volledig herstel pas plaats kan vinden nadat de generatie van het volgende seizoen de sloten weer koloniseert. In tegenstelling tot de eendagsvliegen hebben watervlooien geen mogelijkheid om zich actief van het ene geïsoleerde watersysteem naar het andere te verplaatsen omdat dit strikt watergebonden dieren zijn. Toch herstellen zij zich snel na blootgesteld te zijn geweest aan toxische chloorpyrifosconcentraties. Dit is mogelijk doordat watervlooien korte generatietijden hebben en eveneens een ongevoelig levensstadium bezitten in de vorm van wintereitjes. Als soorten niet op eigen kracht verstoorde systemen kunnen herkoloniseren, en ook geen ongevoelige levensstadia hebben, dan is er een kans dat de soort verdwijnt uit geïsoleerde systemen. Dit is waargenomen bij de vlokreeft *Gammarus pulex*. Bij deze soort is tijdens het groeiseizoen geen herstel waargenomen in proefsloten waar de chloorpyrifosconcentraties de populaties in het voorjaar volledig uitgeroeid hadden.

In Hoofdstuk 4 worden drie microcosmexperimenten beschreven om de vraag te beantwoorden of de levensgemeenschaps-NOEC van 0,1 µg/L chloorpyrifos, die gevonden is in de proefsloten, een reproduceerbare waarde is. De experimenten zijn uitgevoerd in kleine systemen (ca. 15 L), waaraan één dosering van 0 of 0,01 of 0,1 of 1 µg/L chloorpyrifos is toegevoegd. In de microcosms zat alleen fyto- en zoöplankton. Er is voor plankton-gedomineerde systemen gekozen omdat van het zoöplankton bekend is dat hierin gevoelige geleedpotigen voorkomen (o.a. watervlooien en eenoogkreeftjes). De microcosms zijn in het laboratorium geplaatst onder temperaturen, lichtregimes en nutriëntenniveaus die 'gematigde' (1 experiment: temp.16 – 18 °C; licht: 14 uur, 175 µE/m²/s; nutriëntenaanbod: eutroof) en warme 'Mediterrane' experimentele condities nabootsten (2 experimenten: temp. 24 – 28 °C; licht 12 uur, 300 - 400 µE/m²/s; nutriëntenaanbod: 1 experiment eutroof en 1 experiment hypertroof).

De gemiddelde half-waardetijd van chloorpyrifos in het water was 45 uur onder de koele, en ongeveer 30 uur onder de warme experimentele condities. De levensgemeenschaps-NOEC was 0,1 μg/L in alle drie de experimenten (op basis van de piekconcentraties), en daarmee gelijk aan die in de proefsloten en andere studies met ecologisch complexere levensgemeenschappen. Boven de levensgemeenschaps-NOEC verschilden de effecten, de effectketens en de tijdsspanne voor herstel per experiment. In de 'gematigde' systemen waren de larven (nauplii) van de eenoogskreeftjes (copepoden) het gevoeligst. In beide 'Mediterrane' experimenten waren dat de watervlooien, maar per experiment verschillende soorten. Een algenbloei is alleen waargenomen in de twee 'Mediterrane' experimenten. Die bloei kan verklaard worden als een indirect effect tengevolge van het wegvallen van gevoelige zoöplanktongroepen die normaliter de algen begrazen. De algenbloei was het sterkst in de hypertrofe systemen.

De resultaten van de verschillende micro- en mesocosmstudies met chloorpyrifos suggeren dat de ecologische drempelwaarde voor effecten voor een belangrijk deel onafhankelijk is van de schaal en complexiciteit van testsystemen. De relatief eenvoudige testsystemen in het laboratorium blijken toereikend om de veilige drempelwaarde van laag-persistente stoffen zoals chloorpyrifos vast te stellen. In het geval van enkelvoudige toepassingen van chloorpyrifos geeft de robuustheid van de levensgemeenschap-NOEC aan dat deze drempelwaarde waarschijnlijk representatief is voor verschillende zoetwatersystemen.

In Hoofdstuk 5 wordt een experiment beschreven om de vraag te beantwoorden of het tijdstip van toepassing van een insecticide invloed heeft op het ecologisch effect, met name op de drempelwaarde daarvan. Daarvoor zijn slootecosystemen eerst in het voorjaar en daarna in de nazomer met lambda-cyhalothrin behandeld. Er zijn microcosms gemaakt door enclosures (ca. 430 L) in proefsloten te plaatsen. De effecten op de macrofauna, zoöplankton, waterplanten en het levensgemeenschapsmetabolisme zijn in de tijd gevolgd. De macrofauna reageerde het duidelijkst op de behandelingen. Zoals verwacht op basis van toxiciteitsgegevens uit het lab, zijn insecten en kreeftachtigen de gevoeligste organismen. Statistische analyse geeft aan dat de samenstelling van de voor- en najaarslevensgemeenschappen verschillend is. Gevoelige organismen blijken echter ruimschoots aanwezig te zijn in beide seizoenen. De studie laat geen duidelijke verschillen in respons zien tussen het voorjaars- en nazomerexperiment. In beide experimenten zijn kleine, tijdelijke effecten waargenomen op slechts enkele soorten bij het laagste behandelingsniveau van 10 ng/L. Deze waarnemingen suggereren dat drempelwaarden verkregen uit modelecosysteemstudies die vroeger in het jaar uitgevoerd zijn, ook indicatief zijn voor drempelwaarden later in het jaar. De effecten die in onze studies gevonden zijn bij het 10 ng/L-behandelingsniveau, zijn consistent met de waargenomen effectconcentraties van andere modelecosysteemstudies die gedaan zijn met lambda-cyhalothrin.

Hoofdstuk 6 behandelt een experiment in microcosms (ca. 600 L) om de vraag te beantwoorden of de risico-beoordeling voor afzonderlijke stoffen voldoende is om het ecologisch risico van een realistische blootstelling aan meerdere middelen vast te stellen. Het bestrijdingsmiddelenpakket en het behandelingsschema zijn gebaseerd op het bestrijdingsmiddelengebruik in het gewas 'tulp'. Naast lambda-cyhalothrin bestond het pakket uit het fungicide fluazinam en de herbiciden asulam en metamitron. Op basis van toxiciteitsgegevens en berekende blootstellingsconcentraties, is het meest kritische middel ingeschat. Vervolgens zijn de concentraties van de bestrijdingsmiddelen, en effecten op de levensgemeenschap en allerlei dierlijke en plantaardige populaties in de tijd gevolgd. De macrofauna, met name insecten en kreeftachtigen, reageerden het duidelijkst op de behandelingen. Zoals verwacht, zijn de effecten voornamelijk te wijten aan lambda-cyhalothrin. Het laagste behandelingsniveau, waar ook 10 ng/L lambda-cyhalothrin in zat, blijkt de levensgemeenschap-NOEC te zijn. De niet-toxische drempelwaarde en effecten in deze microcosmstudie komen overeen met die in de modelecosysteemstudie beschreven in Hoofdstuk 5 en andere studies die alleen lambda-cyhalothrin onderzochten. In de hier besproken microcosmstudie is de EU-risicoschattingsprocedure voor individuele stoffen afdoende om gevoelige organismen te beschermen tegen een realistisch pakket van bestrijdingsmiddelen.

De in de voorgaande hoofdstukken gepresenteerde studies zijn voorbeelden van micro- en mesocosmexperimenten om hypothesen te testen (bijv. gevoeligheid in het lab ten opzichte van in het veld; warm ten opzichte van koel; voorjaar ten opzichte van najaar) of om een geen-effect-drempelwaarde vast te stellen. Naast deze experimenten zijn er vele andere studies uitgevoerd, met allerlei andere pesticiden en onder een breed scala van experimentele condities. De grootste verschillen tussen studies betreffen de locaties (bijv. klimatologisch en biogeografisch) en het type modelecosysteem (bijv. plankton- of waterplantengedomineerd; binnen of buiten; stilstaand of stromend water). De grote hoeveelheid gegevens die door al deze studies gegenereerd zijn bieden de gelegenheid om de consistentie in de drempelwaarden zoals gevonden in de voorgaande hoofdstukken te evalueren, en om te kijken of er concentratie-effect-relaties of andere algemeenheden af te leiden zijn. Daarom is een literatuurreview uitgevoerd naar modelecosyteemstudies waarin neurotoxische insecticiden (organofosfaten, carbamaten en synthetische pyrethroïden) zijn gebruikt (Hoofdstuk 7). Stoffen dus, die toxicologisch hetzelfde werken als chloorpyrifos of lambda-cyhalothrin. De specifieke doelstellingen van de review zijn (a) om een overzicht te maken van de geen-effect-drempelwaarden van individuele insecticiden, (b) om de levensgemeenschaps-NOECs te vergelijken met waterkwaliteitscriteria voor toelating van bestrijdingsmiddelen en (c) om in te schatten wat de ecologische effecten zijn bij overschrijdingen van de niet-toxische drempelwaarden. Publicaties uit de open literatuur tussen 1980 en 2001 zijn gebruikt in de review en zijn geselecteerd op een aantal criteria. Zo is er onder andere gekeken of de gebruikte modelecosystemen realistische kenmerken hadden van een zoetwaterecosysteem, en of de blootstellings-

concentraties zinnig waren in relatie tot de te verwachten veldconcentraties en of de onderzochte aspecten, zoals de soorten, wel gevoelig zijn voor het middel. De insecticiden zijn vervolgens naar hun werkingsmechanisme ingedeeld (acetylcholine-esterase-remmers of electronentransportverstoorders). Om de verschillende middelen met elkaar te kunnen vergelijken werden hun concentraties uitgedrukt in toxische eenheden (TU: Toxic Unit). De concentratie van 1 TU is daarbij gelijk aan de concentratie van de acute EC50 van het gevoeligste standaardtoetsorganisme (meestal de watervlo *D. magna*). De aspecten die in de studies zijn gerapporteerd zijn in één van de acht door ons gemaakte categorieën ingedeeld, en vervolgens zijn de gerapporteerde effecten ingedeeld naar de intensiteit van het effect.

De stoffen die de meeste aandacht kregen in de literatuur, zijn organofosfaten en synthetische pyrethroïden, en er is meestal naar effecten in stilstaand water gekeken. Als de effecten op basis van de TU's worden uitgezet, dan kan een duidelijke concentratie-respons-relatie in modelecosysteemstudies worden waargenomen. Hierdoor is het mogelijk om met behulp van regressieanalyse de kans te berekenen welke effecten in een modelecosyteemexperiment zouden optreden bij een bepaalde concentratie van neurotoxische insecticiden. Er komt ook naar voren dat in het geval van enkelvoudige toepassingen, uitgevoerd in geïsoleerde wateren, bij concentraties die onder de 0,1 TU blijven gewoonlijk geen effecten waar te nemen zijn. Bij iets hogere concentraties kan men kleine, tijdelijke effecten verwachten. Duidelijke langere-termijn-effecten kan men vanaf 1 TU verwachten. In het geval van herhaalde en chronische blootstellingen is de kans klein dat er duidelijke effecten optreden beneden de 0,01 TU. In het concentratietraject van 0,01 – 0,1 TU zijn korte-termijn-effecten gevonden. Boven 0,1 TU kan men lange-termijn-effecten verwachten. Uitgedrukt in TU's waren de geen-effect-drempelwaarden vaak gelijk voor stoffen met een zelfde werkingsmechanisme.

In het geval van enkelvoudige belastingen - welke voornamelijk uitgevoerd zijn met organofosfaten - blijken de kritische drempelwaarden, zoals vastgesteld in de relatief conservatieve eerste trap van de EU-Uniforme Beginselen, beschermend te zijn. In het geval van herhaalde toepassingen - voornamelijk uitgevoerd met pyrethroïden - zijn geen-effect-drempelwaarden gelijk aan, of tot en factor 5 hoger dan de concentraties uit de eerste trap. De gevonden geen-effect-drempelwaarden uit de modelecosysteemstudies geven aan dat de veiligheidsfactoren in de eerste trap en criteria, zoals beschreven in de EU-Uniforme Beginselen, toereikend zijn om wateren te beschermen tegen effecten van organofosfaten en synthetische pyrethroïden.

Door de gerapporteerde veldconcentraties te normaliseren tot TU's, en door de effecten in klassen in te delen, is het goed mogelijk om studies met stoffen met een overeenkomstig werkingsmechanisme met elkaar te vergelijken. De studies die in de review gebruikt werden zijn op allerlei plaatsen verspreid over de wereld, en onder verschillende experimentele condities uitgevoerd. Desalniettemin blijken ecologische drempelwaarden consistent. Tenminste, als min of meer gelijke blootstellingsregimes

(bijv. enkelvoudige belastingen of meervoudige belastingen) worden vergeleken. Directe effecten op gevoelige soorten blijken concentratie-gerelateerd, en minder afhankelijk van de grootte van de testsystemen of van de geografische locatie.

Binnen de geleedpotigen zijn de gevoeligste soorten voor de neurotoxische insecticiden gevonden. In verschillende typen waterecosystemen, zowel in natuurlijke als modelecosystemen, zijn er altijd wel soorten van deze groep te vinden en vormen over het algemeen zelfs een belangrijk deel van de aquatische levensgemeenschap. Deze alomtegenwoordigheid van voor neurotoxische insecticiden gevoelige soorten in micro- en mesocosm studies verklaart waarom deze studies een bepaalde robuustheid hebben en algemeen indicatief zullen zijn voor het veld. De micro- en mesocosmstudies laten ook zien dat herstel na een bestrijdingsmiddelenbelasting snel kan gaan als: (a) de stof snel uit het milieu verdwijnt, (b) als het abiotische milieu niet door de stof wordt veranderd of als dit milieu snel weer terug is in z'n oorspronkelijke toestand, (c) als gevoelige populaties korte generatietijden hebben en/of (d) als er herkolonisatie vanuit restpopulaties kan plaats vinden.

De gevonden concentratie-respons op basis van de gevoeligste soorten zoals die getest zijn onder semi-veldcondities maakt het mogelijk om de resultaten van micro- en mesocosmstudies te extrapoleren naar een 'kans op effecten' in het veld bij voorspelde of gemeten bestrijdingsmiddelconcentaties.

Het scala aan modelecosysteemexperimenten dat uitgevoerd is met neurotoxische insecticiden toont een zekere consistentie aan in de concentraties waarbij geen tot beperkte effecten optreden (Hoofdstuk 8). Dit geeft aan dat de drempelwaarden voor dit type stoffen, indien verkregen uit goed uitgevoerde micro- en mesocosmstudies, betrouwbaar zijn als indicatoren voor veilige concentraties in het veld. Hoewel ecologisch veilige drempelconcentraties voor directe toxische effecten weinig blijken te varieëren in ruimte en tijd, blijkt dat bij hogere concentraties de mate en duur van effecten (zowel direct als indirect) wél aanzienlijk kunnen verschillen tussen modelecosyteemstudies.

Summary

Model ecosystems, also referred to as micro- or mesocosms, are frequently used research tools in aquatic ecotoxicology. In comparison to natural ecosystems, these test systems are characterised by a reduction in size and complexity, but nonetheless they do include assemblages of organisms (representing several trophic levels) that are usually in equilibrium with their ambient environment.

Registration schemes for pesticides in many jurisdictions require registrants to assess the potential ecological risks of their products using a tiered testing approach. For aquatic ecosystems, acute and chronic standardised laboratory tests with fish, invertebrates and plants (algae and macrophytes) are used to satisfy data requirements at the lower assessment tiers. If these assessments indicate that there may be concerns, further, higher-tier evaluation of the potential risks is required to determine impacts under more environmentally realistic conditions before approvals can further be evaluated. Micro- and mesocosm studies are among the more complex options for conducting higher-tier aquatic testing. Their advantage over other types of higher-tier studies is their ability to integrate realistic exposure regimes with the assessment of endpoints at higher levels of biological integration (e.g. species interactions and indirect effects). They also allow assessment of population and community recovery. Although model ecosystem studies can play an important role in the evaluation of pesticides, one key question is the extent to which spatio-temporal extrapolation of results is possible.

This thesis aims to contribute to the discussion concerning whether micro- and mesocosm studies can serve as adequate models for robust risk assessment of pesticides. For this purpose, results from freshwater micro- and mesocosm experiments conducted under different experimental conditions are presented and compared. Case studies with the relatively well-studied insecticides chlorpyrifos (organophosphate) and lambda-cyhalothrin (synthetic pyrethroid) are used as examples of the variability typical in model ecosystem studies. These studies are placed in the context of other studies to gain insights into the consistency of the outcomes of micro- and mesocosm experiments performed with non-persistent insecticides which act upon the insect nervous system.

To evaluate the validity of standardised laboratory toxicity testing for predicting effects under field conditions, large outdoor mesocosms (ca. 60 000 L) containing complex macrophyte-dominated freshwater ecosystems were used to test a single application of chlorpyrifos under semi-field conditions *(Chapter 2)*. The fate dynamics of chlorpyrifos in the water compartment of the mesocosms was followed. Effects were investigated on macroinvertebrates and zooplankton, and compared with toxicity data obtained in the laboratory. In the mesocosms, acute effects were observed on

arthropods only, which was in line with the laboratory toxicity data. Short-term direct effects could be quantified for seven species. For these species, acute EC50s were in the same order of magnitude as their laboratory semi-static EC50s. The most sensitive standard test species, *Daphnia magna*, was representative of susceptible indigenous species. In the mesocosms, effects were negligible at the 0.1 μg/L treatment level. A safety factor of 10 applied to the 48h-LC50 of *D. magna* would have sufficed to protect almost all of the species in the community of the mesocosms against short-term direct effects. This study showed that, when field exposure concentrations are simulated in the laboratory, effects based on acute toxicity studies are in line with the effects found in the field.

This same mesocosm study was also performed to monitor longer-term community responses, and to gain an insight into the factors determining the recovery of affected populations (*Chapter 3*). Overall in the study, a no observed effect concentration (NOEC) of 0.1 μg/L chlorpyrifos could be derived both at the species and community level. Non-toxic threshold concentrations based on short-term effect observations appeared to protect the entire invertebrate community on the longer term. The recovery of populations of individual species was highly dependent on life-history traits. Important factors are: the number of generations per year (voltinism), the presence of resistant life stages, and the ability to migrate from one system to another. This is illustrated by the responses of two mayflies, cladocerans and an amphipod. Although nymphs of the mayflies *Cloeon dipterum* and *Caenis horaria* were equally sensitive to chlorpyrifos, recovery patterns were different. *C. dipterum* recovered within 12 weeks after treatment at the highest treatment level, whilst *C. horaria* took 24 weeks. This difference can be explained by differences in voltinism. *C. dipterum* is multivoltine, which creates a large window of opportunity for recolonization after non-toxic levels of the insecticide are regained in the mesocosm. *C. horaria* is univoltine, and as a consequence, recovery could only take place when the subsequent year's generation recolonized the mesocosms, as the treatments were in an unfavourable moment in its life-cycle. Unlike mayflies, cladocerans are not able to actively migrate from one isolated system to another as they are largely aquatic during their life-cycle. Nonetheless, they still recovered rapidly after exposure to toxic concentrations. This was possible because cladocerans have short generation times and have resistant life stages in the form of ephippia (resting eggs). If species are not able to recolonize a disrupted system and do not have resistant life stages, the species can become locally extinct in isolated systems. This occurred in the study for the wholey aquatic amphipod *Gammarus pulex*. This species became locally extinct at concentrations resulting in full eradication of the populations.

To test whether the community NOEC of 0.1 μg/L found in the mesocosms and in other model ecosystems was a robust value, three experiments with single applications of chlorpyrifos in small microcosms of ca. 15 L were performed (*Chapter 4*). The microcosms contained plankton communities, as zooplankton are known to

be a susceptible group of arthropods to chlorpyrifos. The microcosms were established in the laboratory under temperature conditions, light regimes and nutrient levels that simulated cool 'temperate' (one experiment: temp.16 – 18 °C; light 14 h, 175 μE/m²/s; productive) and warm 'Mediterranean' experimental conditions (two experiments: temp. 24 – 28 °C; light 12 h, 300 - 400 μE/m²/s; one experiment productive and one highly productive). The mean half-life of chlorpyrifos in the water was 45 h under the 'temperate' and about 30 h under the 'Mediterranean' environmental conditions. All three experiments yielded community NOECs of 0.1 μg/L, similar to the outdoor mesocosm experiment and other more ecologically complex studies. Above this threshold level, responses and chains of effects, and time-spans for recovery differed between the experiments. In the 'temperate' experiment, copepod nauplii were the most sensitive. In both 'Mediterranean' experiments, cladocerans were most sensitive, but there were some differences between species. Algal blooms resulting as an indirect effect from the impact of exposure on sensitive microcrustaceans were only observed in the two experiments under the 'Mediterranean' conditions. The algal bloom in the productive systems was less pronounced than in the highly productive systems. The results of the different microcosm and mesocosm experiments performed with chlorpyrifos suggest that the threshold is largely independent of the scale and complexicity of the test system. The relatively simple indoor test systems appeared to be sufficient for the determination of safe threshold levels for low-persistence insecticides like chlorpyrifos. In the case of single applications of chlorpyrifos, the robustness of the community NOEC indicates that the threshold level is likely to be representative for many freshwater systems.

Analogous to the previous experiment, we varied experimental conditions and applied similar exposure regimes of lambda-cyhalothrin on a drainage ditch ecosystem in spring and late summer (*Chapter 5*). The study investigated whether the results of a model ecosystem study conducted early on in the year may also be indicative for those performed later in the year. For this experiment, microcosms were established by placing enclosures (ca. 430 L) in macrophyte-dominated drainage ditches. Effects on macroinvertebrates, zooplankton, phytoplankton, macrophytes and community metabolism were followed through time. The macroinvertebrate community responded most clearly to the treatments. As anticipated on the basis of laboratory toxicity data, insects and crustaceans were amongst the most sensitive organisms. Statistical analysis showed that the communities were significantly different between the spring and late summer experiments. The most sensitive taxa, however, were abundant in spring as well as in late summer. The study did not show clear differences in the responses of sensitive species between spring and late summer treatments. In both experiments, only slight and transient effects were observed for a few species at the lowest treatment level of 10 ng/L. These observations suggest that threshold levels obtained from early season higher tier studies also are indicative for the threshold level later in the season. The 10 ng/L treatment level approximated the

threshold level for effects and was consistent with other model ecosystem studies with lambda-cyhalothrin.

Indoor microcosms (ca. 600 L) were used in an experiment testing a realistic package of pesticides (*Chapter 6*). The study aimed to assess the potential ecological impact of a realistic exposure regime and to evaluate the protective value of the EU risk assessment procedure, which is based on individual compounds, for realistic exposure events to pesticide combinations. The package and application regime were based on pesticides often used on tulip crops in The Netherlands. Besides lambda-cyhalothrin, the package comprised of the fungicide fluazinam, and the herbicides asulam and metamitron. Based on standard acute toxicity data and target exposure concentrations, the most critical compound was estimated by expressing concentrations as fractions of toxic units (TU) and by applying the concept of concentration addition. The concentrations of the compounds in the water, and effects on phytoplankton, zooplankton, periphyton, macroinvertebrates, macrophytes, decomposition and water quality parameters were assessed. The macroinvertebrate community responded most clearly. Insect and crustaceans were amongst the most sensitive organisms. As anticipated, short-term effects were mainly due to lambda-cyhalothrin. The lowest treatment level, which contained 10 ng/L of lambda-cyhalothrin, was considered the community NOEC. The study showed that the method of TU calculation gives a good indication of primary effects of a package of pesticides on aquatic biota and can be helpful in the initial risk assessment. At the ecosystem level however, where recovery and secondary effects are important evaluation factors, model ecosystem studies are useful for providing further insights into risks at the community and ecosystem level. Although tested under different experimental conditions, the threshold level and effects from this microcosm study were in line with the enclosure studies described in Chapter 5 and other studies conducted with lambda-cyhalothrin alone. In the present model ecosytem study, the lower tier risk assessment procedure for individual compounds was adequate for protecting sensitive populations exposed to a realistic combination of pesticides.

The case studies were examples of micro- and mesocosm studies used to test either hypotheses (e.g., sensitivity lab vs semi-natural systems; cool vs warm; spring vs late summer) or to find no-effect threshold levels. Besides the presented studies many other model ecosystem studies have been conducted. They represent various active ingredients and a wide range of experimental conditions. Major differences between studies were location (e.g. climatological or biogeographical regions) and types of experimental ecosystems used (e.g., plankton or macrophyte-dominated, indoor or outdoor, lentic or lotic). The relatively large amount of data generated by these model ecosystem studies provide the opportunity, on the one hand, to validate the suggested consistency of threshold levels found in the case studies presented in this thesis, and on the other hand, to detect whether there are concentration-effect relationships and/or other generalities to be found in the effect patterns of studies. Therefore, a

review of the open literature was performed focusing on the ecological impact of neurotoxic insecticides (organophosphates, carbamates, synthetic pyrethroids) (*Chapter 7*). Specific objectives of the review were (a) to list ecological threshold values for individual insecticides, (b) to compare ecosystem NOECs with first tier water quality criteria, and (c) to assess the ecological consequences of exceeding the water quality criteria. Publications from 1980 – 2001 were included in the review. Model ecosystem studies were screened on a number criteria. Amongst these were: Do the test systems represent a realistic freshwater community?; Are the exposure concentrations reported and relevant?; Are investigated endpoint sensitive to the substance? Insecticides were grouped according to their mechanism of toxicity (acetylcholineesterase-inhibitors, electron transport inhibitors). To enable comparison of studies using different insecticides, the reported field concentrations were normalised to TU. TU was based on acute toxicity values for the most sensitive standard test species (usually *Daphnia magna*). Endpoints presented in the studies were assigned to one of eight categories representing either structural or functional endpoints. Effects reported on these endpoints were classified into one of five classes. Most studies covered organophosphates and synthetic pyrethroids tested in static water. The probability of effects occurring in model ecosystems was calculated by analysing the data set of the most sensitive endpoints using logistic regression. On the basis of TUs, clear concentration-response relationships could be derived from the model ecosytem studies. In the case of single applications, performed in isolated water systems, effects on the most sensitive endpoints were usually not observed at concentrations below 0.1 TU. At higher doses, slight to clear effects may be expected. Clear longer term effects may be expected at concentrations above 1 TU. For repeated and chronic exposures, it is unlikely that clear effects occur below 0.01 TU. Within the concentration range 0.01 – 0.1 TU, short-term effects are reported. Above 0.1 TU, long-term effects are to be expected. Based on TU, threshold values were equivalent for compounds with a similar mode of action. In the case of single applications - mainly based on studies with organophosphates - first tier concentrations as set by the EU Uniform Principles appear to be protective. For multiple applications - mainly based on pyrethroid studies - NOECs were equal to, or less than a factor of 5 higher than the concentrations derived by the relatively conservative first tier. The established ecosystem NOECs indicated that first tier safety factors and criteria as described in the EU Uniform Principles seem to be adequate for organophosphates and synthetic pyrethroids. Normalisation of reported field concentrations to TU and by using an effect classification system enabled a comparison to be made between studies with insecticides that have working mechanisms in common. The evaluated studies were performed in various parts of the world and under various experimental conditions. Ecosystem threshold levels were still shown to be very consistent, at least when similar exposure patterns were considered. Direct effects on susceptible species are often concentration-related and not dependent on system scale or geographical

location. Arthropods contain the species most sensitive to these compounds. In the different types of ecosystems, both natural and model, sensitive representatives of this group are usually present and generally form a predominant part of aquatic communities. This overall presence of sensitive taxa in micro- or mesocosm studies carried out with these types of insecticides, explains why such studies have a certain robustness and a general predictive value for ecological risk assessment in the field. The micro- and mesocosm studies demonstrated that recovery after pesticide contamination can be expected to be rapid in the field when (a) the compound is non-persistent, (b) the physico-chemical environment is not altered or quickly restored, (c) generation times of vulnerable populations are short, and/or (d) when there is immigration from residual populations. Modeling of the observed responses of sensitive endpoints under semi-natural conditions provides a way of extrapolating results of micro- and mesocosm observations to probabilities of effect occurrences in the field at predicted or measured environmental concentrations

The various model ecosytem experiments performed with neurotoxic insecticides demonstrated that concentrations in the range of no to small transient effects are consistent (*Chapter 8*). For this type of compounds this indicates that threshold levels observed in good quality model ecosystem studies earn confidence as an indicator of non-effective concentrations in the field. Although threshold concentrations for direct toxic effects may vary little in space and time, at higher exposure concentrations the intensity and duration of the responses (directly or indirectly) may vary considerably between different micro- and mesocosm experiments.

1 General introduction

Micro- and mesocosms are frequently used as research tools in aquatic ecotoxicology. They are bounded systems that are constructed artificially with samples from, or portions of, natural ecosystems, or that consist of enclosed parts of natural ecosystems. Although these model ecosystems usually are characterised by a reduction in size and complexity when compared with natural ecosystems, they have to include an assemblage of organisms representing several trophic levels and this assemblage should be in equilibrium with its ambient environment (Crossland and Bennett, 1984; Gearing, 1989; Zieris 1991; Brock et al., 1995; Caquet et al., 2000). The terms microcosm and mesocosm are used more or less loosely when referring to model ecosystems. Following the definitions proposed by Crossland et al. (1993), microcosms are experimental systems containing less than 15 m^3 water volume or experimental streams less than 15 m in length. Mesocosms are experimental systems having more than a 15 m^3 water volume or experimental streams greater than 15 m in length.

The diversity of types of aquatic model ecosystems is large. A major division is that in 'generic' and 'semi-realistic' freshwater model ecosystems. The 'generic' model ecosystems do not mimic any natural system in particular, but rather exhibit some basic properties common to all ecosystems, such as species interaction, production, decomposition and nutrient cycling. These systems are intended to contain only certain defined species and defined abiotic qualities chosen by the experimenter, and they are relatively simple and readily standardized (Taub, 1969; Metcalf et al., 1971; Kersting, 1984). Many aquatic model ecosystems used in ecotoxicology are of the 'semi-realistic' type in that they attempt to mimic real ecosystems. They can be classified according to the type of natural freshwater system that they represent, and whether they are situated indoors or outdoors. In outdoor model ecosystems, a distinction can be made between constructed systems (e.g., concrete tanks in which sediment and water are introduced and that serve as ponds) and enclosed parts of existing ecosystems (e.g., by means of plastic bags, polycarbonate cylinders, or artificially lined limnocorrals). The most frequently used freshwater model ecosystems are those that mimic shallow, static freshwater habitats (e.g., ponds, ditches, littoral zones of lakes).

Model ecosystem studies are frequently performed either for testing ecological hypotheses or for finding ecological threshold concentrations for toxicants. These studies generally focus on interactions in specific test systems and reveal that many factors may influence the outcome of the study in question. Since test conditions and types of communities enclosed influence the results, studies should be considered more or less anecdotal. Another way to gain knowledge from model ecosystem studies

is by trying to generalize observations from a population of individual studies so as to obtain rules-of-thumb. In this thesis I will follow both concepts. First, case studies with micro- and mesocosm experiments are presented. These may be considered typical examples of descriptive model ecosystem studies. Next, I want to show how certain rules-of-thumb can be deduced from the many empirical data now available from micro- and mesocosm studies performed with insecticides.

This thesis concerns risk assessment of insecticides in aquatic ecotoxicology. These type of assessments are closely linked with registration procedures. Registration procedures for pesticides are obligatory in many countries from all over the world and, in many cases, these procedures share more or less equivalent principles and/or regulatory frameworks. Nevertheless, this thesis is written from an European background and therefore mostly relates to the European Union regulatory framework.

The role of micro- and mesocosms for risk assessment of pesticides

Registration schemes for pesticides of many jurisdictions (e.g. EU, USA) require registrants to assess the potential ecological risks of their products using a tiered approach. For aquatic ecosystems, standardised tests with agreed endpoints are used to satisfy data requirements at the lower assessment tiers (e.g. toxicity exposure ratios or risk quotients). If these preliminary assessments indicate that there may be concerns, further evaluation of the potential risks is required to determine impacts under more environmentally realistic conditions before admittance can further be evaluated.

In the EU regulatory framework, a number of approaches may be used to address these concerns (Campbell et al., 1999; European Commission, 2002). They include indoor and outdoor microcosm studies, outdoor mesocosm studies, artificial stream studies and field monitoring. Besides the aim of micro- and mesocosm studies to simulate natural conditions and exposing these systems to environmentally realistic toxicant exposure regimes, these studies normally follow experimental designs to demonstrate causality between treatment and effects, and can also identify concentration-effect relationships. This experimental aspect is more difficult to implement in field monitoring studies, which makes it harder to prove a relation between responses and a toxicant in this type of studies.

Micro- and mesocosm studies are generally considered to be amongst the most complex of higher-tier studies. Studies considered intermediate between laboratory first tier standard aquatic toxicity testing and micro- and mesocosm experiments are: modified exposure studies, species sensitivity distribution studies, population studies, and tests with sensitive life stages (Campbell et al., 1999; European Commission, 2002). The advantage of micro- and mesocosm studies over the other types of higher tier studies is their ability to integrate realistic exposure regimes with the assessment of

endpoints at higher levels of biological integration, and to study species interactions and indirect effects. They also allow assessment of population and community recovery.

Intrinsically for this type investigations, micro- and mesocosm studies yield substantial amounts of data over many endpoints. This makes them difficult to interpret. Specifically in the case of the first model ecosystem studies, which were performed up in the 1970s and '80s, an additional complication arose from the fact that many of the studies were not yet optimally designed for the purpose of risk evaluation (e.g. large variability, presence of fish, demonic intrusions, incomplete data, studies were long and costly, etc.). Because of these difficulties, the US EPA stopped requiring mesocosm studies in 1992. However, the gained knowledge and experience was used for further development and harmonization of micro- and mesocosm studies and resulted in guidance documents from the early nineties onwards (e.g. Guidance Document on Testing Procedures for Pesticides in Freshwater Mesocosms (SETAC-Europe, 1991); Workshop on Aquatic Microcosms for Ecological Assessment of Pesticides (SETAC-RESOLVE, 1992); European Workshop on Freshwater Field Tests (EWOFFT), (Crossland et al., 1993). From the late 1990s onwards, guidance became more focused on the ecological interpretation of studies, on the evaluation of experimental designs and on the implementation of data into risk assessment (Guidance Document on Higher-Tier Risk Assessment of Pesticides (HARAP), (Campbell et al.,1999); Community Level Aquatic System Studies-Interpretation, Criteria (CLASSIC), (Giddings et al., 2002) and eventually culminated in the EU aquatic ecotoxicology guidance document 'Guidance Document on Aquatic Ecotoxicology in the Context of Directive 91/414/EEC' (European Commission, 2002). While implementing and developing more experience with these guidelines, discussion shifted towards subjects like the need of the identification of protection goals and whether all types of water should receive the same level of protection (Van Dijk et al., 2000; Brock, 2003). Another issue concerns the science and the implications of the use of 'Ecologically Acceptable Concentrations' in the EU risk evaluation process (Crane and Giddings, 2004).

Aim of the thesis

Although micro- and mesocosm studies may play an important role in the registration of pesticides (EU, 1997; European Commission, 2002), one of the concerns is the extent by which spatio-temporal extrapolation of results of model ecosystem studies is possible. In nature, aquatic ecosystems and communities are spatially heterogeneous and temporally dynamic (e.g. Giddings et al., 2002; Brock et al., 2005 a,b). This thesis aims to contribute to the discussion concerning whether micro- and mesocosm studies can serve as adequate models for robust risk assessment of pesticides. For this purpose, results of freshwater micro- and mesocosm experiments characterised by

differences in experimental conditions are presented and compared. Case studies with the relatively well-studied insecticides chlorpyrifos and lambda-cyhalothrin are used as examples of the variability in model ecosystem studies and serve as benchmark compounds. Finally, the predictive value of micro- and mesocosm experiments for non-persistent neurotoxic insecticides will be discussed with attention on threshold concentrations for direct effects, recovery and indirect effects.

Profiles of the bench mark compounds

Chlorpyrifos is an organophosphorous compound that displays broad-spectrum insecticidal activity against a number of important pests. The neurotoxicity of chlorpyrifos is caused by the inhibition of the acetylcholine-esterase synthesis (Barron and Woodburn, 1995). It has a low water solubility (1.4 mg/L at 25 ºC) and has a relatively high lipophilicity (log *K*ow: 4.7-5.3) (Barron and Woodburn, 1995; Tomlin, 2000). In aquatic environments, chlorpyrifos is a degradable compound, with hydrolysis as the most important process. Chlorpyrifos shows a field half-life in water of less than 0.08 to 2.4 days (Racke, 1993). It readily sorbs to aquatic macrophytes and sediments (Crum and Brock, 1994). Species sensitivity distribution (SSD) curves based on acute laboratory toxicity data (LC50 and EC50 values) for various groups of aquatic organisms show that arthropods, and to a lesser extend, fish are the most sensitive groups, followed by algae and non-arthropod invertebrates (Fig. 1). The HC5 derived from arthropod SSDs on the basis of laboratory acute toxicity data is 0.08 μg/L (Maltby et al., 2005). The HC5 represents the median hazardous concentration that will affect 5% of the potentially affected species as estimated from species sensitivity distributions. In other words, at a concentration of 0.08 μg chlorpyrifos/L, it is anticipated that at least 95% of the sensitive populations (arthropods) are unlikely to suffer acute effects.

Lambda-cyhalothrin is a synthetic pyrethroid and works by disrupting electron transport in the nerve axons (Clark and Brooks, 1998). Lambda-cyhalothrin has a very low water solubility (5 μg/L at 20 °C) and is highly lipophilic (log *K*ow: 7) (Hand et al., 2001). The compound tends to sorb rapidly and extensively to organic materials in the water column (Maund et al., 1998; Leistra et al., 2003). Half-lives in water under semi-field conditions are less than one, to around one day (Leistra et al., 2003; Van Wijngaarden et al., 2004). In aquatic ecosystems, lambda-cyhalothrin is subject to transformation via biotic and abiotic processes. Alkaline hydrolysis in the water near the surface of macrophytes and algae is considered the most important process (Leistra et al., 2003). Non-arthropods are relatively insensitive to lambda-cyhalothrin (Schroer et al., 2004), whilst arthropods are more sensitive than fish (Fig. 2). Within the arthropods, the taxonomic groups of insects and macrocrustaceans contain the most sensitive species (Maund et al., 1998; Schroer et al., 2004). For lambda-

cyhalothrin, the median HC5 derived from SSDs for arthropods is 0.003 µg/L (Maltby et al., 2005).

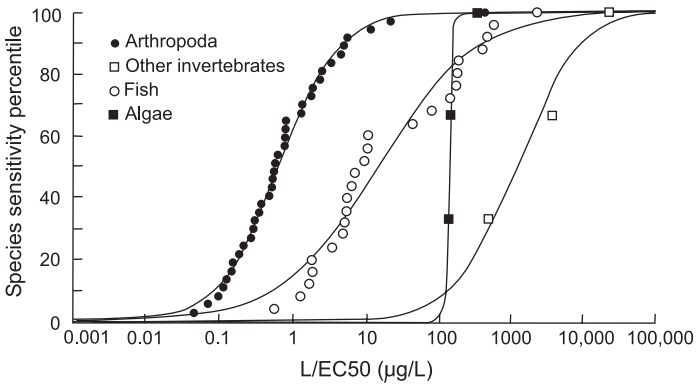

Fig. 1. Species sensitivity distributions based on acute laboratory toxicity data (LC50 and EC50) for various groups of aquatic organisms exposed to the insecticide chlorpyrifos. The data were obtained from Van Wijngaarden et al. (1993), Crommentuijn et al. (1997) and by consulting the AQUIRE data base (http://www.epa.gov/ecotox).

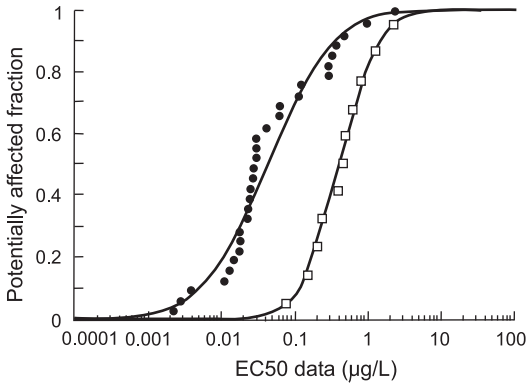

Fig. 2. Species sensitivity distributions based on acute laboratory toxicity data (EC50) for aquatic arthropods (circles) and fish (squares) exposed to the insecticide lambda-cyhalothrin. The data were obtained from Maltby et al. (2002).

Because both compounds rapidly dissipate from the water, it may be expected that organisms are subject to short-term exposures which may cause acute effects. Maltby et al. (2005) compared responses of aquatic organisms exposed to chlorpyrifos or to lambda-cyhalothrin in single-species laboratory toxicity tests with those from model ecosystems. They showed that the cumulative distribution functions for laboratory

and field generated data were practically similar (Fig 3). This indicates that acute effects in aquatic organisms observed in the laboratory, when exposed to similar exposure regimes of these types of neurotoxins, may also be expected under field conditions.

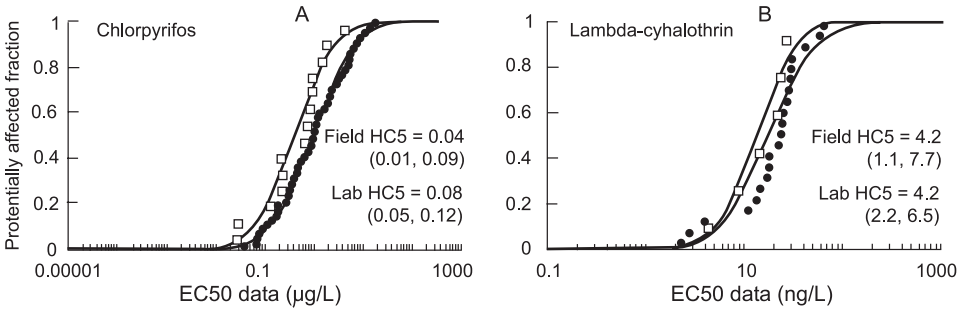

Fig. 3. Species sensitivity distribution curves from single-species laboratory toxicity tests (circles) and single-species responses in model ecosystems (squares) for chlorpyrifos (A) and lambda-cyhalothrin (B). Figure adapted after Maltby et al. (2005).
Data between brackets in the legend are the lower and upper limit of the 95% confidence interval, respectively.

Outline of the thesis

This thesis is divided in three parts. Part I presents case studies with chlorpyrifos and comprises of Chapters 2 - 4.

Chapter 2 evaluates the short-term effects of a single application of chlorpyrifos on the invertebrate populations in experimental ditches, which are relatively large outdoor mesocosms (ca. 60.000 L) that contain complex macrophyte-dominated ecosystems. This study showed that when field exposure dynamics are simulated in the laboratory, acute toxicity data as obtained from the laboratory are in line with those found in the field.

Chapter 3 describes the long-term effects of a single application of chlorpyrifos on the invertebrate community in these experimental ditches. Effects and recovery at the population and community level are discussed. Special attention is given to the relationship between recovery patterns of taxa and their life-history characteristics. This study indicates that, besides the fate dynamics and toxicity of a compound, biological traits are also important in determining the time needed for recovery of populations.

Chapter 4 presents three experiments with single applications of chlorpyrifos in small indoor microcosms (ca. 15 L). Temperature regimes, light regimes and nutrient levels differed considerably between these experiments. Effects on zooplankton, phytoplankton and community metabolism were observed. Despite the differences in

experimental conditions, the community NOECs were similar for all three experiments. Moreover, they also were similar to those found in the experimental ditches and other outdoor model ecosystem studies.

Part II presents case studies with lambda-cyhalothrin and includes Chapters 5 and 6. Chapter 5 presents a study which compares effects of lambda-cyhalothrin on a drainage ditch ecosystem in spring and late summer. In this study, microcosms were established using enclosures (ca. 430 L) in the experimental ditches described in Chapter 2. The responses of the invertebrate community were observed at the community and population level. The community structure was significantly different between spring and late summer. The most sensitive species, however, were present in both seasons. The study did not show clear differences in responses of sensitive taxa between the spring and late summer insecticide treatments. Effect thresholds were similar irrespective of season. The observations suggest that threshold levels obtained from early season higher-tier studies are also indicative for threshold levels later in the season.

Chapter 6. A realistic mixture of pesticides, including lambda-cyhalothrin, was applied to indoor microcosms (ca. 600 L). Based on standard acute toxicity data and target exposure concentrations the most critical compound was estimated by expressing concentrations as toxic units and applying the concept of concentration addition. Effects at the community and population level for the invertebrate community are described. On the basis of laboratory toxicity data available for the different compounds, ecological effect chains are discussed. As anticipated, responses were mainly due to lambda-cyhalothrin. Threshold levels and effects from this study were in line with other studies, which were performed solely with lambda-cyhalothrin.

In part III, I present some rules-of-thumb that emerge from the results obtained in different types of model ecosystems studying neurotoxic insecticides. This last part consists of Chapter 7 and 8. Chapter 7 presents a review on model ecosystem studies with neurotoxic insecticides and presents ecological threshold levels from these studies. These threshold levels are compared with the EU first tier levels, and ecological consequences of exceeding thresholds are evaluated. An effect classification system is used for evaluating effects. Based on toxic units, threshold values are found to be equivalent for compounds with similar modes of action. This also accounts for the nature and magnitude of direct effects at concentrations above threshold level. The community NOEC usually is a factor of 10 or more higher than first tier acceptable concentrations, particularly in the case of single applications and acetylcholine-esterase inhibitors. The consistency of threshold and response patterns found in model ecosystem studies indicates that they are adequate tools for ecotoxicological risk assessment in the case of non-persistent neurotoxic insecticides.

Chapter 8 functions as a synthesis of the previous chapters and goes further into the applicability of micro- and mesocosm studies in relation to higher tier risk evaluations. An attempt is made to derive extrapolation factors for the protection of

aquatic ecosystems on the basis of threshold concentrations derived from micro- and mesocosm studies.

Delimitation of the work

This thesis relates to higher-tier risk evaluation of non-persistent neurotoxic insecticides. Lower-tier risk evaluations indicate that these insecticides mainly pose short-term risks to water organisms after exposure to normal agricultural use of these compounds. Ecological risks caused by long-term or chronic exposures which are more associated with organochlorine insecticides (e.g. DDT, lindane) and several photosynthesis-inhibiting herbicides (e.g. atrazin, diuron) or fungicides, are not discussed in this thesis.

Inherent to the tiered approach of the risk assessment procedure, in the first tier, risks for different taxonomic groups are identified (EU, 1997). In the case of the insecticides studied in this thesis, these were the arthropods (Figs. 1.1 and 1.2). Higher-tier research performed in micro- and mesocosms was therefore primarily focused on the effects on this group of organisms, and not on that of vertebrates such as fishes and amphibians.

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Part I: Case studies with chlorpyrifos

2 Effects of the insecticide Dursban® 4E (Active ingredient chlorpyrifos) in outdoor experimental ditches: I. Comparison of short-term toxicity between the laboratory and the field

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Abstract

Using the insecticide Dursban® 4E (active ingredient chlorpyrifos) as the test compound, results of laboratory acute single-species toxicity tests with indigenous and standard test species were compared with short-term direct effects in outdoor experimental ditches (mesocosms). In the mesocosms a regression experiment was performed with nominal initial chlorpyrifos concentrations of 0.1, 0.9, 6, and 44 μg/L. The mesocosms were sprayed once. Effects were investigated by sampling macroinvertebrates and zooplankton and by doing in situ cage experiments with several species. Chlorpyrifos concentrations showed highest spatial and temporal variation within 2d of treatment. Acute effects were observed on arthropods only and essentially were manifest on day 0. Short-term direct effects in the mesocosms could be quantified by a regression method for seven of 120 species. For these species, 48-and 96-h median effective concentrations (EC50s) ranged from 0.1 to 3.4 μg/L and were in the same order of magnitude as their laboratory EC50s. Susceptibility of the most sensitive standard test species (*Daphnia magna*; 48-h median lethal concentration [LC50], 1 μg/L) was more or less representative of susceptible indigenous species. In the mesocosms effects were negligible at the 0.1-μg/L treatment level. A safety factor of 0.1 (48-h LC50 of *Daphnia magna*) may have protected almost all of the species in the community in the mesocosms against short-term direct effects. A safety factor of 0.01 probably protected the most susceptible taxa we found (laboratory 96-h EC10 for *Gammarus pulex,* 0.02 μg/L; no-observed-effect concentration for Copepoda, < 0.1 μg/L). The question remains, however, whether long-term (in)direct effects on the populations or the community may occur at the 0.1-μg/L treatment level.

Keywords: Chlorpyrifos; Mesocosm; Safety factor; Effective concentration; Extrapolation

Introduction

Procedures to determine safe concentrations of pesticides in aquatic ecosystems are usually based on standardized laboratory toxicity tests [1,2]. However, extrapolating laboratory data to the field may be difficult for several reasons. Standardized single-species toxicity tests investigate only a limited number of species, usually species that are easily kept in the laboratory. In addition, concentrations of the pesticide remain relatively constant in the laboratory, whereas under field conditions pesticide levels usually show substantial variation in space and time [3,4].

To evaluate the usefulness of standardized laboratory tests for predicting the fate and effects of a pesticide at the ecosystem level, we performed several types of laboratory studies and an outdoor mesocosm experiment with the organophosphorus insecticide Dursban® 4E (active ingredient chlorpyrifos). The test systems we used showed increasing ecological complexity (laboratory single-species toxicity tests, generic microecosystems and semirealistic microcosms in the laboratory, outdoor mesocosms) [5].

This article compares laboratory single-species toxicity tests with the short-term fate and effects of chlorpyrifos in our most complex test system. Mesocosms resembling drainage ditches were treated with a single application of chlorpyrifos in a regression design. Comparisons between laboratory single-species toxicity tests and test systems of intermediate ecological complexity have already been dealt with [6–10].

The aims of this article are to describe the experimental design of the mesocosm study, to report on the dynamics and spatial variation in chlorpyrifos concentrations in the water column of the mesocosms, to compare short-term toxicity in the laboratory between standard test species and indigenous taxa from the mesocosms, to compare the short-term response of the indigenous species in laboratory toxicity tests with that in the mesocosm study, and to discuss the safety factor by which the toxicity parameter of the most susceptible standard test species should be multiplied to obtain safe concentrations in the surface water of the mesocosms after a single application of the insecticide.

This article is part I of a series of three. In part II, which is also in this issue [11], long-term effects on the invertebrate community structure and the recovery of affected populations are described. Part III will focus on functional aspects such as oxygen and community metabolism.

Materials and methods

Experimental setup

Laboratory experiments. Acute toxicity tests were performed with standard species and with several species indigenous to the mesocosms. These tests have been described by Kersting and Van Wijngaarden [6] and Van Wijngaarden et al. [12].

Mesocosms. We used experimental ditches (length, 40 m; width at water surface, 3.4 m; water volume, 60 m^3). The ditches had a 0.25-m sediment layer of sandy loam and a water column that was 0.5 m deep. The ditches were lined with a water-tight, nontoxic polyvinyl chloride (PVC) layer to prevent leakage of water to the surrounding environment (see Drent and Kersting [13] for details).

Biocoenosis development. The sediment was an important initial source of benthic and pelagic organisms. Before the experiment, for over 2 years the mesocosms became dominated by macrophytes and developed a community typical of shallow ponds and ditches. In the months before and during treatment, the vegetation covered almost the entire sediment area (mean cover 2 weeks before treatment, 85 ± 17%; n = 12). Dominant macrophyte species during the experiment were *Elodea nuttallii* (Planch.) St. John, *Chara* sp., and *Ranunculus circinatus* Sibth. Mean biomass (dry weight) of the macrophytes at time of treatment was about 0.25 kg/m^3 [11].

About 8 months prior to the insecticide treatment we introduced 20 to 30 individuals of each of the crustaceans *Asellus aquaticus* L. and *Gammarus pulex* (L.) into each mesocosm. We did so because these species appeared to be absent in the mesocosms even though they usually occur in drainage ditches in the Netherlands.

Insecticide treatment. Chlorpyrifos was applied as Dursban® 4E, which forms an emulsion in water. The mode of action and physicochemical properties of the insecticide have been described by Marshall and Roberts [14].

On May 8, 1990, the insecticide was sprayed over eight mesocosms in a single treatment. Nominal chlorpyrifos concentrations for the duplicates were 0.1, 0.9, 6, and 44 μg/L. Four mesocosms were used as controls. Treatments were assigned randomly to 12 mesocosms. Spraying of the insecticide was done by means of a spray boom mounted with eight split nozzles (Tee Jet® XR-110-08; air pressure, 1.25 bar; droplet size, 150 to 700 μm). Doses were evenly distributed over the water surfaces by moving the spray boom at a constant speed over the total length of the mesocosms. Spraying took place under almost windless conditions (mean wind speed, 1.3 ± 0.32 m/s; measurements between 6 a.m. and noon).

Sampling locations. Within each mesocosm, measurements and samplings were assigned to specific locations (Fig. 1) to avoid mutual influences or disruption of the individual sampling programs.

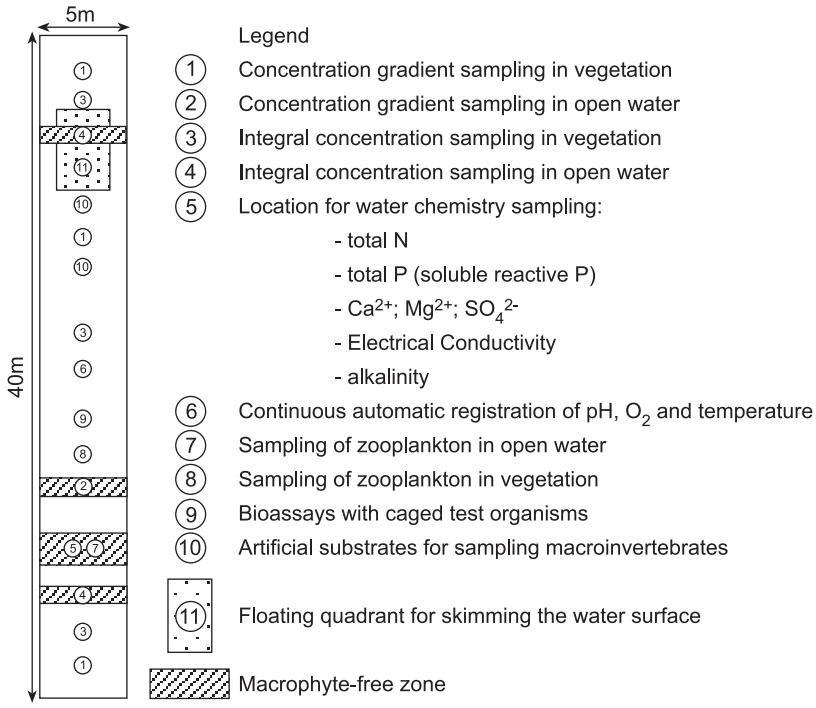

Fig. 1. Scheme of sampling sites for each of the experimental ditches for studying fate and effects following a single chlorpyrifos application.

Chlorpyrifos residues in water. Nominal chlorpyrifos concentrations were based on the known amount of sprayed Dursban® 4E assuming instantaneous mixing over the water column. Actual chlorpyrifos concentrations were estimated by measuring the stratification and by taking depth-integrated water samples [15].

Stratification of chlorpyrifos was investigated from day 1 up to and including day 4 posttreatment by placing four gradient sampling devices in one 44-µg/L treatment mesocosm. Three devices were placed in macrophyte-dominated locations, and one was placed in a segment cleared of vegetation (open-water sampling) (Fig. 1). These devices were used to take water samples simultaneously at 0.1, 0.25, and 0.45 m below the water surface [15]. A detailed description of the sampling device and technique is given in Brock et al. [8].

Depth-integrated water samples were taken at five locations in each mesocosm, three in macrophyte-dominated and two in open-water locations (Fig. 1).

Chlorpyrifos was extracted with hexane. Analytical procedures have been described by Crum and Brock [15].

Table 1. Volumes of water and sampling locations for investigating zooplankton communities

Zooplankton*	Location	Sample volume (L)
Macro	Macrophyte-free	10
Macro	Macrophytes	5
Micro	Macrophyte-free	1

* Macro, zooplankton >300 μm; micro, zooplankton <300 μm.

Sampling of invertebrates. Zooplankton was sampled with Perspex® tubes (length, 0.4 m; volume, 0.8 L) in locations with and without macrophytes (Table 1). Samples from both locations were treated separately. Zooplankton was concentrated by passing the water through a 300-μm-mesh net to restrain Cladocera, Copepoda, and Ostracoda (macrozooplankton). Part of the filtered water was collected for the identification and counting of the microzooplankton (Amoeba, Ciliata, and Rotatoria).

Concentrated macrozooplankton samples were preserved with formalin (4%). After coloring with lugol, the total number of individuals of each taxon was counted under a Wild-5MA stereo microscope (25x magnification). The cladocerans were identified to species level. For copepods a distinction was made between nauplii and more mature stages. Ostracods were not identified any further.

Ten milliliters of lugol was added to the microzooplankton samples. Subsequently, the sedimented microzooplankton was concentrated to a known volume and preserved with 4% formalin. Microzooplankton was counted under a Zeiss microscope (100x magnification) with a 1-ml Sedgewick-Rafter counting chamber. The microzooplankton taxa were identified to species level where possible.

Macroinvertebrates from each mesocosm were sampled by means of two types of artificial substrate. One type was made of nylon gauze (mesh size, 1 mm; 0.3 x 0.25 m) stitched to two stainless-steel frames (0.15 x 0.25 m each) in such a way that the gauze could be folded like a book cover. The other type of artificial substrate consisted of a wire frame filled with pebbles (diameter, 32 to 64 mm). The pebble basket was 0.15 m long x 0.15 m wide x 0.075 m high; the mesh size of the wire was 0.012 x 0.012 m.

Two gauze substrates, one at a water depth of 0.25 m and one at the sediment surface, and a pebble basket were placed at two sites in each mesocosm. The artificial substrates were left to be colonized for 4 weeks. Macroinvertebrates present on the artificial substrates were then removed and pooled into one sample. After identification (to species level where possible) and following the nomenclature of Mol [16], the organisms in each sample were counted.

To monitor short-term direct effects on several aquatic insect species, a floating 3 x 5-m frame was lowered onto the water surface of each mesocosm. The water surface within the frame was skimmed at preset time intervals during 1 week

posttreatment. Numbers of affected individuals of each species were counted. Organisms were considered to be affected when they showed abnormal locomotory behavior, including mortality.

Cage experiments. In order to quantify effects under controlled conditions (i.e., fixed numbers and relatively constant concentrations during the 48-h exposure period), we carried out in situ bioassays.

At day 6 and day 21 larvae of the midge *Chaoborus obscuripes,* nymphs of the mayfly *Cloeon dipterum,* and adults of the crustaceans *A. aquaticus* and *G. pulex* were introduced into cages. Those for *C. obscuripes* consisted of 195-ml glass jars with openings that were covered with stainless-steel gauze (mesh size, 0.7 mm). Ten or 20 specimens were placed in each jar. The cages for *C. dipterum, A. aquaticus,* and *G. pulex* consisted of a glass Petri dish (diameter, 0.12 m) closed with a cover of stainless-steel gauze (mesh size, 0.7 mm). Each cage contained eight to 11 specimens of a particular test species. Our intention was to place a set of two cages in each mesocosm. Sometimes, however, we had to make do with one cage due to lack of test organisms. Cages were positioned on the sediment surface. At the end of a 48-h exposure period, the number of specimens that had died or were immobile was scored. Mean scores for a set of cages were calculated for each mesocosm.

Comparison of effects in the laboratory and the field

Outline of the problem. It is common practice in laboratory toxicity experiments to expose a fixed number of organisms to a toxicant at a range of constant concentrations. The effect of the toxicant is described by a concentration–response model like that shown in Figure 2A. This model can be characterized by two effect concentration (EC) values, the EC10 and the EC50. In the field, the exposure regime as well as the number of organisms exposed are not the same as in the laboratory. For instance, concentrations vary over time; the numbers of individuals exposed are not known a priori and will differ between replicates. Therefore, the concentration–response model for the field will be different from the one based on a fixed concentration and number of individuals exposed.

Concentration–response model for the field. It was hypothesized that the exposure concentration is best characterized by an average exposure concentration (AEC) estimated by the area under the curve. The *y* axis plots the numbers of a particular species showing the response. This number does not have a fixed upper limit. Although scales for concentration and response in the laboratory and field concentration–response models are different, we assumed that direct toxic effects, summarized as EC10 and EC50, are equal in the field and the laboratory. The hypothetical concentration–response relationship would look like Fig. 2B. This model can also be characterized by its EC values. The assumption to be tested was that laboratory EC10 and EC50 values differ from those in the mesocosms.

Comparing EC10 and EC50 values in the laboratory and the field. The mesocosm study provided data on population densities in experimental ditches treated with various doses of chlorpyrifos (cf. Fig. 2C). The question now was whether these observations fitted the hypothetical model described above (Fig. 2B). To answer this question we fitted a similar type of model to the data. Due to stochastic variation, observed mesocosm ECs may differ from the known EC values of the hypothetical model. If these differences are small we conclude that mesocosm ECs and laboratory ECs are similar.

Calculation of AECs in mesocosms. The AEC for each mesocosm was calculated by combining the results of measurements at several sampling locations within the mesocosm. These concentrations were then used to calculate an average concentration over a certain period of time. For each location, the concentration was the average from the depth-integrated water samples in the macrophyte-dominated and macrophyte-free locations. Averaging over time was done using the measurements carried out on days 0 to 2. The choice of a 2-d time span was based on the results of the skimming. These indicated that effects of intoxication mainly occurred within the first 2 d posttreatment (see "Acute effects in mesocosms" in the Results section), which suggests that the actual concentrations within this time span were responsible for the acute effects on the arthropod species.

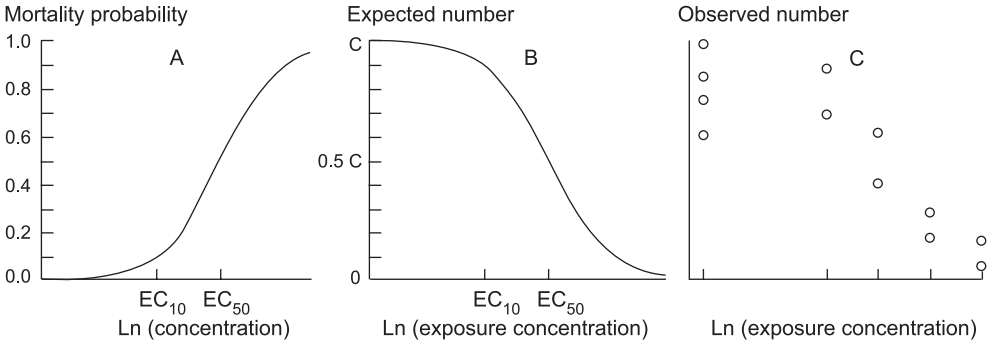

Fig. 2. (**A**) Concentration–response curve as for laboratory single-species toxicity tests. Curve shows logistic relation between probability of scored effect (e.g., mortality) and ln(concentration). (**B**) Hypothetical model describing the expected decrease in numbers of individuals in a field population in relation to increased exposure concentrations of a toxicant. Shape of curve is based on laboratory EC10 and EC50 values for corresponding species. This model assumes that equal exposure concentrations in the laboratory and the field result in equal effects. c = expected number of individuals in controls. (**C**) Response (observed number) of a hypothetical species to increasing exposure concentrations of a toxicant. Circles represent numbers observed at certain concentrations.

We used an integration method for averaging over time. This led to the following formula for calculating the AEC for each mesocosm:

$$\mathrm{AEC} = \frac{\sum_{i=1}^{n} \bar{c}_i \times \Delta t_i}{\sum_{i=1}^{n} \Delta t_i}$$

Where

$\bar{c}_i$ = $(c_i + c_{i-1})/2$, $\Delta t_i = t_i - t_{i-1}$, and $i = \{1 \ldots 3$;

t_1 = 0.03, t_2 = 1, and t_3 = 2 d posttreatment; and

c_i = average of chlorpyrifos concentrations measured in depth integrated water samples at t_i (n = 5).

Fitting a model to the observations in mesocosms. In order to obtain ECs for the mesocosms we fitted the following regression model for several arthropod taxa. We used abundances (macroinvertebrates on artificial substrates and zooplankton in water columns) measured 7 d posttreatment. Numbers were assumed to be quasi-Poisson-distributed [17] and to depend on the AEC in the following way:

$$\text{Expected number} = c/(1 + e^{-b(\ln(\mathrm{AEC}) - a)})$$

This model resulted in a sigmoid concentration–response curve for ln(AEC), with the parameters c = expected number in the control mesocosms; a = log of the concentration (ln[AEC]) at which expected numbers will have been reduced by 50%; and b = slope parameter.

The value of e^a is denoted by the mesocosm EC50. The mesocosm EC50 and mesocosm EC10 are defined as the AECs at which expected numbers will have been reduced by 50 and 10%, respectively.

Effects in cage experiments. The results of the in situ cage experiments were also used to estimate EC10 and EC50 values.

Because of the fixed number of test organisms and the relatively constant exposure concentrations, these were most similar to the corresponding laboratory EC values. Cage ECs can therefore be considered intermediate between mesocosm and laboratory ECs.

The effect scored was immobility, including mortality. The cage ECs and their 95% confidence intervals (CIs) were calculated using a log concentration–logit effect regression method [18]. Within the regression, calculated ECs were adapted for immobility and/or mortality in the controls [19]. Concentrations used as input for the regression model were estimated by calculating the geometric mean of chlorpyrifos concentrations at the start and at the end of the 48-h exposure period. Effects and

concentrations of the day 6 and day 21 cage experiments were pooled into one regression analysis.

The regression models for mesocosm and cage ECs were programmed in GENSTAT [20].

Protection level for the mesocosms

Several authors have advocated the use of low effect concentrations instead of no-observed-effect concentrations (NOECs) for establishing safe values [21–23]. We calculated mesocosm nominal EC10s ($EC10_{nom}s$) for several taxa. Calculation of mesocosm $EC10_{nom}s$ is similar to that of mesocosm EC10s, but concentrations are nominal initial concentrations. We calculated mesocosm $EC10_{nom}$ since regulatory bodies in the European Union use nominal concentrations in their risk assessment procedures for acute toxicity. Of the mesocosm $EC10_{nom}s$ we hypothesized the lowest to be a safe concentration as regards short-term direct effects of chlorpyrifos for all populations in the mesocosms.

To see whether mesocosm $EC10_{nom}s$ performed differently from conventional NOECs, we determined NOECs based on initial nominal concentrations ($NOEC_{nom}s$) by means of the Williams test [24,25]. Since testing on homogeneity of variance and normality cannot be done in a study with two replicates per treatment, we assumed that we met these criteria after a log transformation. Abundance values were $\ln(10x + 1)$ transformed [26]. The Williams test was applied to the 7-d-post-treatment data. Testing was done with the computer program Community Analysis, version 3.5 [27]. The short-term $NOEC_{nom}$ for the tested species was regarded as the highest treatment level at which numbers of that species did not differ significantly ($p < 0.05$) from those in the control mesocosms.

Table 2. Average and nominal chlorpyrifos concentrations in duplicate mesocosms

	Chlorpyrifos concn. (µg/L)			
Average	0.1	0.9	6.0	44.0
Nominal	0.10, 0.14	0.73, 0.99	4.94, 6.57	38.7, 48.4

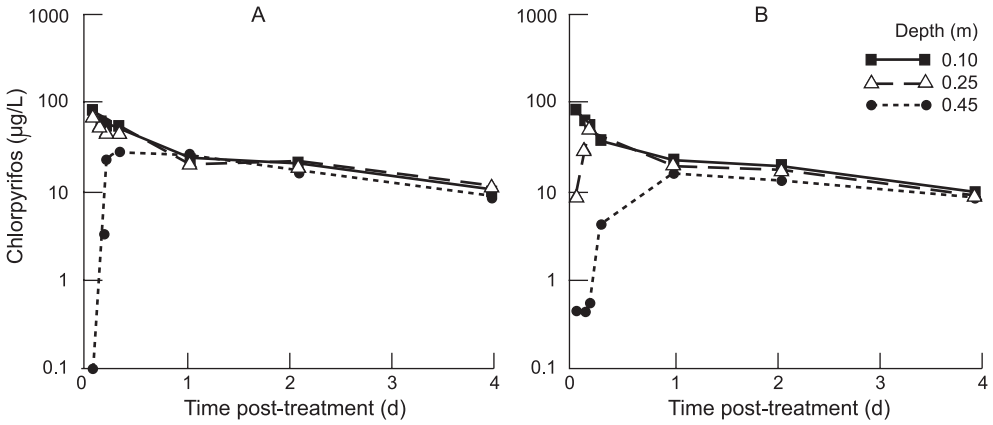

Fig. 3. (**A**) Spatial and temporal distribution of chlorpyrifos in a mesocosm. Initial nominal chlorpyrifos concentration, 44 µg/L. Samples collected in open water. (**B**) Spatial and temporal distribution of chlorpyrifos in a mesocosm. Initial nominal chlorpyrifos concentration, 44 µg/L. Samples collected in macrophyte-dominated locations.

Results

Chlorpyrifos residues in water

Variation in nominal concentrations between duplicates of the treatment levels ranged by a factor of 1.25 to 1.4 (Table 2). Treatment levels were considered the average nominal concentrations of the duplicate mesocosms. Within mesocosms, spatial variation in chlorpyrifos concentrations, both vertically and horizontally, were largest within 2 d posttreatment. As a result of the surficial spraying, the highest concentrations were initially found at the top of the water column, and the lowest were found at the bottom (Fig. 3A,B). In open-water locations, complete mixing of chlorpyrifos was recorded within 1 d (Fig. 3A). Stratification was maintained for as long as 2 to 4 d in macrophyte-dominated locations (Fig. 3B). The variation along the length of the mesocosms also decreased after spraying (Fig. 4), from 62% at 15 min to 12 to 14% at 1 d.

The dynamics of the mean chlorpyrifos concentrations in the integral water column is shown in Figure 5. Mean concentrations declined from about 40 to 50% of nominal concentrations 1 d posttreatment to 1 to 3% after 28 d. Rates of dissipation of chlorpyrifos from the water column were found to be more or less similar for all treatment levels. After the initial period of partitioning over environmental

compartments [15], the half-life of chlorpyrifos in the water column was estimated to be 10 to 18 d [28].

Toxicities for standard and indigenous species in the laboratory

The most susceptible standard species for chlorpyrifos was *Daphnia magna* (48-h LC50, 1.0 μg/L [6]). *Daphnia magna* was at the low end of the susceptibility range of the indigenous arthropods we tested (Table 3). The 48-h LC50 of the most susceptible species in our tests, *G. pulex,* was lower by a factor of 10 than that of *D. magna* (Table 3).

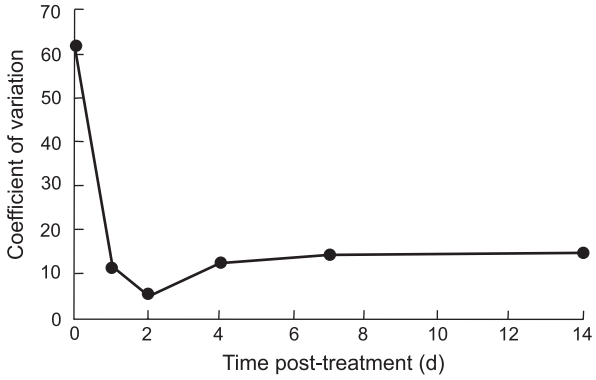

Fig. 4. Mean (n = 8) coefficient of variation (CV) of measured chlorpyrifos concentrations within depth-integrated water samples taken over the entire length of mesocosms in relation to time.

Table 3. Laboratory toxicity of chlorpyrifos for species indigenous in mesocosms

Species	48-h LC50 (μg/L)	95% CI (μg/L)
Gammarus pulex	0.08	0.05–0.14
Daphnia longispina	0.8	0.6–1.0
Simocephalus vetulus	0.8	0.7–0.9
Cloeon dipterum	1.0	0.8–1.4
Corixa punctata	6.0	4.2–8.5
Caenis horaria	>3	
Proasellus coxalis	>20	

CI = confidence interval; LC50 = lethal concentration.
Data from Van Wijngaarden et al. [12].

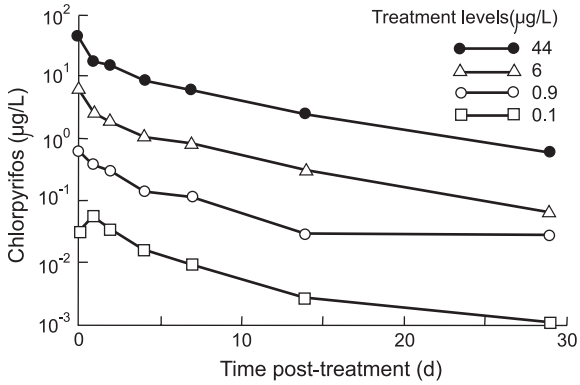

Fig. 5. Dynamics of mean chlorpyrifos concentrations in depth-in-tegrated water samples in mesocosms treated with nominal concentrations of 0.1, 0.9, 6, and 44 µg/L.

Acute effects in mesocosms

Acute effects mainly occurred within 24 h posttreatment (Fig. 6). The number of skimmed individuals increased with increasing treatment level. At the 0.1-µg/L treatment level results were similar to those in the control mesocosms. Skimming yielded only macroinvertebrates. Within this group, acute effects were observed only in insect taxa (Table 4).

Chlorpyrifos ECs in mesocosms

From 1 week before treatment to day 7, 120 macroinvertebrate and zooplankton taxa were collected [11]. Quantification of the effects of chlorpyrifos, based on mesocosm ECs, was achieved for only seven relatively abundant species (Table 5).

All taxa for which mesocosm or cage ECs could be calculated belonged to either the insect or the crustacean classes. All taxa except *Ablabesmyia phatta* and/or *A. monilis* midges had mesocosm EC10 and EC50 values <1 µg/L (Table 5). Of the four species that were caged and introduced for 48 h into the mesocosms, the isopod *A. aquaticus* showed the greatest resistance. The amphipod *G. pulex,* the mayfly (insect) *C. dipterum,* and the midge (insect) *C. obscuripes* again showed more or less similar susceptibilities, with cage EC10 and EC50 values <1 µg/L (Table 5).

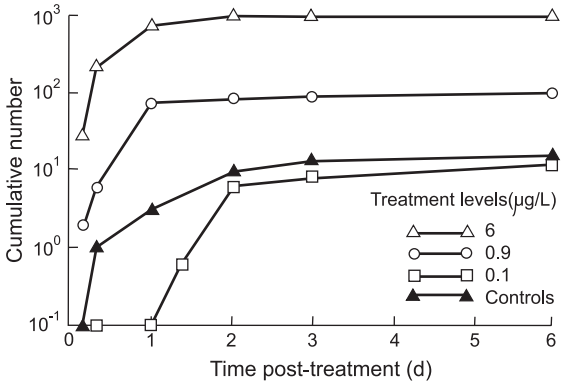

Fig. 6. Numbers of affected individuals of insects collected at water surface after chlorpyrifos treatment. Mean numbers (controls, $n = 4$; treated, $n = 2$) for treatment levels are used. Sampling times on day 1 were at 4 and 8 h posttreatment. Data for the 44-µg/L treatment level are not available.

Table 4. Mean numbers of affected individuals of taxa (all Insecta) found in skimming samples. Numbers are the accumulated results of 4-, 8-, and 24-h posttreatment samplings. Chlorpyrifos nominal treatment levels (0.1, 0.9, and 6) in micrograms per liter. Data for the 44-µg/L treatment level are not available

	Mean number affected			
Taxa	Controls	0.1	0.9	6
Notonecta spp.	0	0	1	48
Corixidae	0	0	1	347
Cloeon dipterum	0	0	2	258
Caenis horaria	<1	0	2	38
Coleoptera	<1	<1	0	30
Ceratopogonidae	0	0	<1	4
Chaoborus obscuripes	<1	0	61	440
Chironomidae	0	0	0	1
Odonata	0	0	<1	1
Trichoptera	0	0	1	2

Protection level for the mesocosms

For the seven species tested, mesocosm $EC10_{nom}$s were $\geq$0.03 µg/L (Table 6). The high variation in number of individuals per species tested was reflected by the (extreme) width of the 95% CIs in five of the seven cases (Table 6). We included covariables in the regression model for calculating ECs to correct for pretreatment differences in abundance between mesocosms by using the abundances at 7 d before treatment. As regards the 95% CIs, estimates of mesocosm $EC10_{nom}$ did not improve (Table 6). Furthermore, results for Coenagrionidae and *S. vetulus* indicated that the

respective models had not stabilized (Table 6). Given these results, we could not decide on a lowest mesocosm $EC10_{nom}$ and, consequently, on a presumably safe concentration nom for the mesocosms. A $NOEC_{nom}$ could be determined for 13 taxa. The lowest NOEC was <0.1 μg/L for Copepoda (Table 6). For all other taxa, $NOEC_{nom}$s were >0.1 μg/L.

Table 5. Chlorpyrifos effect concentrations (and 95% confidence intervals) in mesocosms and in the laboratory for several taxa indigenous in the mesocosms

	Chlorpyrifos effect concn[a] (μg/L)					
Taxon	x	Mesocosm ECx		96-h ECx	Laboratory Cage 48-h ECx	Laboratory 48-h ECx
Cloeon dipterum	10	0.2	(0.07–0.74)	0.1 (0.1–0.2)	0.1 (0.04–0.40)	0.3 (0.2–0.4)
	50	0.3	(0.17–0.50)	0.2 (0.2–0.2)	0.4 (0.21–0.60)	0.4 (0.3–0.4)
Caenis horaria	10	0.3	(0.13–0.54)	0.3 (0.3–0.6)	NA	NA
	50	0.4	(0.25–0.50)	0.5 (0.4–0.5)	NA	NA
Asellus aquaticus	10		NA	1.8 (1.4–3.0)	1.2 (0.56–3.71)	2.0 (1.2–4.3)
	50		NA	2.7 (2.1–3.6)	3.4 (2.22–5.13)	4.3 (3.3–5.6)
Gammarus pulex	10		NA	NA	0.1 (0.08–0.34)	NA
	50		NA	NA	0.3 (0.24–0.45)	NA
Coenagrionidae spp.	10	0.1	(0.00–33.3)	NA	NA	NA
	50	0.5	(0.02–12.8)	NA	NA	NA
Ablabesmyia spp.[b]	10	2.7	(1.08–7.00)	NA	NA	NA
	50	2.8	(1.41–5.75)	NA	NA	NA
Chaoborus obscuripes	10	0.4	(*–*)	0.3 (0.2–0.6)	0.4 (0.05–1.96)	0.6 (0.4–1.2)
	50	0.4	(*–*)	0.7 (0.6–0.8)	0.5 (0.17–1.60)	1.4 (1.1–1.7)
Mystacides spp.[c]	10	0.01	(0.00–1.98)	NA	NA	NA
	50	0.1	(0.01–2.01)	NA	NA	NA
Simocephalus vetulus	10	0.3	(0.00–23.9)	0.3 (0.2–0.4)	NA	NA
	50	0.6	(0.02–16.7)	0.4 (0.3–0.5)	NA	NA

NA = no data available.
– = no confidence intervals could be calculated.
[a] Effect concentrations (ECxs) in the mesocosms were obtained from logistic regression and from in situ cage experiments. Standard laboratory 48-and 96-h ECxs were obtained from Van Wijngaarden et al. [12].
[b] *Ablabesmyia* spp. consisted of *A. phatta* and *A. monilis*.
[c] *Mystacides* spp. consisted of *M. longicornis* and *M. nigra*.

Table 6. Chlorpyrifos 10% effect concentrations (Mesocosm $EC10_{noms}$) and no-observed-effect concentrations ($NOEC_{noms}$) (with 95% confidence intervals) for several indigenous taxa[a]

	Mesocosm $EC10_{nom}$		
Taxon	Without covariables	With covariables	$NOEC_{nom}$[b]
Cloeon dipterum	0.5 (0.20-1.40)	0.6 (0.39-0.84)	0.1
Caenis horaria	0.6 (0.34-1.08)	0.8 (0.26-2.22)	0.9
Coenagrionidae	0.05 (<0.001-80)	5.6 (*–*)	6
Ablabesmyia spp.[c]	1.3 (0.002-1.118)	1.5 (<0.001-4.534)	0.9
Chaoborus obscuripes	0.9 (*–*)	0.8 (*–*)	0.9
Mystacides spp.[d]	0.1 (0.002-6.7)	0.1 (0.015-0.62)	0.9
Simocephalus vetulus	0.03 (<0.001-25)	No convergence[e]	0.9
Hygrotus versicolor	NA	NA	0.9
Ceratopogonidae	NA	NA	0.9
Ostracoda	NA	NA	0.9
Ciliata[f]	NA	NA	0.1
Copepoda	NA	NA	<0.1
Copepod nauplii	NA	NA	0.9

NA = not analyzed

– = confidence limits could not be calculated.

[a] Exposure concentrations (μg/L) were based on initial nominal concentrations

[b] Determined by the Williams test

[c] *Ablabesmyia* spp. consisted of *A. phatta* and *A. monilis.*

[d] *Mystacides* spp. consisted of *M. longicornis* and *M. nigra*

[e] Regression model did not converge to optimal fit

[f] Ciliata were mainly *Hateria* spp.

Discussion

Comparison between laboratory and field results

Daphnia magna proved to be almost as sensitive as the more sensitive indigenous species. The data for *D. magna,* divided by a safety factor, could yield values likely to protect the more susceptible species. The difference between the laboratory EC50s of chlorpyrifos for *D. magna* and that for the most susceptible indigenous species we found (*G. pulex*) was a factor of ca. 10 (*D. magna,* 1.0; *G. pulex,* 0.08 μg/L). However, if one aims at minimizing effects on the mesocosms (e.g., 10 or 5% effect levels), then a safety factor of 100 should be applied since the 48-h LC10 for *G. pulex* is 0.03 μg/L [12].

A comparison between the short-term responses of indigenous species in laboratory toxicity tests and the mesocosm study indicated that laboratory EC values were good estimations of ECs in the mesocosms. Laboratory and mesocosm ECs differed by less than a factor of three for the seven species studied. The cage experiments confirmed this similarity between laboratory and field results. We considered differences of a factor of two to three between laboratory and mesocosm

ECs to be nonsignificant. It is known that even under standardized laboratory conditions the EC50s of a toxicant can vary by considerably more than a factor of three within a single species [29,30].

We aimed at estimating fair exposure concentrations (AECs), meaning that concentrations in a certain exposure period could be considered responsible for caused effects. We therefore averaged over a short period of time (2 d). Averaging over a longer period yields lower exposure concentrations, which results in lower values for mesocosm ECs. In practice, however, the choice of the length of the exposure period seemed not to be very critical; differences between mesocosm ECs resulting from AECs over periods of 1, 4, and even 7 d were relatively small (Table 7). Furthermore, in spite of considerable chlorpyrifos concentration gradients in space and time (Figs. 4A,B, and 5), mesocosm ECs gave results similar to the ECs measured at constant concentrations. This indicated that, in the case of a single pulse immision with chlorpyrifos, variable and constant exposure regimes led to comparable effects.

The effect scores consisted of observations at day 7. Seven-day effect scores could be used for measuring short-term direct effects, if we could assume that these were not yet influenced by recovery and/or recolonization processes. We therefore looked whether this assumption was justified for the daphnid *S. vetulus*. Of the species tested, this was the only one with a short reproductive cycles. None of the treatment levels indicated an increase in numbers within week 1 (Table 8).

The results of both the mesocosm and cage ECs show that acute laboratory single-species toxicity tests can be used to estimate short-term direct effects in the field for populations of the same species. This agrees with other studies with chlorpyrifos and other pesticides [31–34].

Our pragmatic approach in calculating AECs and using laboratory EC values yields a simple method to link the fate of and responses to a pesticide in field situations. In cases where measured concentrations are not available, fate models that estimate AECs can be used for the calculation of effects in the field if toxicity data for indigenous species are available. However, it has been pointed out that, at present, model prediction of exposure is still associated with many uncertainties [35].

Safety factor for the mesocosms

One of our aims was to establish an $EC10_{mesocosm}$ based on the mesocosm $EC10_{nom}$ of the most susceptible indigenous species. Our results, however, did not allow us to draw firm conclusions on a lowest mesocosm $EC10_{nom}$ because of the low accuracy of these estimations (Table 6). Only seven of the 120 collected species could be considered for analysis by the regression method for calculating mesocosm EC_{nom}s. Of these, only two gave reliable results (Table 6). The low number of species tested and the large uncertainties associated with their mesocosm $EC10_{nom}$ estimations were due to the limited number of dominant species as well as to the high variation in

numbers within mesocosms (in time) and between mesocosms (in space). An example of a data set which was typical of the abundances of species in the mesocosms is given in Table 9, with the resulting model shown in Fig. 7. In addition to this high variation, another problem is that no optimal concentration range and in-between intervals can be chosen for all taxa in a field study [36]. This also results in wider CIs for the species concerned. In general, the regression approach has some important statistical advantages (quantification of an effect percentile, ECx may lie outside the concentration range, indication of quality of estimation by CIs, and highest analytical flexibility) [36,37]. In our case, however, the limited number of experimental units, the high biological variation observed, and the adopted concentration range restricted a successful use of this approach for estimating a safe concentration for the mesocosms.

The use of the Williams test allowed us to establish conventional NOECs. Except for one case, the lowest $NOEC_{nom}$s we found were at the 0.1-μg/L treatment level (Table 6), with lowest $LOEC_{nom}$s at 0.9 μg/L. A serious drawback of the analysis of variance (ANOVA) approach, in this case the Williams test, is its lack of information on the amount of effect still occurring at the NOEC (i.e., the lack of power of the test [21]). In our study, effects were probably negligible at the $NOEC_{nom}$ of 0.1 μg/L; skimming results at the 0.1-μg/L treatment level were similar to those in the controls (Fig. 6), while the lowest cage EC10s were 0.1 μg/L (Table 5). This would suggest that a factor of 0.1 times the toxicity parameter of the most susceptible standard species (*D. magna*; 48-h LC50, 1.0 μg/L) would have yielded a safe concentration of chlorpyrifos in the mesocosms.

Taking into account the most susceptible taxa we found in the mesocosms (Copepoda spp.; $NOEC_{nom}$, <0.1 μg/L) and in the laboratory (*G. pulex*; 96-h LC10, 0.02 μg/L), an extra safety factor of about 0.1 should be applied. Hence, factors of 0.1 and 0.01 times the 48-h LC50 of *D. magna* provide, respectively, a liberal and a conservative safe chlorpyrifos concentration for the mesocosms.

Pusey et al. [38] found a treatment level of 0.1 μg/L applied for 6 h (single pulse) to have a nonsignificant impact on running waters. Our observations suggest that this treatment level also produces a negligible effect concentration in standing waters. The present report deals only with short-term direct effects. The question remains, however, of whether at the 0.1-μg/L treatment level, long-term direct or indirect effects on populations or the community that went unnoticed at a short-term observation may occur. These categories of effects of chlorpyrifos on the population and community level will be the subject of the second article in this series [11].

Table 7. Calculated chlorpyrifos effect concentrations (ECxs) in mesocosms (with 95% confidence intervals) for several indigenous taxa. Effect scores were based on sampling at 7 d posttreatment for all mesocosm ECs. Exposure concentrations were based on average exposure concentrations over time periods of 1, 2, 4, and 7 d posttreatment. Average exposure concentrations were estimated by an integration method.

		Mesocosm ECx (µg/L)			
Taxon	x	1-d exposure	2-d exposure	4-d exposure	7-d exposure
Cloeon dipterum	10	0.3 (0.08–0.89)	0.2 (0.07–0.74)	0.1 (0.04–0.48)	0.1 (0.03–0.47)
	50	0.3 (0.20–0.60)	0.3 (0.17–0.50)	0.2 (0.11–0.32)	0.2 (0.08–0.29)
Caenis horaria	10	0.3 (0.15–0.65)	0.3 (0.13–0.54)	0.2 (0.08–0.34)	0.1 (0.06–0.45)
	50	0.4 (0.29–0.60)	0.4 (0.25–0.50)	0.2 (0.15–0.32)	0.2 (0.12–0.30)
Coenagrionidae spp.	10	0.1 (<0.01–51)	0.1 (0.0–33.3)	<0.1 (0.00–20.4)	<0.1 (0.00–16.0)
	50	0.7 (0.02–18.9)	0.5 (0.02–12.8)	0.3 (0.01–8.82)	0.3 (0.01–6.29)
Ablabesmyia spp.	10	3.9 (*–*)	2.7 (1.08–7.00)	2.0 (*–*)	1.5 (0.66–3.25)
	50	4.0 (*–*)	2.8 (1.41–5.75)	2.1 (*–*)	1.5 (0.82–2.75)
Chaoborus obscuripes	10	0.5 (*–*)	0.4 (*–*)	0.3 (*–*)	0.2 (*–*)
	50	0.5 (*–*)	0.4 (*–*)	0.3 (*–*)	0.3 (*–*)
Mystacides spp.	10	0.01 (<0.01–6.66)	0.01 (<0.01–1.98)	<0.1 (0.00–1.04)	<0.1 (0.00–1.03)
	50	0.1 (0.01–2.69)	0.1 (0.01–2.01)	0.1 (0.01–1.17)	0.1 (0.00–1.05)
Simocephalus vetulus	10	0.3 (<0.00–40.0)	0.3 (0.00–23.9)	0.2 (0.00–14.89)	0.2 (0.03–16.0)
	50	0.8 (0.02–26.6)	0.6 (0.02–16.7)	0.4 (0.01–10.86)	0.3 (0.01–8.62)

– = no confidence limits could be calculated.

Table 8. Mean numbers of *Simocephalus vetulus* at various single-dose treatment levels of chlorpyrifos at 1, 2, and 7 d posttreatment. Percentages are relative to the control mesocosms

Treatment level (µg/L)	Mean number (percentage)		
	1d	2d	7d
0	13.1 (100)	18.8 (100)	16.6 (100)
0.1	14.3 (109)	21.8 (116)	6.7 (40)
0.9	9.2 (70)	14.4 (77)	11.6 (70)
6	0.8 (6)	0.8 (4)	0.1 (1)
44	0.4 (3)	0.5 (3)	0 (0)

Table 9. Abundance of Coenagrionidae spp. collected on artificial substrates in mesocosms

Week posttreatment	Abundance											
	Controls				0.1[a]		0.9		6		44	
-4	6	1	4	4	6	5	2	0	11	2	5	2
-1	30	18	12	3	24	6	18	3	3	15	7	26
1	21	34	12	3	26	2	18	2	4	2	0	0
2	13	17	8	3	26	0	22	1	1	3	0	0

[a] Chlorpyrifos treatment levels in micrograms per liter.

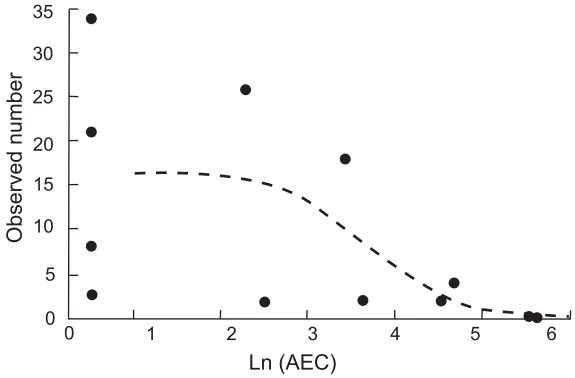

Fig. 7. Observed numbers of Coenagrionidae on artificial substrates versus ln (averaged exposure concentration) (points), and estimated regression model versus ln (averaged exposure concentration) (line).

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3 Effects of the insecticide Dursban® 4E (Active ingredient chlorpyrifos) in outdoor experimental ditches: II. Invertebrate Community Responses and Recovery

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Abstract

This article describes the long-term effects on the macroinvertebrate and zooplankton community in outdoor experimental ditches after a single application of the insecticide chlorpyrifos. Nominal concentrations of 0.1, 0.9, 6, and 44 μg/L of chlorpyrifos were applied to two mesocosms each, while four served as controls. Both macroinvertebrates and zooplankton were sampled from 4 weeks before to 55 weeks after treatment. The macroinvertebrate and zooplankton data sets were combined into one data set and analyzed using the multivariate ordination technique "redundancy analysis." The method provided a clear description of the effects on the invertebrate community in time while still showing the effects at the species level. Crustacea and Insecta showed a rapid, concentration-dependent decrease in numbers after insecticide application (direct effects). An increase in gastropods and Oligochaeta was found, suggesting indirect effects. The start of recovery of the invertebrate populations affected was found to depend not only on the susceptibility of the taxa but also on ecological characteristics, such as the length of the life-cycle. A no-observed-effect concentration of 0.1 μg/L could be derived both at the species and the community level. Safe concentrations, based on no-observed-short-term-effect levels for some characteristic indigenous taxa susceptible to chlorpyrifos, also appeared to protect the total invertebrate community in the long term. The invertebrate community at all treatment levels was considered to have recovered after 24 weeks posttreatment.

Keywords: Mesocosms; Invertebrate community; Multivariate ordination; techniques; Recovery; NOEC

Introduction

Model ecosystems that mimic freshwater ecosystems are often used to assess the potential ecotoxicological hazards of pesticides [1–3]. A major advantage of these experimental freshwater ecosystems is their simulation of realistic pesticide exposures to aquatic organisms in a complex ecosystem. Thus, effects on and recovery of a wide array of species can be studied while allowing interactions between the various populations of a community.

This article is the second of a series of three dealing with the impact of a single application of the insecticide Dursban® 4E (active ingredient chlorpyrifos) on the ecology of outdoor mesocosms. The studies presented in this series were initiated in order to evaluate the significance of standard laboratory tests for predicting effects of a pesticide in aquatic ecosystems. The first article compared acute toxicity to indigenous species in the laboratory with short-term effects in the mesocosms. It also proposed a safe concentration for the mesocosms based on short-term effects observed in these systems [4]. The third article will deal with the effects of the insecticide on ecosystem functioning and oxygen metabolism in particular.

The aims of this article are to describe long-term effects of a single application of the insecticide chlorpyrifos on invertebrate populations and the invertebrate community of outdoor experimental ditches, to evaluate the rate of recovery of susceptible populations and the invertebrate community, and to set safe threshold values for susceptible indigenous populations and the invertebrate community.

Materials and methods

Experimental design On May 8, 1990, the organophosphorus insecticide Dursban® 4E was applied once by means of a spray boom to eight outdoor experimental drainage ditches (mesocosms). Four dose levels were applied to two mesocosms each, while four other systems served as controls. Each mesocosm had the following characteristics: length, 40 m; width at water surface, 3.4 m; water volume, 60 m^3; and mean water depth, 0.5 m. Details of the construction and equipment of the mesocosms can be found in Drent and Kersting [5]. The aquatic community in the mesocosms resembled that of macrophyte-dominated drainage ditches.

The nominal concentrations of the active ingredient chlorpyrifos, calculated from the amounts of insecticide sprayed and the water volume of the mesocosms, were 0.1, 0.9, 6, and 44 μg/L. These concentrations are related to agricultural application in the sense that the lowest treatment level is considered a safe standard concentration, while the highest corresponds to a "realistic worst case" scenario. Common agricultural application of chlorpyrifos in the Netherlands results in predicted environmental concentrations (PECs) of 0 to 64 μg/L (authors'

calculations). Detailed information on the experimental design can be found in the first article of this series [4].

Invertebrate community sampling and analysis

The invertebrate data set. From week -4 through week 56 the zooplankton and macroinvertebrate communities were sampled 15 times. These communities were sampled in both macrophyte-dominated and macrophyte-free locations. The sampled individuals were identified in the laboratory, to species level if possible. The sampling and identification methods are described in detail in part I [4].

To evaluate the effects of the insecticide at the level of the invertebrate community, all zooplankton and macroinvertebrate data sets had to be combined into one. Abundances of macro-zooplankton (>300 μm) in macrophyte-free and macrophyte-dominated locations were lumped. The lumped data set was then used to calculate average numbers for each mesocosm. The averages (numbers per liter) of the macrozooplankton and the data set of the microzooplankton were combined into a single zooplankton data set. As was described in detail in part I, the macro-invertebrates were sampled in both macrophyte-free and macrophyte-dominated locations by means of artificial substrates [4]. Samples of the two locations were also lumped and average numbers calculated. Abundance data for zooplankton (numbers per liter) and macroinvertebrates (numbers per substrata) were ln (10x + 1)-transformed (for the rationale of this transformation see Van den Brink et al. [6]) and subsequently standardized. The following formula was used for standardization:

$$\text{abundance values data set Macroinv.}_{\text{standardized}} = \sqrt{\frac{tss_{\text{data set Zoopl.}}}{tss_{\text{data set Macroinv.}}}} * \text{abundance values dataset Macroinv.}$$

where where *tss* is the total sum of squares of the corresponding macroinvertebrates (Macroinv) and zooplankton (Zoopl) data sets. This standardization was needed to make both data sets equally important in terms of amount of variance. In our case, the "square root term" in the formula resulted in a factor of 0.98. As a consequence, the log-transformed abundance values of the macroinvertebrate data set were multiplied by 0.98. All statistical analyses were performed using the invertebrate data set thus obtained.

Multivariate analysis of treatment effects. Effects at the community level can be analyzed by means of multivariate regression techniques such as principal component analysis (PCA) [7] and redundancy analysis (RDA). Redundancy analysis is the constrained form of PCA and has the advantage of allowing effects of explanatory variables to be expressed and can be combined with a Monte Carlo permutation test

for statistical analysis [8]. These techniques have a limited and comprehensible output, even when starting with complex and large data sets. They provide a clear overview of temporal and treatment effects on a community and can indicate recovery of this community [8].

In the present study, the responses and recovery in time of the invertebrate community after the Dursban® 4E treatment were analyzed using RDA. The sampling periods, comprising weeks -4 through 24 and weeks 42 through 55 (before and after the winter season, respectively), were analyzed separately.

Principal component analysis and RDA are based on a linear response model. This means that they calculate a linear regression line from the abundance data of all samples. This regression line represents a fraction of the total variance in the data set and is presented in a diagram as the first axis (see Fig. 3). A second regression line is extracted from the remaining variance, representing the second axis of the diagram. In extracting the regression lines, PCA takes into account all variance of a data set. In contrast to PCA, RDA is constrained to the fraction of the total variance that is explained by the explanatory variables. These explanatory variables are fixed upon the analysis a priori.

The percentage of the total variance of the data set explained by the explanatory variables is called the sum of all canonical eigenvalues. The axes in an RDA (e.g., Fig. 3) represent a percentage of this sum. The higher these percentages, the more variation is explained by the axes. Values of about 30 to 40% are quite common in ecological applications [9]. For more theoretical background information and technical details see Ter Braak [9–11]. Specific details on the application of RDA to the results of model ecosystem experiments are given in Van Wijngaarden et al. [8].

Redundancy analysis was performed using the CANOCO computer program, version 3.14 [10]. In the RDA, the factors "treatment" and "sampling week," plus their interaction, were used as combined dummy explanatory variables since we wanted to focus on the relevant variance of the invertebrate data set (i.e., only that variance which can be attributed to time or treatment). Since macrophytes play an important role in structuring the aquatic invertebrate community and since the macrophyte biomass at the time of application is an important factor influencing the fate and effects of Dursban® 4E in aquatic ecosystems [12], macrophyte biomass was used as a covariable to correct for possible systematic differences between the mesocosms. In order to obtain a good macrophyte biomass estimate for the period comprising weeks -4 through 24, the mean of the macrophyte biomasses sampled in weeks -2 and 13 was used as a covariable. Macrophyte biomass for these sampling weeks was estimated (in kilograms dry weight per m^2) by sampling the macrophytes in five 1-m^2 plots in each mesocosm. The macrophyte biomasses of the mesocosms, used as covariables, are given in Table 1. Only one mesocosm showed a deviant biomass, one replicate of the 0.9-µg/L treatment. Because no macrophyte biomass estimations were available for the period consisting of weeks 42 through 55, covariables were used only in the

first analysis (weeks -4 through 24). Within CANOCO, we opted for scaling 1 (euclidean distances) since dummy explanatory variables were used [13]. Apart from this, the default options were chosen.

Table 1. Macrophyte biomass used as covariable in the redundancy analysis for weeks -4 through 24. The mean biomass values for the sampling weeks -2 and 13 are shown.

Replicate number	Macrophyte biomass (kg dry wt./m²)				
	Control mesocosms	0.1 µg/L Treatment mesocosm	0.9 µg/L Treatment mesocosm	6 µg/L Treatment mesocosm	44 µg/L Treatment mesocosm
1	0.26	0.22	0.26	0.27	0.24
2	0.26	0.26	0.14	0.26	0.28
3	0.24	-	-	-	-
4	0.26	-	-	-	-

To check whether treatment-related differences shown in the RDA diagrams were statistically significant, Monte Carlo permutation tests, incorporated in CANOCO, were carried out. General concepts of Monte Carlo permutation testing, combined with ordination, have been described in Ter Braak et al. [10,14,15]. The permutation tests used in the present study have been described in Van Wijngaarden et al. [8].

Before testing, treatment levels were log-transformed. We did so because dose–response curves are intrinsically sigmoid [16], and this allowed us to fit the dose–response curve as closely as possible to the linear response model in the RDA. Because of the limited options for permutation, permutation testing of each treatment separately against the controls was useless. Therefore, all treatments were tested jointly with controls. The tests were performed for each sampling week, with $\ln(20x + 1)$-transformed nominal concentrations as log–dose, where x is the nominal concentration (for the rationale see Van den Brink et al. [6]).

No-observed-effect concentration calculations. To study effects on and recovery of separate taxa, univariate analyses were performed on the 19 most discriminant species of the RDA analysis of the period comprising weeks -4 through 24. These analyses used the Williams test [17], which assumes an increasing effect for an increasing dose. This test allowed us to establish a no-observed-effect concentration (NOEC) ($p < 0.05$) for each sampling week for each taxon. The Williams test was performed using the Community Analysis computer program, version 3.5 [18].

Before the $NOEC_{community}$ could be obtained, a variable had to be calculated that best summarized the community variance. Redundancy analysis is not suitable for providing this variable because it uses explanatory variables, which are a priori-related to the toxicant. In PCA, however, the entire unconstrained variance of the data set is

taken into account. Therefore, PCA was used to calculate the first principal component, which is the single variable that summarizes the community variation best; it is a linear combination of the species data, not a priori-related to the toxicant. Principal component analysis was performed on the invertebrate data set for each sampling week using the CANOCO computer program. When the principal component of the samples (coordinates of the first PCA axis) was analyzed with the Williams test, we tested whether these coordinates represented the treatment regime. These analyses resulted in an $NOEC_{community}$ for each sampling week.

Analysis of functional groups. It may be questioned whether effects on individual species are reflected in the properties of the community. We therefore evaluated effects on functional feeding groups of macroinvertebrate taxa. Five groups can be distinguished: shredders, scrapers, predators, collector filter-feeders, and collector gatherers [19,20]. Zooplankton was excluded since no information on functional groups was available. The original macroinvertebrate data set was used for the analysis. All abundance values of taxa belonging to the same functional group were added up; if a taxon belonged to two or three functional groups, its abundance value was divided evenly over these functional groups. From these summations, the relative share of each functional group could be calculated. These calculations were done for three periods: weeks -4 through -1, weeks 1 through 4, and weeks 47 through 51.

Results

General sampling results for the invertebrate community

A total of 189 taxa were identified and their abundance determined (59 zooplankton and 130 macroinvertebrate taxa). In terms of the numbers of taxa, the most important taxonomic groups were Insecta (103), Rotatoria (36), Crustacea (22), and Gastropoda (15).

In the first week after insecticide treatment the number of arthropod taxa decreased substantially at the two highest treatment levels (Fig. 1A), unlike the number of nonarthropod taxa (Fig. 1B).

Before treatment, no differences in functional group composition or in absolute numbers of macroinvertebrate individuals sampled were observed between treatments (Fig. 2A). Compared to the controls, numbers of macroinvertebrates were significantly lower at the 0.9-μg/L treatment level and higher (Fig. 2B). At these treatment levels, ratios of the functional groups had shifted; shares of collector gatherers decreased and shares of collector filterers increased (Fig. 2B). One year after treatment the relative share of collector gatherers and scrapers was found to have decreased in all treatments (Fig. 2C). At all treatment levels except the highest, the share of shredders had increased in the year after treatment (Fig. 2C).

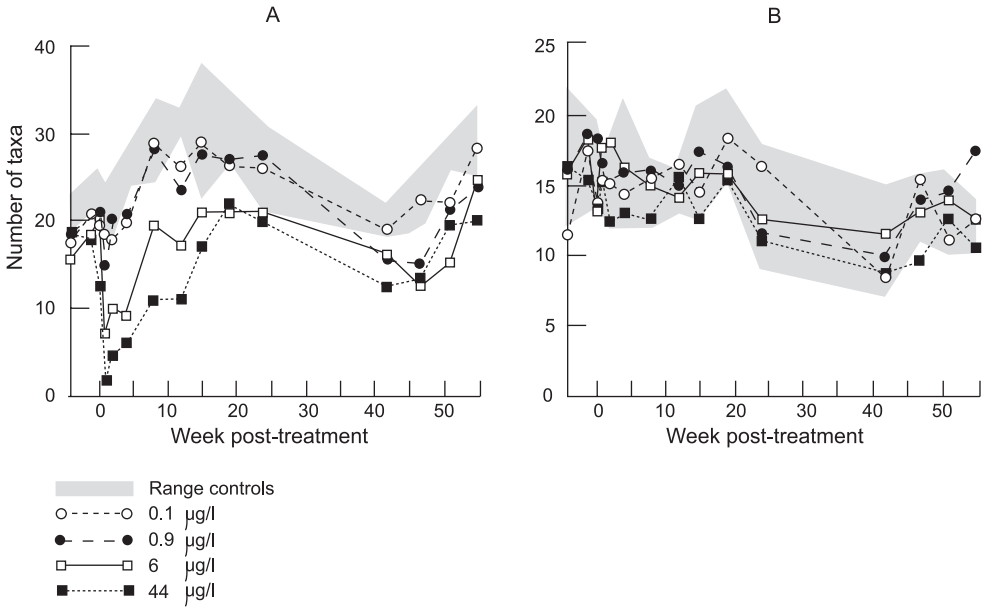

Fig. 1. Dynamics of numbers of arthropod (**A**) and nonarthropod (**B**) taxa. Shaded areas represent the minimum and maximum numbers collected in the control mesocosms. The lines represent the average number of taxa collected per treatment.

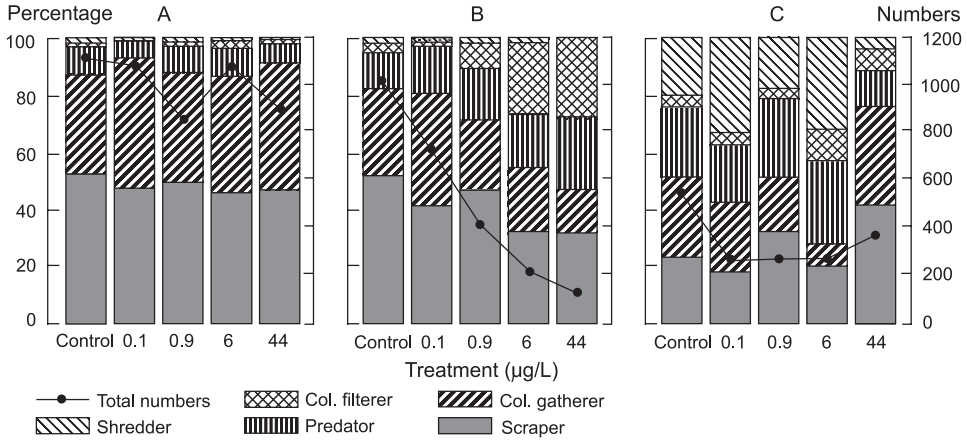

Fig. 2. Relative shares (%) of the macroinvertebrate individuals sampled for the functional feeding groups averaged over three periods. (A) Before treatment (weeks -4 and -1). (B) Weeks 1 through 4. (C) Weeks 47 through 51. The absolute numbers of sampled individuals per treatment are also indicated.

Multivariate and univariate analysis

Sampling period of weeks -4 through 24. The RDA diagram (Fig. 3) summarizes the treatment effects in the data set while still showing the approximate species composition for all samples. In the diagram, samples with nearly identical species composition lie close together, while samples with very different species composition lie far apart. If an imaginary line is drawn through a species point and the origin of the plot, the relative abundance of this species in all samples can be derived by perpendicularly projecting the sample point on this imaginary line. The samples projecting on the "species line" far away from the origin but on the same side of the origin as the species point contain relatively high numbers of this species. The greater the distance between the projection of a sample and the origin, the more abundant this species is in this sample. If a sample point projects on the other side of the origin compared to the species point, numbers of this species are relative low in this sample. In the diagram, the species *Cloeon dipterum* is relatively abundant in all control samples and (almost) absent from the samples of weeks 1, 2, and 4 for the highest treatment level. To limit the number of taxa shown in the diagram, only the 45 most discriminant taxa in each analysis are presented. The 45 most discriminant taxa are defined as the 45 taxa with the highest fractions of variance explained by the axes.

The RDA indicated pronounced effects of the insecticide application on the invertebrates (Fig. 3). The diagram reveals a dose–effect relationship; the magnitude of the effect of the treatment decreases in the order 44 > 6 > 0.9 > 0.1 μg/L ≈ controls. The clustering of all pretreatment samples indicates minor differences between the mesocosms at the start of the experiment. The shift of the control samples from the left to the right indicates a time vector in this direction. The line representing the 0.1-μg/L treatment level is situated closest to the control line and most closely resembles its pattern. All week 24 samples of the treated mesocosms are situated close to the corresponding control samples, indicating that differences at 24 weeks posttreatment were minor. This suggests recovery of the invertebrate community in all treated mesocosms. The direction of the treatment vector is from the upper left quadrant to the lower right quadrant (Fig. 3). Those taxa affected negatively by the treatment are situated in the upper left quadrant and above the line representing the control treatment. Insusceptible and positively affected taxa are situated below this line. The treatment resulted in a decrease in the numbers of most arthropods, especially ephemeropterans, dipterans, coleopterans, zygopterans, trichopterans, megalopterans, amphipods, cladocerans, copepods, and ostracods. Nonarthropods showing a decreasing tendency included Ciliata (mainly *Halteria* sp.), and the mollusks Sphaeriidae and *Armiger crista.* The RDA diagram indicates a positive correlation between the numbers of gastropods (*Bithynia tentaculata* and *Radix peregra*), the leech *Erpobdella octoculata*, and oligochaetes on the one hand and treatment levels on the other.

Table 2. Results of the Williams test ($p < 0.05$) of the discriminant taxa of the redundancy analysis. The no-observed-effect concentration (NOEC) of each taxon is given per sampling week. Only those taxa that showed a significant response in two consecutive sampling weeks are presented

		Sampling week[b]														
Taxon	Effect[a]	-4	-1	0.1	1	2	4	8	12	15	19	24	42	47	51	55
Annelida																
Oligochaeta	+	>	>	>	>	>	0.9	6	>	>	>	0.9	>	>	>	>
Stylaria lacustris	+	>	>	>	>	>	0.9	0.9	>	>	>	>	>	>	>	>
Arthropods																
Crustacea																
Simocephalus vetulus	-	>	>	0.9	0.9	0.9	0.9	6	>	>	>	>	>	>	>	>
Daphnia galeata	-	n.p.	n.p.	n.p.	>	>	0.1	0.1	6	6	>	>	n.p.	n.p.	>	>
Ostracoda	-	n.p.	L-	6	0.9	6	6	0.9	6	6	6	>	n.p.	L-	>	>
Copepoda (mature stages)	-	>	>	>	L-	6	>	0.9	>	>	>	>	>	>	>	>
Copepoda (nauplii)	-	>	>	0.9	0.9	0.9	0.9	6	>	n.p.	>	n.p.	n.p.	>	>	>
Gammarus pulex	-	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0.1	L-	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Insecta																
Caenis horaria	-	>	>	6	0.9	0.9	0.1	0.9	6	0.9	6	0.9	>	0.1	L-	0.9
Caenis luctuosa	-	n.p.	n.p.	6	n.p.	0.9	0.1	0.9	>	>	n.p.	n.p.	n.p.	n.p.	L-	L-
Cloeon dipterum	-	>	>	0.9	0.1	0.1	0.1	6	>	>	>	>	>	>	>	>
Coenagrionidae	-	>	>	>	6	6	6	>	>	>	>	>	>	>	0.1	0.1
Sialis lutaria	-	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0.1	n.p.	0.1	0.1	n.p.	0.1	n.p.	L-	n.p.
Hygrotus versicolor	-	n.p.	n.p.	>	0.9	0.9	n.p.	6	0.9	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.
Mystacides longicornis and *M. nigra*	-	>	>	0.9	0.9	L-	0.1	>	6	6	6	>	n.p.	>	>	n.p.
Ablabesmyia phatta and *monilis*	-	>	>	6	0.9	0.9	0.9	6	6	>	0.9	n.p.	n.p.	0.1	0.9	n.p.
Ceratopogonidae	-	>	>	>	0.9	>	L-	6	6	>	>	>	n.p.	n.p.	>	n.p.
Chaoborus obscuripes	-	L-	>	0.1	0.9	0.1	0.9	6	6	6	>	6	>	>	>	>
Chironomus	-	n.p.	n.p.	n.p.	>	n.p.	>	>	6	6	>	>	n.p.	n.p.	n.p.	>

[a] + indicates a significant increase in numbers in treated mesocosms relative to controls: - indicates a significant decrease.

[b] L- indicates an NOEC < 0.1 µg/L; n.p. indicates that the Williams test was not performed because of the absence of the taxon from two or more controls (this criterion was used only when the effect of the treatment was negative); > indicates a NOEC of >44 µg/L.

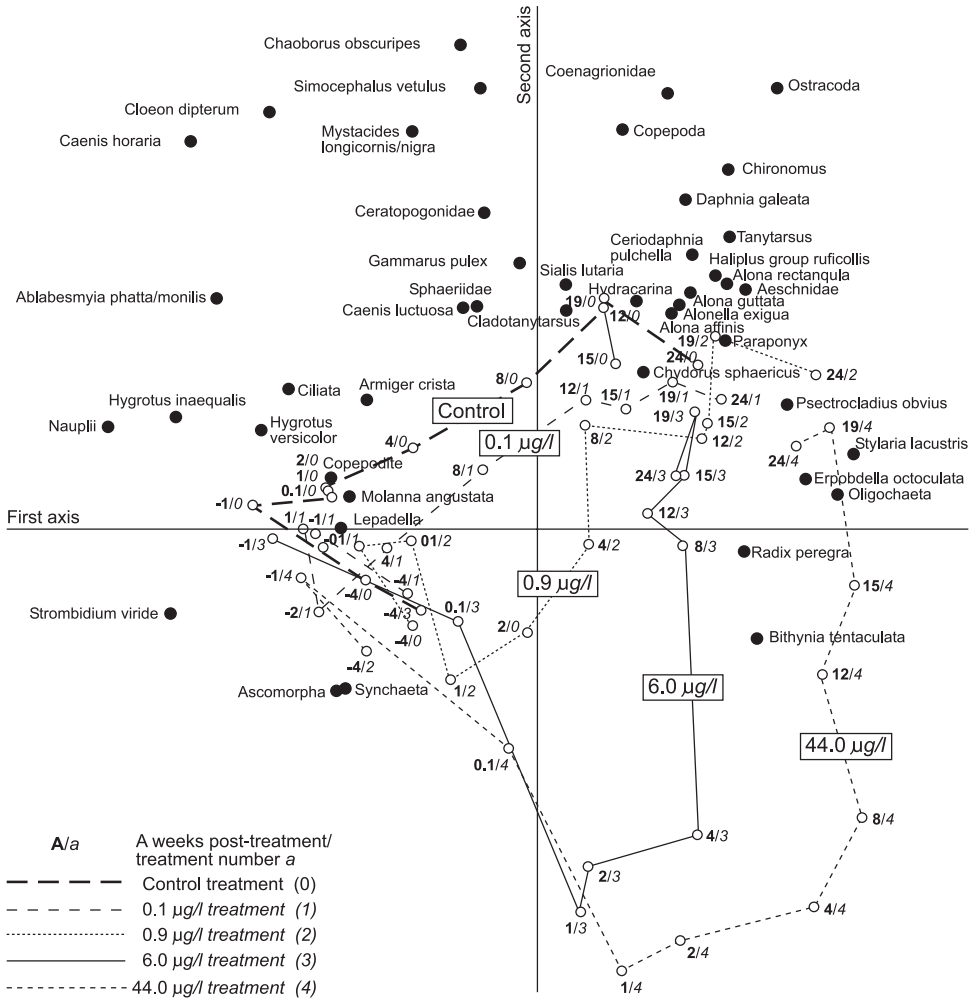

Fig. 3. Ordination diagram (redundancy analysis [RDA]) indicating effects of a single application of the insecticide chlorpyrifos on zooplankton and macroinvertebrates. The sampling period covered weeks -4 through 24. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables, macrophyte biomass was taken as a covariable. The lines represent the course of the treatment levels in time. Of all variance, 55% can be attributed to the explanatory variables. Of this explained variance, 37% is displayed in the diagram. Only those 45 species most discriminant for the diagram are shown.

No-observed-effect concentrations are presented for those taxa that showed a consistent response, i.e., a significant response on two or more consecutive sampling weeks (Table 2). Negative effects were most pronounced from weeks 0.1 through 4. Most taxa recovered within 24 weeks. *Caenis horaria* and *Gammarus pulex* failed to recover fully within the first 24 weeks posttreatment. The statistical analysis indicates that Oligochaeta spp. and *Stylaria lacustris* were significantly more abundant in the high treatment levels than in the controls (Table 2). *Cloeon dipterum* showed a decrease in numbers in the 0.9-, 6-, and 44 µg/L treatments compared to the controls. The 0.9- and 6-µg/L treatments returned to control abundance values within 8 weeks posttreatment; the 44-µg/L treatment, within 15 weeks. In contrast to *C. dipterum*, *C. horaria* failed to return to control abundance values in the 6-and 44-µg/L treatments within 24 weeks (Table 2 and Fig. 4).

Sampling period of weeks 42 through 55. The RDA over the sampling period of weeks 42 through 55 indicates treatment-related differences in species composition (Fig. 5), with the effect of the treatment decreasing in the order 44 ≈ 6 ≈0.9 > 0.1 µg/L ≈ controls. The direction of the treatment vector in the RDA diagram (Fig. 5) is from the top to the bottom. The direction of the time vector is from left to right. Taxa less abundant in the treated mesocosms are situated at the top, and the insusceptible and positively affected taxa are situated at the bottom. *Gammarus pulex, C. horaria*, and Coenagronidae spp. occurred in significantly lower numbers at the highest treatment levels (Table 2). *Gammarus pulex* was almost absent from the 0.9-µg/L and completely absent from the 6-and 44-µg/L mesocosms in week 55 (Fig. 6). Taxa that occurred in higher densities than in the controls (though no significant differences could be demonstrated) included the *Agrypnia/Dasystegia/Phryganea* complex and *Bithynia tentaculata* (see Fig. 5 and Table 2).

Monte Carlo permutation and $NOEC_{community}$. No significant differences between the invertebrate communities could be demonstrated before treatment (Table 3). After insecticide application, the permutation tests showed the treatment to have a significant effect on the invertebrate community until week 24. After week 42 the effect became significant again. However, when *G. pulex* was omitted, no significant effects could be demonstrated after 24 weeks posttreatment. The lowest $NOEC_{community}$ found was 0.1 µg/L (Table 3).

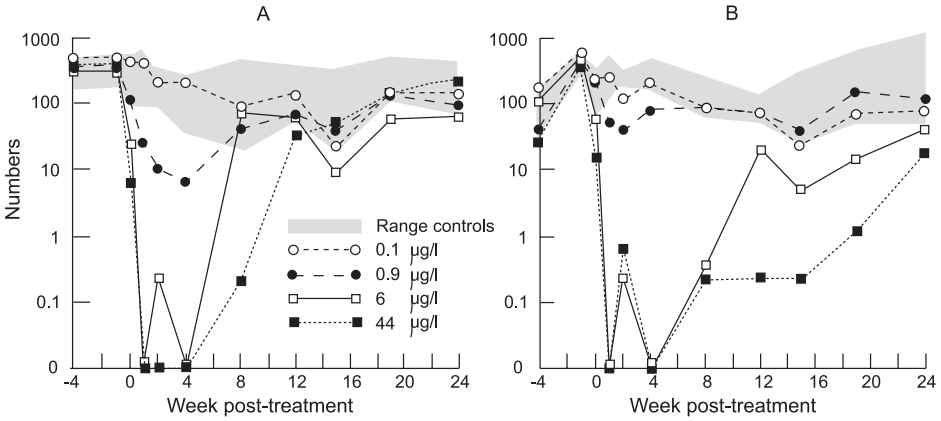

Fig. 4. Dynamics of numbers of the ephemeropterans *Cloeon dipterum* (**A**) and *Caenis horaria* (**B**). Shaded areas represent the minimum and maximum numbers collected in the control mesocosms. The lines represent the average numbers collected per treatment.

Discussion

Overall ecological effects

The application of the higher treatment levels of chlorpyrifos in our mesocosms resulted in a pronounced decrease in the number of arthropod species (Fig. 1A,B) and in a reduction of all arthropod populations abundant at the time of application (Table 2). The RDA diagram (Fig. 3) can be seen as a mean response pattern of all susceptible arthropod populations, suggesting a concentration-dependent negative effect during the first week after treatment and (the start of) recovery within 24 weeks. However, caution should be exercised in the interpretation of locations of taxa in the RDA diagram in terms of susceptibility to chlorpyrifos only. Seasonal aspects, such as natural succession, should also be taken into account. For example, the most susceptible species according to laboratory tests. *G. pulex* [21], was collected in most mesocosms (including controls) at the time of treatment (Table 2). Numbers increased in the controls and 0.1-µg/L mesocosms in the course of the experiment and failed to do so at the two highest treatment levels (Fig. 6). This is why the position of this species in the RDA diagram is not really extreme (in view of the treatment level), in contrast to relatively less or equally susceptible species, such as *C. dipterum* and *Chaoborus obscuripes* [21], that were abundant at the time of insecticide treatment. Nevertheless, the significantly lower numbers of *G. pulex* in the 0.9-, 6-, and 44-µg/L treatments compared to the controls (Fig. 6 and Table 2) can be explained from its susceptibility to chlorpyrifos (96h lethal concentration [LC50] of 0.07 µg/L [21]). In general, the negative effects on arthropod populations observed in our study are in

accordance with results of single-species toxicity tests [21] and other aquatic model ecosystem studies performed with chlorpyrifos [12,22–31].

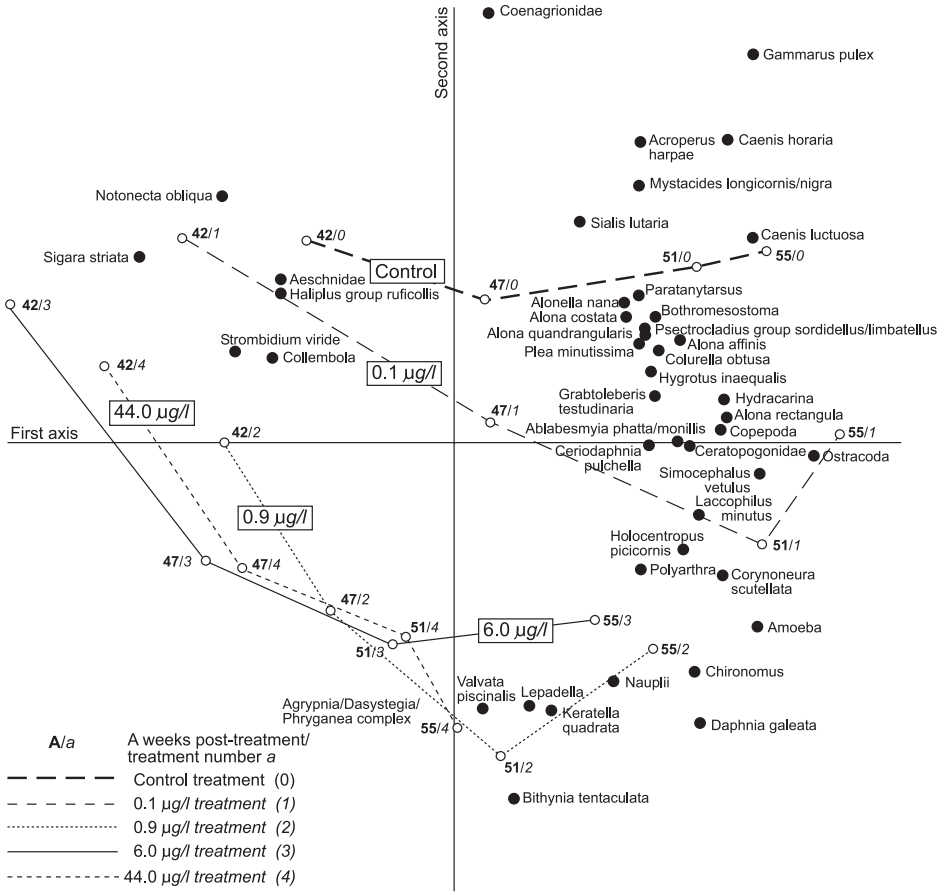

Fig. 5. Ordination diagram (redundancy analysis [RDA]) indicating effects of a single application of the insecticide chlorpyrifos on zooplankton and macroinvertebrates. The sampling period covered weeks 42 through 55. Sampling week and treatment level, as well as their interactions, were taken as explanatory variables. The lines represent the course of the treatment levels in time. Of all variance, 49% can be attributed to the explanatory variables. Of this explained variance, 40% is displayed in the diagram. Only those 45 species most discriminant for the diagram are shown.

The loss of arthropod populations in the first week posttreatment (direct effects) did not result in many detectable indirect effects on other invertebrate populations. Significant effects on nonarthropod populations of zooplankton (Rotatoria, Ciliata) could not be demonstrated. Of the macroinvertebrates, only the oligochaete worms (Oligochaeta spp., *S. lacustris*; Table 2) showed significant increases in abundance. A

similar indirect effect of chlorpyrifos on *S. lacustris* was observed in one of our experiments in indoor microcosms and was explained by the increased supply of food in the form of periphytic algae after the loss of arthropod grazers [28]. In the three indoor microcosm experiments performed within the same research program, in which the communities of drainage ditches were simulated [27,32], indirect effects of a nominal chlorpyrifos treatment of 35 μg/L were much more diverse than those of the highest treatment level (44 μg/L) in the mesocosms. In the microcosms it was the invertebrate populations of Rotatoria, Turbellaria, Hirudinea, Oligochaeta, Mollusca, and Isopoda which showed indirect effects. The structure of the community in the indoor microcosms, however, was less complex than that of the outdoor mesocosms. Apparently, a structurally more diverse and complex ecosystem includes more redundant populations and feedback mechanisms, so indirect effects are harder to detect.

An understanding of the trophic structure of the community in the mesocosms is important in assessing the impact of chlorpyrifos stress. Before treatment, differences between the treatment levels in the distribution of macroinvertebrate individuals over functional groups were found to be small (Fig. 2A). Many of the susceptible arthropod taxa found appeared to be generalists rather than specialists with regard to their food habits [20]. In addition, the susceptible populations of Insecta in particular comprised all functional groups. Nevertheless, the share of collector gatherers showed a dose-dependent decrease in the first month after treatment due to the loss of susceptible taxa such as *C. horaria* and *C. dipterum* (Figs. 2 and 4). At the same time, the share of collector filterers increased, partly due to the (nonsignificant) increase in numbers of the snail *B. tentaculata* and the significant increase in oligochaete worms at the two highest treatment levels (Figs. 3 and 2B). Both functional groups use fine particulate organic matter (FPOM) as their food resource [20], so the loss of collector gatherers can explain the increase in collector filterers. One year after chlorpyrifos treatment, consistent differences between treatments in the relative shares of collector gatherers and collector filterers could no longer be demonstrated (Fig. 2C). In all treatments except the 44-μg/L mesocosms, the share of shredders was relatively high compared with the previous periods. This can be attributed to the increased abundance of the amphipod *G. pulex* in the controls and 0.1-μg/L treatment and of the isopod *Asellus aquaticus* in the 0.9- and 6-μg/L treatments (results not shown). Given that in the 44-μg/L treatment shredders were almost absent 1 year after chlorpyrifos treatment and that the most important shredders in freshwater ecosystems are usually arthropods, this functional feeding group should be considered at least potentially susceptible to insecticide contamination (low redundancy). This is in accordance with observations of a decrease in shredder populations and breakdown of plant litter in microcosms treated with 35 μg/L chlorpyrifos [27,32] and with observations by Wallace et al. [33], who reported similar effects in a headwater stream treated with methoxychlor.

Table 3. *p*-values calculated with the Monte Carlo permutation tests and no-observed-effect concentration ($NOEC_{community}$) values calculated by the Williams test for two data sets, the total invertebrate data set and the total invertebrate data set except for *Gammarus pulex*

Technique and Data set	Sampling week[a]														
	-4	-1	0.1	1	2	4	8	12	15	19	24	42	47	51	55
Monte Carlo permutation (*p* value)															
Invertebrates	>	>	0.01	0.01	0.01	0.01	0.01	0.01	0.03	0.01	>	>	0.03	0.02	0.01
Invertebrates without *G. pulex*	>	>	0.01	0.01	0.01	0.01	0.01	0.01	0.05	0.02	>	>	>	>	>
Williams test ($NOEC_{community}$)															
Invertebrates	>	>	>	0.1	0.1	0.1	6	>	6	6	>	>	6	0.1	>
Invertebrates without *G. pulex*	>	>	>	0.1	0.1	0.1	6	>	>	6	>	>	>	>	>

[a] > indicates *p* values >0.05 and NOECs >44 µg/L.

Table 4. Reported no-observed-effect concentration ($NOEC_{ecosystem/community}$) values for chlorpyrifos in freshwater model ecosystems

(µg/L)	Type of system	Dose regime	Reference
<0.5	Lotic, outdoor, mesocosms	Chronic (100 d)	[29]
<0.1	Lentic, indoor, microcosms	Chronic (50 d)	[6]
<0.1	Lotic, outdoor, mesocosms	Chronic (21 d)	[39]
<0.5	Lentic, outdoor, mesocosms	Acute	[26]
<0.5	Lentic, indoor, microcosms	Acute	[30]
<1.7	Lentic, outdoor, microcosms	Acute	[24]
0.1	Lotic, outdoor, mesocosms	Acute (6 h)	[31]
0.1	Lentic, outdoor, mesocosms	Acute	This study

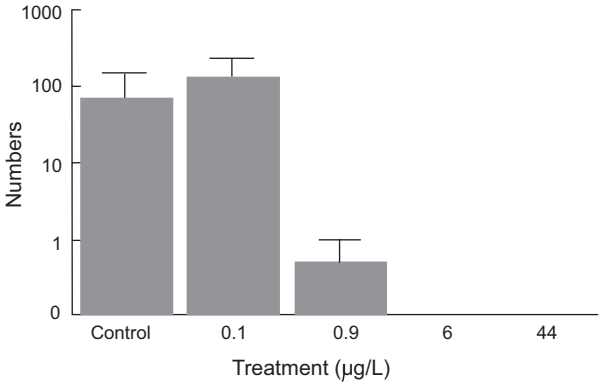

Fig. 6. Number of *Gammarus pulex* (average ± SD) per treatment level sampled in week 55.

Recovery

In this article we consider a susceptible population to be recovered from chlorpyrifos stress when, over a prolonged period of time, significant differences in abundance between control and treated mesocosms can no longer be demonstrated.

In considering the recovery of arthropods at the species level, it is convenient to distinguish between populations of Crustacea, which complete their life-cycle strictly in water, and populations of Insecta, which usually have distinct aquatic and terrestrial life phases. Of the Crustacea in our mesocosms, representatives of Cladocera (*Simocephalus vetulus, Daphnia galeata*), Ostracoda, and Copepoda (including nauplii) showed a relatively rapid recovery within 12 to 24 weeks, even at the highest treatment level (Table 2). The relatively rapid recovery of microcrustaceans can be explained by their short life-cycle and/or high reproductive capacity. In addition, pesticide-insensitive resting stages may be of importance (e.g., ephippia of daphnids). These properties allow a rapid development to normal population densities starting from a few surviving individuals or viable diaspores or after a few propagules happen to enter the treated systems after the insecticide concentration has dropped to below critical threshold levels. The lack of recovery of *G. pulex,* even a year after chlorpyrifos application (Fig. 6 and Table 2), can be explained by the fact that this species has no resistant life stages and by the complete extermination of the population at the higher treatment levels. In addition, recolonization of this strictly aquatic amphipod was apparently restricted by the lack of connections between the mesocosms.

In aquatic insects, which are characterized by an adult terrestrial life stage and an ability to fly, the isolated position of the mesocosms probably does not restrict recolonization. It can be argued that the generation time is one of the important factors influencing rate of recovery in insects. For example, *C. dipterum* and *C. horaria* are more or less equally susceptible to chlorpyrifos (with 96-h effective concentration [EC50] values of 0.2 µg/L and 0.5 µg/L, respectively [21]), yet *C. dipterum* showed a

rapid recovery, while *C. horaria* showed a delayed recovery (Table 2 and Fig. 4). *Cloeon dipterum* produces two or more generations per year [34] and can thus recover rapidly. *Caenis horaria,* however, produces only one generation per year [34] and consequently has a much smaller "time window" for recovery. Even 1 year after treatment this taxon was less abundant at the highest treatment levels compared to the controls (Table 2 and Fig. 4B). In the RDA diagram the species therefore occupies an extreme position for this treatment level (Fig. 5, upper right quadrant). Delayed recovery due to a relatively long generation time could also have occurred in *Mystacides longicornis* and *M. nigra,* Coenagrionidae, *Caenis luctuosa,* and *Sialis lutaria,* all of which situated in the upper right quadrant of Figure 5. Coenagrionidae and *S. lutaria* are reported to have one generation every 1 or 2 years [35,36]. *Mystacides longicornis, M. nigra,* and *C. luctuosa* have two generations per year, but the second generation is smaller in number than the first [37,38]. Caution should be exercised, however, in interpreting the position of these taxa in the RDA plot in terms of effects and recovery. At the end of the experiment, these taxa occurred in low numbers in the controls as well (mean abundance, <10 individuals).

The above examples of recovery at the species level show that the start of recovery cannot be predicted by simply calculating the time when the concentration of the insecticide becomes less than the laboratory NOEC or EC10 for the species concerned. Life-cycle characteristics must also be taken into account. This makes recovery at the species level hard to predict. Hence, toxicity and ecological data at the species level are needed to explain, and eventually predict, recovery.

At the level of the invertebrate community as a whole, results of the Monte Carlo permutation test suggest a recovery at the start of the winter season (Table 2 and Fig. 3). However, when all taxa are taken into account, the effect becomes significant again in week 47 (the following spring). When *G. pulex* is not taken into account, the treated mesocosms remain indistinguishable from the controls. Since the lack of recovery of this amphipod can be regarded as an artefact due to the isolated position of the mesocosms, the invertebrate community of the treated mesocosms was judged to have (potentially) recovered after 24 weeks.

Safe threshold levels

Although occasionally an NOEC <0.1 μg/L was calculated for some taxa in our mesocosm study, it seems reasonable to set the overall safe threshold level for susceptible species at 0.1 μg/L (Table 2). In the case of *C. obscuripes* and Ostracoda, the occasional NOECs <0.1 μg/L occurred during the pretreatment period, indicating systematic differences between mesocosms not related to the treatment or perhaps type I errors. In the case of *C. luctuosa,* an NOEC <0.1 μg/L was calculated for two consecutive sampling weeks (weeks 51 and 55; Table 1). This species, however, was present in very low numbers only. As a consequence, absence data are less indicative

of the effects of chlorpyrifos. Furthermore, the most severe direct effects were expected in weeks 0 through 4, when chlorpyrifos concentrations were highest [4]. The occasional NOECs <0.1 μg/L found for Copepoda, Ceratopogonidae, and *Mystacides* spp. in this initial period might be a result of a type I error or indeed a direct effect of chlorpyrifos. In any case, the power of these NOEC values is limited because they were not always consistent with those of the preceding and subsequent sampling weeks. However, we are well aware that by leaving these incidental NOECs out of consideration, small transient effects on these taxa may be overlooked.

At the level of the invertebrate community, the chlorpyrifos treatment resulted in a concentration-dependent response. Figure 3 and the Williams test performed on the coordinates of the first PCA axis allowed us to determine an $NOEC_{community}$ value of 0.1 μg/L. In part I of the present series of articles, a short-term NOEC of 0.1 μg/L was reported for susceptible indigenous species [4]. The present mesocosm study thus shows safe concentrations based on no observed short-term effects on susceptible taxa to be adequate for protection of these taxa and the invertebrate community in the long term.

Several other model ecosystem studies have attempted to set safe threshold levels for chlorpyrifos at the community or ecosystem level (Table 4). In most of these studies, however, even the lowest concentrations tested showed effects, which meant that NOECs could not be determined. This seems to be a general problem in most of the microcosm and mesocosm studies that have been performed with pesticides. Hence, if one aims at better estimates of safe threshold values for ecosystems, it will be necessary to include lower test concentrations in future model ecosystem experiments.

The safe threshold value for chlorpyrifos of 0.1 μg/L that we found in our mesocosm study corresponds with the outdoor model stream experiment of Pusey et al. [31]; both experiments were characterized by an acute exposure regime (Table 4). In our study, chlorpyrifos concentrations declined relatively fast after the single application [4].

Two studies in which chronic exposure concentrations of chlorpyrifos were maintained for 50 and 21 d [6,39] found that a level of 0.1 μg/L resulted in significant effects. Thus, in estimating safe threshold levels for ecosystems it seems wise to differentiate between acute and chronic exposure regimes. In the case of chlorpyrifos in Dutch drainage ditches, an acute exposure regime is more realistic because of the limited number of applications to agricultural crops and the relatively rapid decrease in bioavailability (rapid hydrolysis and sorption to organic matter) [40].

Evaluation of data analysis

Ordination was found to be a powerful tool for evaluating effects at the community level in ecotoxicological experiments [8]. In the present study the RDA ordination

technique provided a clear description of the effects at the invertebrate community in time while still showing the effects at the species level. The great advantage of RDA over other multivariate techniques used in ecotoxicology [41] is that species and samples are analyzed simultaneously, so a "feedback" toward the species level is relatively easy. The RDA diagram allows hypotheses about ecological interactions to be made.

Another advantage of the implementation of ordination in ecotoxicology is the ability of statistical testing for the significance of effects at the community level [8]. The Monte Carlo permutation test has the advantage of testing all variance of a community. This test, however, has low power when few replicates per treatment are used. Testing the coordinates of the first PCA axis with the Williams test has the benefit of providing $NOEC_{community}$, but it takes only a fraction of the total variance into account.

Conclusions

The chlorpyrifos treatment resulted in a reduction in those arthropod invertebrate taxa which were abundant at the time of application. Based on long-term observations, NOECs of 0.1 μg/L could be determined for the most susceptible species in the mesocosms and for the invertebrate community. This safe threshold level is similar to that established in the first part of this series [4], suggesting that, in the case of a single application, safe concentrations based on short-term observations are sufficient to protect communities in the long term. When a taxon starts to recover depends not only on the actual chlorpyrifos concentrations but also on its life-cycle characteristics and on infrastructural aspects of the ecosystem (e.g., the degree of isolation). The RDA ordination technique provided a clear description of the effects on the invertebrate community.

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4 Effects of chlorpyrifos in freshwater model ecosystems: the influence of experimental conditions on ecotoxicological thresholds

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Abstract

Three experiments to determine the impact of the insecticide chlorpyrifos (single applications of 0.01 to 10 µg a.i. litre^{-1}) in plankton-dominated nutrient-rich microcosms were conducted. The microcosms (water volume approximately 14 litres) were established in the laboratory under temperature, light regimes and nutrient levels that simulated cool 'temperate' and warm 'Mediterranean' environmental conditions. The fate of chlorpyrifos in the water column was monitored and the effects on zooplankton, phytoplankton and community metabolism were followed for 4 or 5 weeks. The mean half-life ($t_{½}$) of chlorpyrifos in the water of the test systems was 45 h under the 'temperate' conditions and about 30 h under the 'Mediterranean' environmental conditions. Microcrustaceans (cladocerans and copepod nauplii) were amongst the most sensitive organisms. All three experiments yielded community NOECs (No Observed Effect Concentrations) of 0.1 µg a.i. litre^{-1}, similar to those derived from more complex outdoor studies. Above this threshold level, responses and effect chains, and time spans for recovery, differed between the experiments. For example, algal blooms as an indirect effect from the impact of exposure on grazing organisms were only observed under the 'Mediterranean' experimental conditions. The relatively simple indoor test system seems to be sufficient to provide estimates of safe threshold levels for the acute insecticidal effects of low-persistence compounds such as chlorpyrifos. The robustness of the community NOEC indicates that this threshold level is likely to be representative for many freshwater systems.

Keywords: aquatic; pesticide; risk assessment; microcosms; community NOEC

Introduction

Aquatic microcosms and mesocosms are relatively complex test systems which are regularly employed in the higher-tier risk evaluation of pesticides. Often these test systems have their own unique characteristics (e.g. dimensions, location, community composition) and the outcome of tests may depend on these and other factors. Though microcosm and mesocosm studies provide more realistic risk assessments than lower-tier single-species tests, the results are more difficult to interpret and extrapolation to other types of ecosystems may be problematic.[1] For these reasons, evaluation and interpretation of such studies have become the subject of wide-ranging discussions.[2 - 4] The present study was initiated following the questioning of whether mesocosm studies conducted with chlorpyrifos in North Western Europe (eg [5 - 7]) were valid for Mediterranean regions because of differences in environmental conditions.

Because temperatures and light intensities are generally higher during the growing season in southern Europe, it may be expected that physico-chemical processes might here lead to higher dissipation rates of chemicals and so reduce the bioavailability of these compounds.[8] On the other hand, temperature and toxicity are positively correlated for most chemicals: toxicity increases as temperature increases.[9] These counteracting factors make it difficult to predict the relative sensitivity of test systems under warmer conditions compared to those in ecosystems tested under more temperate conditions. Additionally, Mediterranean water systems in agricultural areas often contain relatively high amounts of nutrients and can be considered as highly eutrophic. This might result in more severe direct or indirect effects compared to those under milder temperate conditions.

In the present study, we tested whether the ecological threshold levels and effects on plankton communities associated with exposure to the insecticide chlorpyrifos differed under cool 'temperate' and warm 'Mediterranean' environmental conditions. Because of their convenience and controllability, in combination with their simplified ecosystem traits, we used indoor semi-realistic microcosms which are considered an intermediate between laboratory standard toxicity tests and mesocosm or field studies.[1, 3]

The purpose of the present paper is twofold. Firstly, we compare threshold levels for chlorpyrifos generated in the microcosm studies under different experimental conditions and, secondly, we compare these with the threshold levels reported for complex outdoor test systems.

Materials and methods

Experimental set-up

Three experiments were carried out, which will be referred to as Experiment 1 (Expt. 1_{Med}) and Experiments 2A (Expt. $2A_{temp}$) and 2B (Expt. $2B_{Med}$). The latter two were conducted simultaneously. The experiments were performed in microcosms situated in a water bath for temperature regulation in a climate-controlled room (Fig. 1). The microcosms simulated plankton-dominated nutrient-rich freshwater systems. They consisted of glass cylinders (diameter 0.25 m, height 0.35 m, volume 18 litre), and contained a sediment layer of approximately 0.02 m and a water layer of 0.3 m (water volume c. 14 litre). Sediment and water were collected from an uncontaminated eutrophic ditch (Sinderhoeve Experimental Station, Renkum, The Netherlands). The systems were seeded with zooplankton and phytoplankton from uncontaminated waterbodies at the same experimental station and from a pond at the Alterra institute (Wageningen, The Netherlands). In Expts $2A_{temp}$ and $2B_{Med}$, the microcosms were also incubated with *Daphnia* gr. *galeata* originating from a temporary laboratory culture at Alterra.

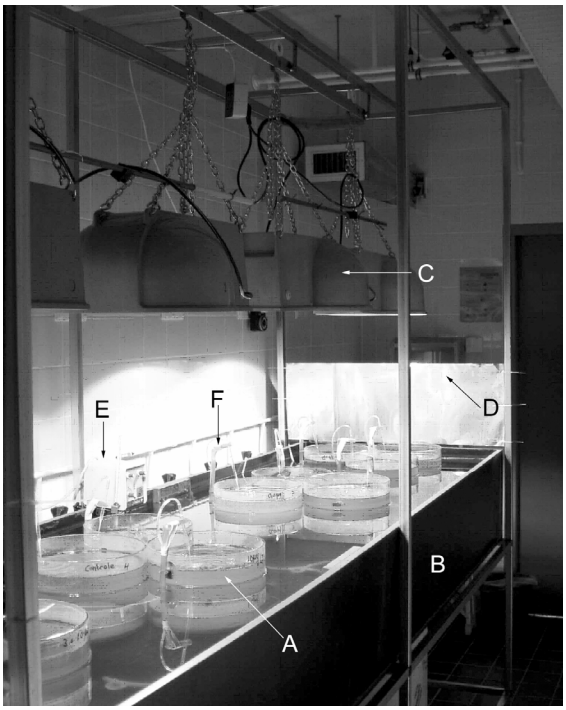

Fig. 1. Microcosms in a temperature-controlled water bath. A: microcosm (glass cylinder), B: water bath, C: lamps providing artificial daylight, D: reflecting shield, E: thermostat, F: aeration system

Conditions for phytoplankton growth were stimulated by adding nutrients (NH_4NO_3 and KH_2PO_4) to the microcosms twice a week, starting two weeks before the chlorpyrifos treatments. Nutrient levels were chosen to simulate productive to highly productive aquatic agro-ecosystems (Table 1). To suppress periphyton growth, five snails (*Lymnaea stagnalis*) per system were introduced. Additionally, in Expts $2A_{temp}$ and $2B_{Med}$ the walls of the microcosms were brushed once a week.

Table 1. Experimental conditions.

	Experiment		
	1 'Mediterranean'	2A 'temperate'	2B 'Mediterranean'
Type	warm, productive	cool, productive	warm, highly productive
Mean temp. (°C)	24 –28	16 -18	25 –28
Mean light (µE m^{-2} s^{-1})	295 (12 h)	175 (14 h)	399 (12 h)
Nutrients (mg N $litre^{-1}$)	0.09	0.09	2.0
(mg P $litre^{-1}$)	0.015	0.015	0.2
Micronutrients	no	no	yes
Treatment (µg a.i. $litre^{-1}$)	0, 0.01, 0.1, 1, 10	0, 0.01, 0.1, 1	0, 0.01, 0.1, 1
Replication[a]	n = 4 (c), n = 2 (t)	n = 3 (c & t)	n = 3 (c & t)

[a] c: controls; t: treatments

Test system conditions

Water temperature, light intensity and duration of illumination period differed amongst the experiments (Table 1). Experiment 2A represented temperate conditions, and Experiments 1 and 2B represented 'Mediterranean' conditions. Temperatures increased during the day due to the lamps (Philips HPI-T 400WE 40 high-pressure metal halide lamps) providing artificial daylight. Light intensity was measured by means of a Li.cor LI-185B light meter.

To prevent growth of a bacterial layer on the water surface of the microcosms and to stimulate some water movement, compressed air was used to provide a light air-flow (0.5-1.5 litre min^{-1} $vessel^{-1}$) over the water surface. Water losses due to evaporation were replenished with demineralised water.

Treatment and fate of test substance

Single applications of chlorpyrifos in the form of Dursban® 480 (Dow AgroSciences, UK), an emulsifiable concentrate (EC) formulation containing 480 g a.i. $litre^{-1}$, were made to give concentrations from 0.01 - 10 µg a.i. $litre^{-1}$ (Table 1) in the microcosm water.

Before application, sub-samples were taken from the stock solutions for determination of initial nominal concentrations. Next, appropriate aliquots of these stock solutions were evenly distributed over the water surface of the microcosms and

gently stirred to promote equal concentrations throughout the complete water column.

Concentrations of chlorpyrifos in the water were determined 1 day before and 1, 4, 8 hours and 1, 2, 4, 7, 14, and 28 days after application of the test substance. Analysis was stopped when chlorpyrifos was below the detection limit (about 20 ng a.i. litre^{-1}) on two successive sampling dates.

Depth-integrated water samples were taken in duplicate from microcosms by means of a Perspex® tube (length 50 cm, diameter 3.9 cm) and transferred into glass flasks. Of these samples, 100 ml was used for chlorpyrifos analysis. Directly after collection, flasks were weighed for determination of the exact mass of the volume of water samples. Next, redistilled hexane (35 ml) was added. Water and hexane were mixed on an orbital shaker (circa 175 rpm) for at least 0.5 h for extraction of chlorpyrifos into the hexane layer. A quantified amount of the hexane was collected in a tube. For further concentration of the extract, the hexane was evaporated in a water bath (40 °C) with air. The air-dried samples were dissolved in hexane (1.5 ml) and shaken on a vortex mixer. Chlorpyrifos was determined by splitless injection (1 µl) on a HP 5890 gas chromatograph equipped with an electron-capture detector (ECD) and an HP 6890 auto-sampler. GLC operating parameters for the column: wide-bore WCOT fused silica capillary, coated with CP Sil 5CB, length 25 m, diameter 0.25 mm, film thickness 1.26 µm. The injection and detector temperature were 240 °C and 325 °C, respectively. The carrier gas was helium (1 ml min^{-1}). The temperature programme was: initial oven temperature was 150 °C (1.5 min), the temperature was raised by 40 °C min^{-1} to a final temperature of 250 °C (7 min). The flow rate of nitrogen through the detector was 60 ml min^{-1} (as auxiliary gas). Standards with concentrations of 2.5 to 100 µg litre^{-1} were injected to construct the calibration curve. Retention time of chlorpyrifos: about 7 min. Detection limit: about 2.5 pg. Chlorpyrifos recovery efficiency from water was 88.5 ± 7.2 % (mean ± SD, n = 49). Measured concentrations were not corrected for recovery.

Half-lifes (t½) were based on measured concentrations within the first 200 h and assuming first-order kinetics. The dissipation coefficient was calculated by applying linear regression on the Ln-transformed concentrations by means of the computer program Microsoft® Excel version 2002 SP-2.

Phytoplankton and zooplankton

The species composition of the phytoplankton and zooplankton was determined to the lowest practical taxonomic level. Samples were taken on 8 days and 1 day before application, and on a weekly basis after application of Dursban® 480. Water (approx. 2 litres) collected from several positions in the microcosms was filtered through a plankton net (mesh width, 20 µm). The filtered water was returned to the microcosm.

Collected plankton was preserved with formalin (*ca.* 37 g litre^{-1} formaldehyde, as final volume).

Phytoplankton was identified and counted under a microscope. At first, a sub-sample was checked in order to get an impression of the taxon composition and of the diversity of cell types within or between samples. Where colony-forming algae were found, the number of colonies was counted and converted to numbers per ml.

Zooplankton was identified under a microscope, numbers of micro-zooplankton (i.e. Rotifera, copepod nauplii) being determined by counting a sub-sample of known volume. Macro-zooplankton (i.e. Cladocera, adult and copepodit stadia of Copepoda, Ostracoda) was quantified by counting all the sample using a binocular microscope.

Chlorophyll-a

The chlorophyll-a content of the phytoplankton was determined 8 days and 1 day before application, and on Days 2, 4, 7, and thereafter on a weekly basis after application of chlorpyrifos. When relevant, samples were taken simultaneously with those for phyto- and zooplankton to avoid a dilution effect of the returned filtered water from these samplings. Water samples were taken randomly from each microcosm by means of a Perspex® tube (length 40 cm, diameter 4 cm) and samples (0.1-0.5 litre) were filtered through a Whatmann glassfibre filter (GF/C, diameter 4.7 cm, mesh size 1.2 μm), using a vacuum pump. Filters containing phytoplankton were stored below –20 °C for a maximum of 1.5 months. Surplus water and filtrates were returned to the appropriate microcosms. Extraction of chlorophyll-a was performed using the method of Moed and Hallegraeff.[10] Measurement of chlorophyll-a content was carried out using a Beckman DU-64 spectrophotometer.

Community metabolism

Dissolved oxygen (DO), electrical conductivity, temperature and pH were measured at mid-water depth. The measurements were carried out in the morning, just before or around the start of the photoperiod when lowest DO levels occur, and after 15.00 hrs of that same day when DO levels were expected to have reached their maximum levels. Measurements were performed in each microcosm, 8 days and 1 day before application, and twice a week after application of the insecticide. Dissolved oxygen and temperature were measured using a YSI model 58 oxygen meter; pH was measured using a WTW-pH 323 meter, equipped with a Sentix 81 pH electrode. Conductivity was measured using a WTW LF96 electrical conductivity meter, equipped with a TetraCon 96 electrode.

Data analysis

Prior to univariate and multivariate analyses, zooplankton and phytoplankton abundance data were Ln(10x+1) and Ln(0.001x+1) transformed, where x stands for the abundance value. This was done to down-weight high-abundance values and to approximate a normal distribution of the data.

Univariate analysis. No Observed Effect Concentrations (NOEC) calculations at taxon or parameter level ($p \leq 0.05$) were carried out using the Williams test (ANOVA).[11] The test assumes that the mean response of the variable is a monotonic function of the treatment, thus expecting increasing effects with increasing dose. The analyses were performed with the Community Analysis computer program,[12] resulting in an overview of NOECs in each sampling week. Where we suspected a non-monotonic response of endpoints, we checked the outcome of the Williams test with the Dunnett t-test (computer program SPSS, version 8). Since the Dunnett tests confirmed the outcome of the Williams tests, data are only presented for the latter.

Multivariate analysis: The effects of the chlorpyrifos treatment on the zooplankton and phytoplankton communities were analysed by the Principal Response Curves method (PRC). The PRC method is a multivariate technique specially designed for the analysis of data from model ecosystem experiments. The method is based on the redundancy analysis ordination technique, which is the constrained form of principal component analysis.[13] PRC diagrams are interpreted as follows: Figure 5 indicates that, compared to the controls, the largest deviations in species composition occurred at the treatment of 10 μg litre^{-1}. Smaller deviations were found at the other treatments. The species weighting (b_k) shown on the axis on the right-hand side of the diagram can be interpreted as the affinity of each species with the response shown in the diagram. Thus, the cladoceran, *Daphnia* gr. *galeata*, which has the highest weighting with the diagram, is indicated to have decreased at both 1 and 10 μg litre^{-1}. The negative weighting of the rotifer, *Anuraeopsis fissa*, with the diagram indicates that its numbers have increased at the higher treatment levels. A full description of the PRC method is given by Van den Brink and Ter Braak.[13,14] The statistical significance of treatment effects at the community level is tested by using Monte Carlo permutation tests. In addition to the overall significance of the effects of a treatment regime on a community, we also determined which treatments differed significantly from the control, so as to infer the NOEC at the community level. The NOEC calculations were performed by applying the Williams test to the sample scores of the first principal component of each sampling date in turn.[6] Monte Carlo permutation tests and NOEC calculations were also performed per sampling date, allowing the significance of the effects of a treatment regime to be tested over time.

The NOECs obtained were further analysed in relation to statistical artefacts and biological significances. In the first instance, effects were considered consistent when they showed statistically significant deviations in the same direction for at least two consecutive sampling points. Statistical deviations were further evaluated in relation to (1) magnitude of measured counts; (2) whether counts were evenly distributed over the samples or if they were of a scattered nature, and (3) whether there was a treatment-related concentration response or a clear causality with community interactions or timing.

In order to compare across the three experiments amongst each other, we summarized observed effects into effect classes and placed the studied endpoints in one of the endpoint categories as proposed by Brock et al. (2000).[15] Following this method we placed results in four effect classes:

- Effect Class 1: no effects observed.
- Effect Class 2: slight effects. Effects only observed on individual samplings, especially shortly after treatment.
- Effect Class 3: clear short-term effects. Effects observed at some subsequent sampling dates. Full recovery occurred within the study period.
- Effect Class 4: clear effects, no full recovery within study period. Clear effects were observed, but study was too short to reach control levels.

Endpoint categories used in the present paper were 'microcrustaceans' which included the endpoints Cladocera and Copepoda; 'rotifers' which included Rotifera; 'algae' which included the phytoplankton and chlorophyll-a measurements, and 'community metabolism' which included EC, DO, and pH measurements. Within each endpoint category, the most sensitive endpoint was decisive for the placement in one of the four effect classes.

Results

Chlorpyrifos concentrations

Mean nominal concentrations of chlorpyrifos in the water were 95 ± 8.6 % (± SD) of the target concentrations. Measured concentrations in the integral water column 1 h after application, were 107 ± 24 % of the target concentrations, indicating high spatial variability in exposure concentrations immediately after application. Dissipation under the temperate environmental conditions was slower than under the warmer environmental conditions (mean half-life values: 45 h Expt. $2A_{temp}$ compared to 30 – 32 h for Expts 1_{Med} and $2B_{Med}$).

Zooplankton

In all three experiments, rotifers formed the majority of the number of taxa (8 – 16 taxa). Crustaceans (Cladocera and Copepoda) were also abundant in the experiments

(7 – 11 taxa). Populations of cladocerans showed consistent reductions at the 10 and 1 μg litre^{-1} treatment levels (Fig. 2). The reductions were specifically in populations of *Daphnia* gr. *galeata* and *Simocephalus vetulus* (Table 2).

Table 2. LOECs (Williams test, $p < 0.05$) per sampling date for zooplankton populations in microcosms under cool and warm conditions. Only taxa for which LOECs on at least two consecutive sampling days were calculated are shown. Treatments resulted in significant increases (↑) or reductions (↓). Blank fields indicate that LOECs were above highest tested concentration.

	LOEC (μg litre^{-1})				
Day	7	14	21	27/28	35
Experiment 1 (warm)					
CLADOCERANS					
Daphnia gr. *galeata*		10(↓)	10(↓)		10(↓)
Simocephalus vetulus	1(↓)	1(↓)	1(↓)	10(↓)	10(↓)
COPEPODS					
Calanoida		1(↑)	1(↑)	0.1(↑)	10(↑)
Cyclopoida		1(↑)	1(↑)	1(↑)	0.1(↑)
ROTIFERS					
Anuraeopsis fissa		10(↑)	10(↑)	10(↑)	10(↑)
Experiment 2A (cool)					
COPEPODS					
Nauplii	1(↓)	1(↓)			--[b]
Calanoida		1(↑)	1(↑)		--
ROTIFERS					
Polyarthra remata	0.01(↑)[a]	1(↑)			--
Synchaeta sp.	1(↑)	1(↑)			--
Experiment 2B (warm)					
CLADOCERANS					
Daphnia gr. *galeata*	1(↓)	1(↓)	1(↓)	1(↓)	--

[a] No clear dose-response relationship
[b] --: not studied.

In Expt. $2A_{temp}$, effects of chlorpyrifos on densities of cladocerans in the 1 μg litre^{-1} treatment were not significantly different from those in the controls due to the lack of a clear concentration-response relationship. Nevertheless, densities in the 1 μg litre^{-1} treatment clearly deviated from those in the 0.01 and 0.1 μg litre^{-1} treatment levels (Fig. 2). Besides cladocerans, copepod nauplii also showed consistent treatment-related reductions (Table 2). At the highest treatment level, geometric mean numbers

were reduced to 36 % and 29 % of control levels at Days 7 and 14, respectively, and were back to control levels after 21 days. In Expt. $2B_{Med}$, *D.* gr. *galeata* had not fully recovered within the study period at the 1 µg litre^{-1} treatment level (Table 2).

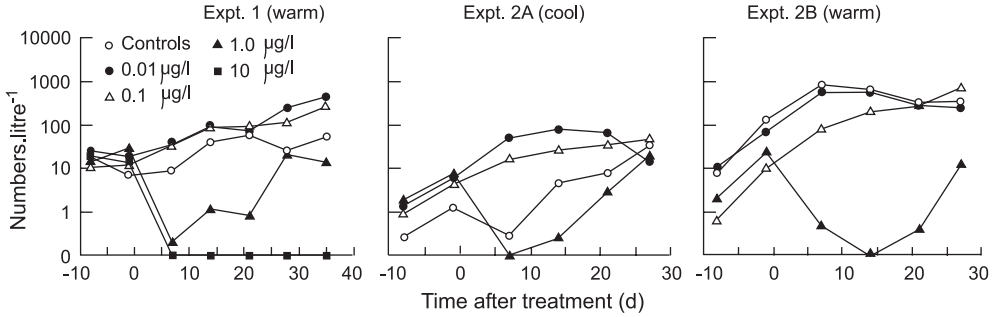

Fig. 2. Geometric mean numbers of cladocerans in microcosm studies treated with chlorpyrifos.

Statistical analysis indicated that copepod populations (Calanoida and Cyclopoida) and rotifer populations had consistently increased compared to control levels at treatment concentrations of 1 and 10 µg litre^{-1} (Table 2), indicating indirect effects. Graphical presentation shows that these increases were only apparent for copepods in Expt. 1_{Med} (Fig. 3) and for rotifers in Expts 1_{Med} and $2A_{temp}$ (Fig. 4).

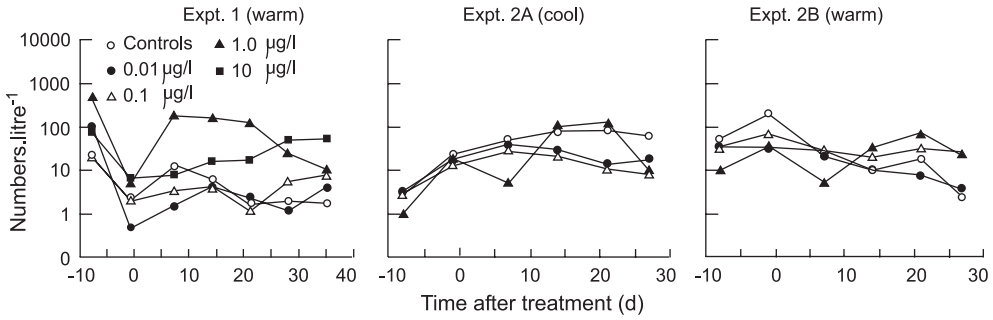

Fig. 3 Geometric mean numbers of copepods in microcosm studies treated with chlorpyrifos.

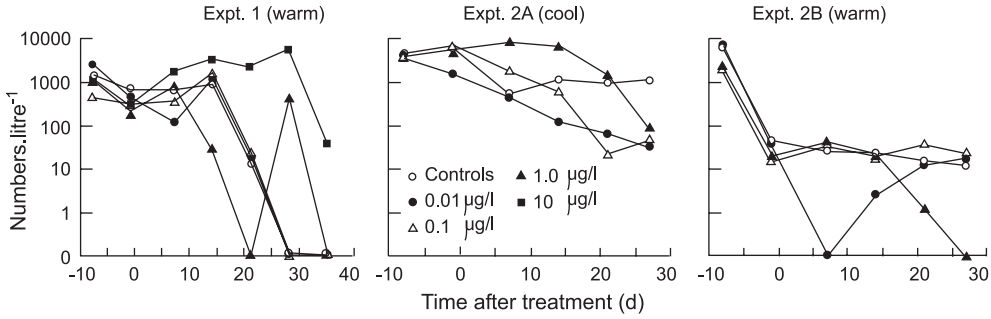

Fig. 4. Geometric mean numbers of rotifers in microcosm studies treated with chlorpyrifos.

The consistent NOEC for the most sensitive population was 0.1 µg litre^{-1} in all three experiments. At this concentration level, however, some small short-term increases of copepods in Expt. 1_{Med} were observed (Table 2, Fig. 3).

The multivariate analyses also reflected the treatment-related effects on zooplankton at the 10 and 1 µg litre^{-1} treatment levels, resulting in community NOECs of 0.1 µg litre^{-1} for all three experiments (Table 3). The PRC analyses indicated that, in Expt. 1_{Med}, 48 % of all variance could be attributed to the treatment regime. Of this variance 49 % is displayed on the vertical axis of Fig. 5. Of all variance in Expt. $2A_{temp}$, 27 % could be attributed to the treatment regime. In the case of Expt. $2B_{Med}$, this was 22 %. Of this variance, 54 % and 47 % is displayed on the vertical axes of Fig. 6 for Expt. $2A_{temp}$ and Expt. $2B_{Med}$, respectively.

Responses of the cladocerans, most explicitly in the form of *D.* gr. *galeata*, were positively correlated with the treatment regime (i.e., they decreased in numbers) in all three experiments (Figs 5 and 6). Rotifers and copepods (Calanoida and Cyclopoida) increased to some extent compared to control levels as they generally showed negative species weighting (b_k) in Figs 5 and 6. At the 10 µg litre^{-1} treatment level, the zooplankton community had not yet recovered at the end of Expt. 1_{Med} (Fig. 5, Table 3). At the 1 µg litre^{-1} treatment level, test systems under the warm conditions (Expts 1_{Med} and $2B_{Med}$) had not yet recovered within 4 weeks (Day 27) (Figs 5 and 6B). In Expt. $2B_{Med}$, the deviation at the 1 µg litre^{-1} treatment level at the last sampling date (Fig. 6B) is not confirmed statistically (Table 3). In Expt. $2A_{temp}$, recovery had occurred within 4 weeks (27 days (Fig. 6A, Table 3).

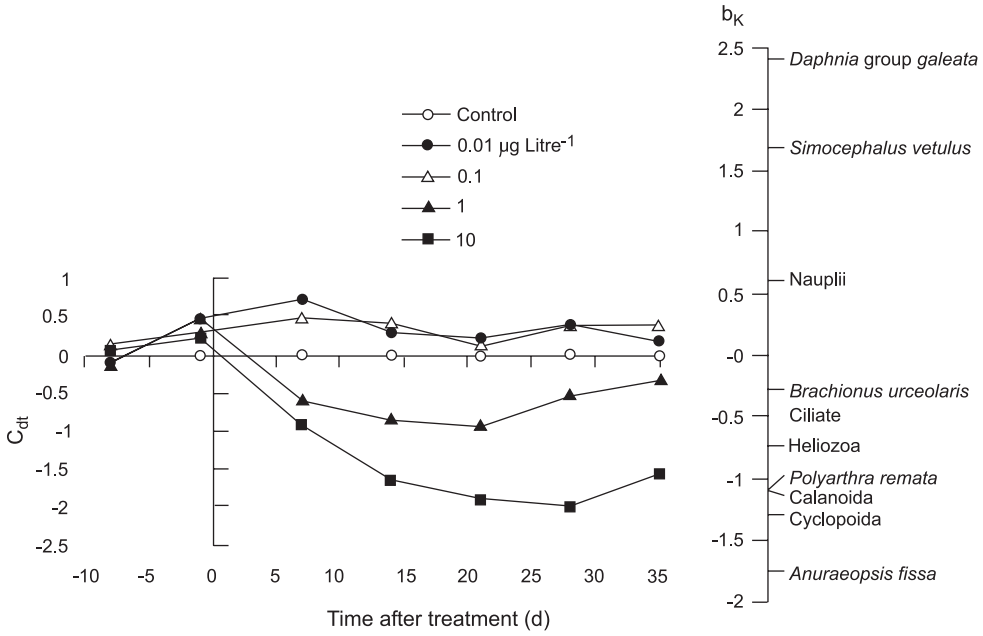

Fig. 5. Principal Response Curves for the zooplankton data of Experiment 1 (Expt. 1_{Med}), indicating the effects of the insecticide chlorpyrifos on the zooplankton community. The vertical axis represents the differences in community structure between treatments and the controls expressed as regression coefficients (c_{dt}) of the PRC model. The species weight (b_k) can be interpreted as the affinity of the taxon to the PRC.

Table 3. Significance of effect of chlorpyrifos treatment on the zooplankton community in the three microcosm experiments determined for each sampling date (Monte Carlo permutation tests, *p*-values) and the corresponding $NOEC_{community}$ (Williams test, $p < 0.05$).

	Experiment					
	1 'Mediterranean'		**2A 'temperate'**		**2B 'Mediterranean'**	
Day	*p*-value	NOEC (µg litre^{-1})	*p*-value	NOEC (µg litre^{-1})	*p*-value	NOEC (µg litre^{-1})
-8	> 0.05	≥ 1	> 0.05	≥ 1	> 0.05	≥ 1
-1	< 0.05	0.01	> 0.05	≥ 1	> 0.05	≥ 1
7	0.02	0.1	0.014	0.1	0.007	0.1
14	0.01	0.1	0.049	0.1	0.003	0.1
21	< 0.05	0.1	> 0.05	≥ 1	0.029	0.1
27	0.01	0.1	> 0.05	≥ 1	> 0.05	≥ 1
35[a]	0.04	1	--	--	--	--

[a] --: not studied.

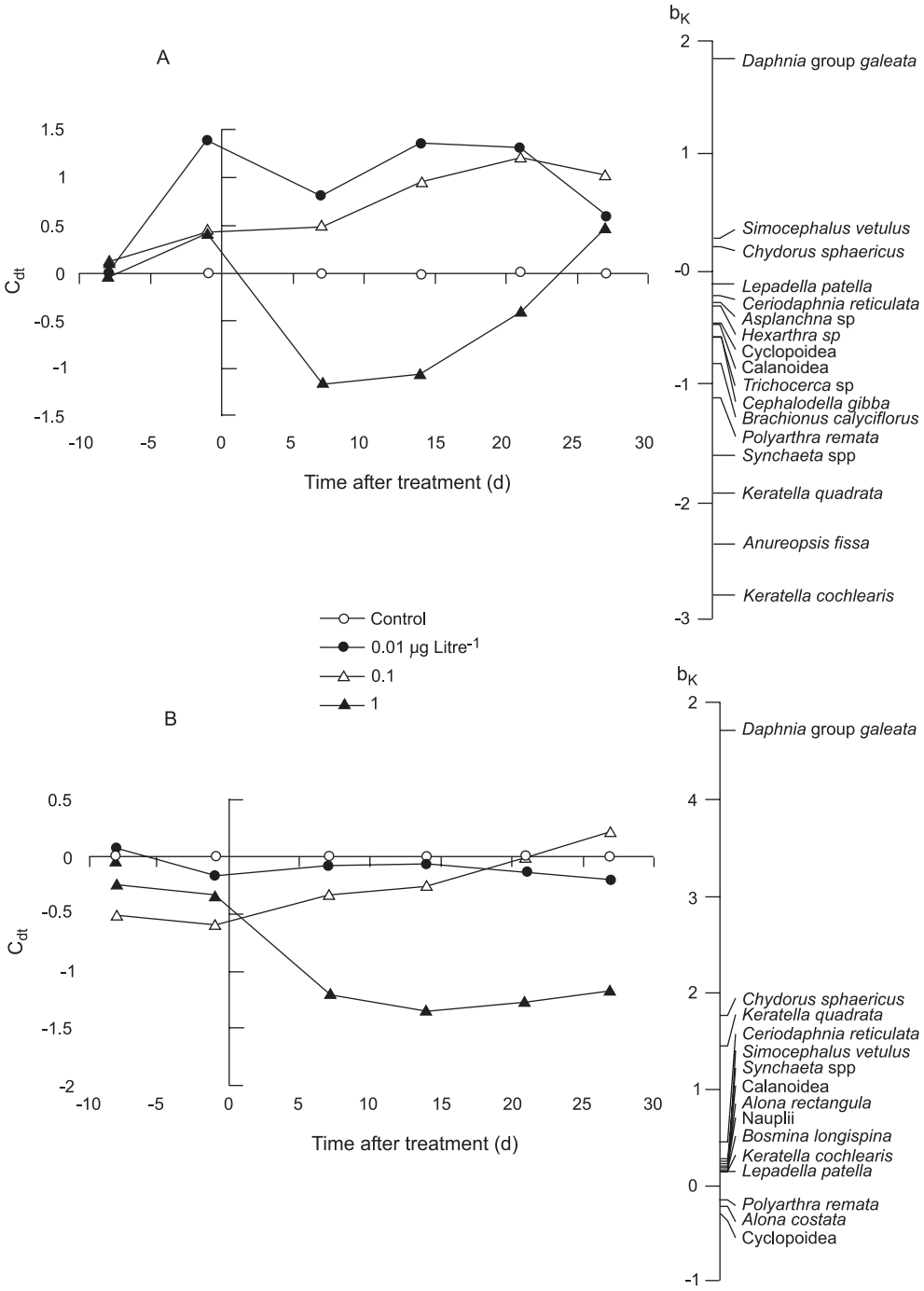

Fig. 6. Principal Response Curves for the zooplankton of Expt. $2A_{temp}$(A) and Expt. $2B_{Med}$ (B), indicating the effects of chlorpyrifos on the zooplankton communities. See Fig. 5 for explanation of axes.

Phytoplankton

In the three experiments, the phytoplankton communities consisted of taxa mostly belonging to the green algae (Chlorophyta), followed by blue-greens (Cyanophyta). The PRC analysis indicated that, in Expt. 1_{Med}, 48 % of all variance could be attributed to the treatment regime. Of this variance 49 % is displayed on the vertical axis of Fig. 7. Of all variance in Expt. $2A_{temp}$, 27 % could be attributed to the treatment regime. In the case of Expt. $2B_{Med}$, this was 29 %. Of this variance, 29 % and 45 % is displayed on the vertical axes of Fig. 8 for Expt. $2A_{temp}$ and Expt. $2B_{Med}$, respectively. Overall, the phytoplankton community showed apparent treatment-related effects at the 1 and 10 μg litre^{-1} treatment levels (Figs 7 and 8). Community NOECs were at the 0.1 μg litre^{-1} treatment level for the experiments under the warm environmental conditions (Expts 1_{Med} and $2B_{Med}$). For the experiment under the cooler, temperate conditions the $NOEC_{community}$ was ≥ 1 μg litre^{-1} (Table 4). In Expts 1_{Med} and $2B_{Med}$, recovery did not occur within 4 wk (27 d) at the 1 μg litre^{-1} treatment level. Chlorophyll-a was increased at treatments of 1 μg litre^{-1} and higher (Fig. 9). In the highly productive systems of Expt. $2B_{Med}$, mean chlorophyll-a concentrations over the complete treatment period were 325 μg litre^{-1}, while they were 32 μg litre^{-1} in Expt. 1_{Med} at the 1 μg litre^{-1} treatment level. NOECs based on chlorophyll-a measurements were very similar to those for abundance (Table 4).

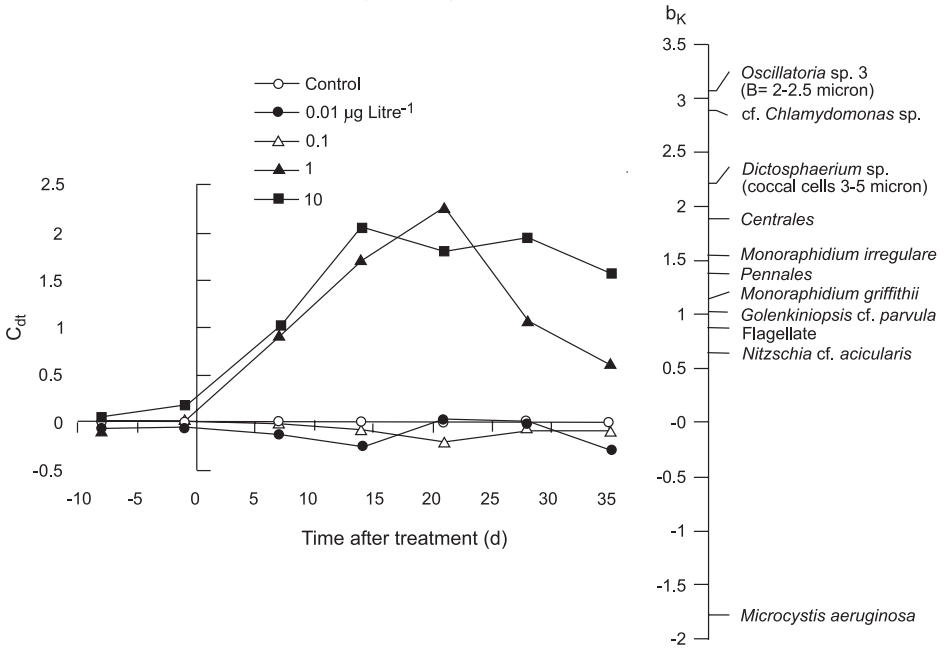

Fig. 7. Principal Response Curves for the phytoplankton of $Exp1_{Med}$, indicating the effects of the insecticide chlorpyrifos on the phytoplankton community. See Fig. 5 for explanation of axes.

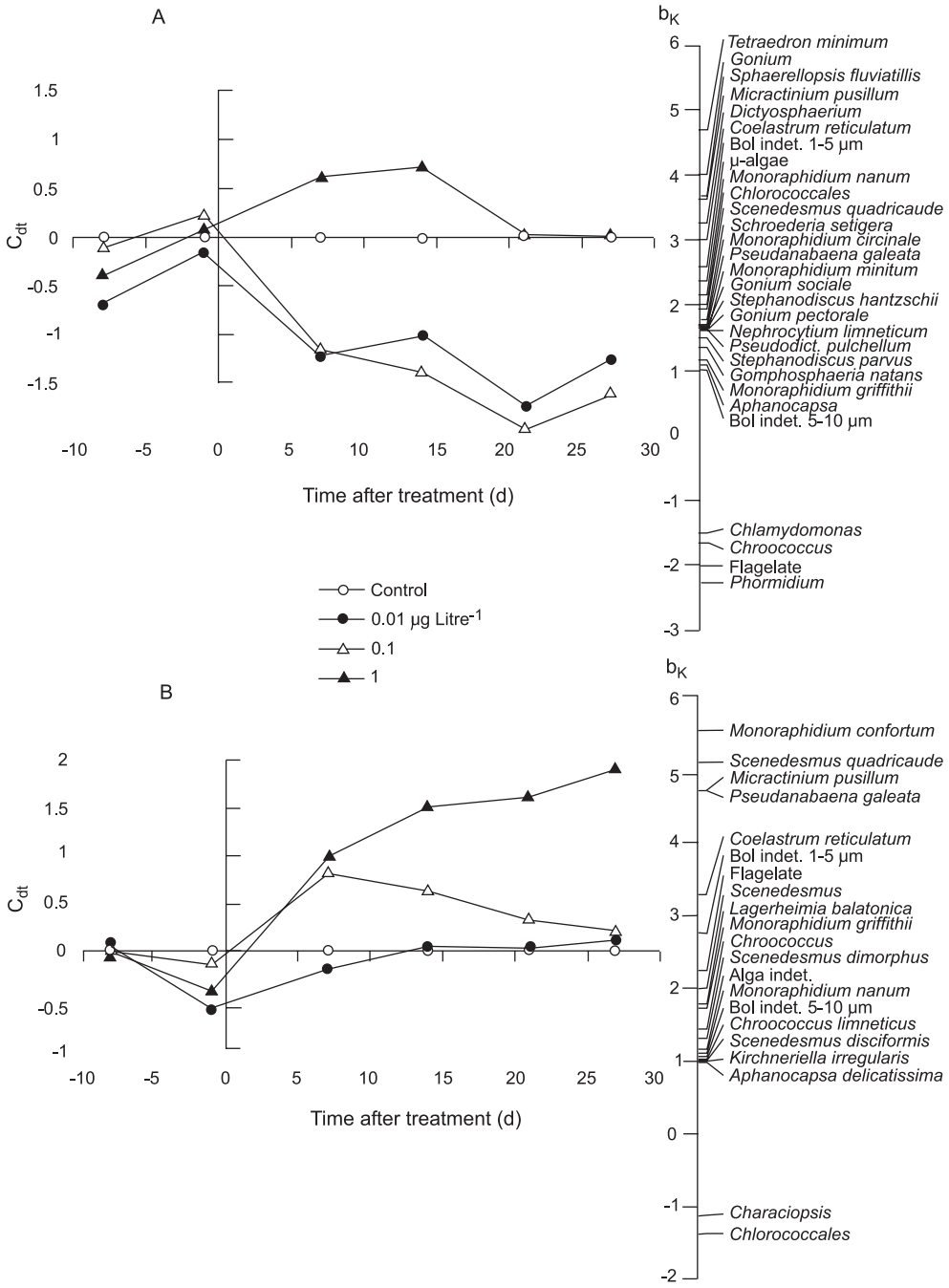

Fig. 8. Principal Response Curves for the phytoplankton of Expt. $2A_{temp}$ (A) and Expt. $2B_{Med}$ (B). See Fig. 5 for explanation of axes.

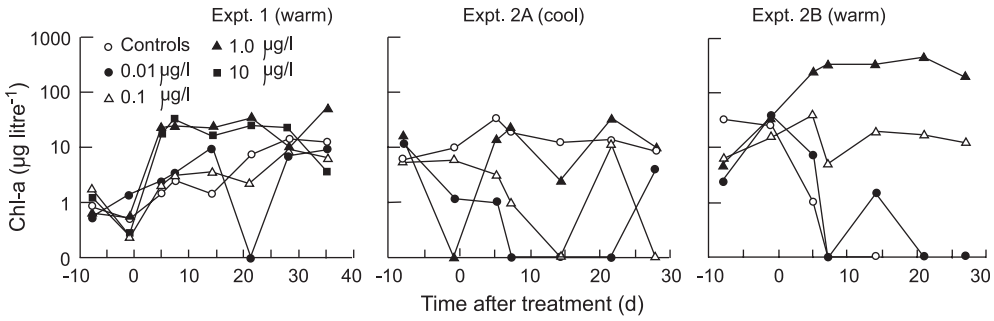

Fig. 9. Geometric mean concentrations of phytoplankton chlorophyll-a in microcosm studies treated with chlorpyrifos.

Table 4. NOECs for the phytoplankton community and for chlorophyll-a by sampling date (Williams test, $p < 0.05$).

	NOEC (µg litre^{-1})					
	Phytoplankton			Chl-a		
Day	Expt. 2A cool	Expt. 1 warm	Expt. 2B warm	Expt. 2A cool	Expt. 1 warm	Expt. 2B warm
-8	≥ 1	≥ 1	≥ 1	≥ 1	≥ 1	≥ 1
-1	≥ 1	≥ 1	≥ 1	≥ 1	≥ 1	≥ 1
7	≥ 1	0.1	0.1	≥ 1	0.1	0.1
14	≥ 1	0.1	0.1	≥ 1	0.1	0.1
21	≥ 1	0.1	0.1	≥ 1	0.1	0.1
27	≥ 1	0.1	0.1	≥ 1	≥ 10	0.1
35[a]	--	1	--	--	≥ 10	--

[a] --: not studied

Community metabolism

Overall, community metabolism endpoints were affected less severely by the chlorpyrifos treatments. In cases where deviations occurred, they were of small magnitude. Electrical conductivity typically was around 140 - 170 µS cm^{-1} in Expts 1_{Med} and $2A_{temp}$, and 220 µS cm^{-1} in Expt. $2B_{Med}$ but was not found to be affected by the chlorpyrifos treatments.

Minimum dissolved oxygen (DO) levels typically were around 7 – 8 mg litre^{-1} in the three experiments, indicating that critical anoxic conditions for organisms did not occur. DO showed increased concentrations at the higher treatment levels in Expts 1_{Med} and $2B_{Med}$ (Table 5). In Expts 1_{Med} and $2A_{temp}$, DO increased to about 10 ± 1 mg

litre^{-1}, while maximum DO levels were around 17 ± 3 mg litre^{-1} in Expt. $2B_{Med}$. DO concentrations were back to control levels within 27 d (Table 5).

Minimum and maximum pH values ranged between 8 and 9 (10 in the case of Expt. $2B_{Med}$). In Expts 1_{Med} and $2B_{Med}$, pH values in treated systems were significantly higher within the first one to two weeks after treatment and were most apparent in Expt. 1_{Med} (Table 5). In contrast, pH values in Expt. $2A_{temp}$ were significantly lower (Table 5). A consistent NOEC of < 0.01 µg litre^{-1} was calculated for this endpoint (Table 5). In all cases, deviations of pH were within 1 pH unit and are therefore considered of minor ecological importance.

Table 5. LOECs (Williams test, $p < 0.05$) per sampling date for community metabolism endpoints in microcosms. Endpoints were dissolved oxygen (DO) and pH. max – min: evening DO values minus morning DO values. Treatments resulted in significant increases (↑) or reductions (↓). Blank fields indicate that LOECs were above highest tested concentration.

		LOEC (µg litre^{-1})									
Day		1	5	8	12	14/15	19	22	26/27	29	33
Expt. 1 (warm)											
DO	*morning*[a]										
	afternoon		0.1(↑)	1(↑)	10(↑)	10(↑)					
	max–min			10(↑)	1(↑)	10(↑)	1(↑)	1(↑)			
pH	*morning*		0.1(↑)	0.01(↑)	0.01(↑)	0.01(↑)					
	afternoon		0.1(↑)	0.01(↑)	0.01(↑)	0.1(↑)					
Expt. 2A (cool)											
DO[a]											--[b]
pH	*morning*					0.01(↓)	0.01(↓)	0.1(↓)			--
	afternoon[b]										--
Expt. 2B (warm)											
DO	morning[a]										--
	afternoon[a]										--
	max–min						1(↑)	1(↑)			--
pH	*morning*[a]										--
	afternoon		1(↑)	1(↑)							--

[a] One or two isolated significant deviations only.
[b] --: not studied.

Effect classes and NOECs

Summarizing the three experiments in terms of 'effect classes', clear effects (Class 3 and Class 4) occurred in all three experiments at the 1 μg litre^{-1} treatment level (Table 6). Effects in Expt. $2B_{Med}$ generally tended to be of a longer duration as effects on many endpoints were considered to be of Class 4 (Table 6). Class 4 effects also occurred at the 10 μg litre^{-1} treatment level in Expt. 1_{Med} (Table 6).

Table 6. Summary of effects observed in microcosms under differing test conditions and treated with chlorpyrifos. 1 = no effect; 2 = slight effects; 3 = clear short-term effects, full recovery observed within study; 4 = clear effects, no full recovery observed at the end of the experiment. ↓ = decrease of endpoint; ↑ = increase of endpoint; ↓↑ decrease and increase of endpoint. PRC: Principal Response Curves analysis. --: not tested.

	Endpoint	**Treatment levels (μg litre^{-1})**			
		0.01	**0.1**	**1.0**	**10**
Expt. 1	Zooplankton community (PRC)	1	1	3	4
(Warm)	Microcrustaceans	1	1-2↑[a]	3↓↑	4↓↑
	Rotifers	1	1	1	4↑
	Phytoplankton community (PRC)	1	1	3	4
	Algae (chl-a)	1	1	3↑	3↑
	Community metabolism	1[b]	1[b]	2↑	3↑
Expt. 2A	Zooplankton community (PRC)	1	1	3	--
(Cool)	Microcrustaceans	1	1	3↓↑	--
	Rotifers	1	1-2↑[a]	3↑	--
	Phytoplankton community (PRC)	1	1	1	--
	Algae (chl-a)	1	1	1	--
	Community metabolism	1[b]	1[b]	1[b]	--
Expt. 2B	Zooplankton community (PRC)	1	1	3-4	--
(Warm)	Microcrustaceans	1	1	4↓	--
	Rotifers	1	1	1	--
	Phytoplankton community (PRC)	1	1	4	--
	Algae (chl-a)	1	1	4↑	--
	Community metabolism	1	1	3↑	--

Remarks related to the placing of responses into effect classes:
[a] Isolated deviations, causality with treatments unclear.
[b] Small deviations of pH; not considered ecologically significant.

The 0.1 μg litre^{-1} treatment level did not indicate any negative effects in these experiments. The incidental transient density increases in the endpoint categories 'microcrustaceans' and 'rotifers' did not show clear causality due to treatments (Fig. 3, Expt.1_{Med} and Fig. 4, Expt. $2A_{temp}$). At concentrations as low as 0.01 μg litre^{-1} statistical deviations occurred for the endpoint category 'community metabolism' (Table 6). These concerned minor deviations in pH and were considered to fall in effect class 1.

All three experiments yielded community NOECs of 0.1 μg litre^{-1} for structural endpoints regardless of environmental conditions (Table 6). Because no consistent and biologically significant effects were observed at the 0.1 μg litre^{-1} treatment level and lower, the 0.1 μg litre^{-1} treatment level is considered to be the overall microcosm NOEC for all three experiments, i.e. for both 'temperate' and 'Mediterranean' conditions.

Discussion

Community interactions

Phytoplankton community responses in the three experiments typically showed a reciprocal pattern to that of zooplankton (e.g., compare Figs 5 and 7). The pattern reflected the reduced grazing pressure in the treatments of 1 and 10 μg litre^{-1} caused by reductions in populations of sensitive microcrustaceans (mainly cladocerans). In Expt. $2A_{temp}$, increased grazing pressure by cladocerans in the 0.01 and 0.1 μg litre^{-1} treatments resulted in relatively low densities of many algal taxa (compare Figs 6A and 8A). In both warm systems (Expts 1_{Med} and $2B_{Med}$), changed grazing pressure led to signs of eutrophication in the form of significant increases of chlorophyll-a concentrations and abundance numbers of several algal taxa at treatment levels of 1 μg litre^{-1} and 10 μg litre^{-1}. Signs of eutrophication were also seen in the form of significant increases of DO and pH levels, especially in Expt. 1_{Med} (Table 5). Increases in copepods and rotifers coincided with reductions of cladocerans (Table 2). Their responses are also considered secondary effects and can be explained by release from food competition or release from mechanical filtering. Similar indirect effects within freshwater communities are described in numerous other studies.[16, 17]

All three experiments yielded a $NOEC_{community}$ of 0.1 μg litre^{-1}. Above this threshold level, responses and effect chains differed between experiments. At the highest treatment level in common (1 μg litre^{-1}), taxa within the microcrustaceans, which differed in each experiment, were temporarily reduced (Table 2). Time spans between recovery of sensitive endpoints varied per experiment. Recovery of endpoints was most rapid in the experiment conducted under the cool conditions (Expt. $2A_{temp}$) (Table 3). Additionally, phytoplankton densities significantly increased in the warm systems (Expts 1_{Med} and $2B_{Med}$), but not in the 'temperate' system. The algal bloom in

Expt. 1_{Med} was milder than in Expt. $2B_{Med}$ as mean chlorophyll-a levels over the complete treatment period at the 1 μg litre^{-1} treatment level were a factor of 10 lower than in Expt. $2B_{Med}$. In Expt. 1_{Med}, algal densities tended to recover within the experimental period as chlorophyll-a concentrations returned to control levels within 4 weeks in this experiment while this was not the case for Expt. $2B_{Med}$ conducted under the more eutrophic Mediterranean conditions (Table 4).

Representativeness

The present experiments were conducted with zoo- and phytoplankton collected from populations originating from a temperate climate zone. This raises the question whether the organisms used are representative of plankton communities in warmer climates. Major taxonomical groups like cladocerans, copepods and rotifers, and also the algae, have cosmopolitan distributions. At lower taxonomical levels, however, restrictions to specific biogeographical regions and even endemism are common within these groups.[18] Although not necessarily the same species, representatives of these major zooplankton groups are therefore to be expected in freshwater systems all over the world. More specifically for the Mediterranean region, the same representatives of the groups sensitive to chlorpyrifos (cladocerans, i.e. *D.* gr. *galeata*, *S. vetulus* and copepod nauplii) can be found here.[19, 20, 21]

Positive correlations between temperature and toxicity found in numerous studies[9, 22, 23] led to the assumption that organisms from warm climates will be more sensitive than those from cold climates.[24,25] However, very few studies have compared the sensitivity of temperate and (sub-)tropical invertebrates to environmental contaminants. Maltby et al.[26] compared species sensitivity distributions for temperate and tropical freshwater arthropods exposed to the insecticides fenitrothion, carbofuran and chlorpyrifos. They reported a tendency for tropical arthropods to be more sensitive, but this difference was not statistically significant. This indicates that there are no, or only minor, differences in sensitivity distributions to be expected for chlorpyrifos between the plankton communities in temperate and warmer freshwater systems. Maltby et al.[26] and Hose and Van den Brink[27] found no evidence to indicate that the use of northern-hemisphere temperate species in hazard assessment places tropical or southern-hemisphere freshwater ecosystems at undue risk from insecticides.

Community responses are not dependent on the sensitivity of the organisms alone. They result from the combination of sensitivity to a biologically active compound and the bioavailability of that compound. Microcosm and mesocosm studies integrate these two aspects and the outcomes of these studies may be compared. Outdoor studies, also involving single applications of chlorpyrifos, all yielded community NOECs of 0.1 μg litre^{-1}. The $NOEC_{community}$ value of 0.1 μg litre^{-1} from the three indoor test systems are in agreement with those of the more complex

outdoor studies (Table 7). It appears that threshold levels are largely independent of the scale and complexity of the test system. Hence, relatively simple indoor test systems appear to be sufficient for the determination of safe threshold levels for low-persistence organophosphate insecticides such as chlorpyrifos. This can be explained by the fact that microcrustaceans, and cladocerans in particular, are amongst the most sensitive species to organophosphate exposure and that these species are usually abundant in both small and large test systems. In the case of single applications of chlorpyrifos, a substance which has a relatively short DT_{50} in the water phase (*ca.* 7 d or less), the robustness of the $NOEC_{community}$ of 0.1 μg litre^{-1} indicates that this threshold level is likely to be representative for freshwater systems in general.

Table 7. Mesocosm and microcosm studies receiving single applications of chlorpyrifos.

Location		Study	NOEC (μg litre^{-1})	LOEC (μg litre^{-1})
Outdoor test systems				
USA (Kansas)	standing water	Biever et al.[28]	0.1	0.3
Netherlands	standing water	Van den Brink et al.[6]	0.1	0.9
Australia	running water	Pusey et al.[29]	0.1	5.0
USA (Minnesota)	standing water	Brazner et al.[30]	---	0.5
Microcosms (plankton-dominated)				
Indoor	warm, productive	Expt. 1 (this paper)	0.1	1.0
Indoor	cool, productive	Expt. 2A (this paper)	0.1	1.0
Indoor	warm, highly productive	Expt. 2B (this paper)	0.1	1.0
Indoor	mixed flask cultures and standardized aquatic microcosms	Stay et al.[31]	---	0.5

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Part II Case studies with lambda-cyhalothrin

5 Ecological effects of spring and late summer applications of lambda-cyhalothrin in freshwater microcosms

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Abstract

The aim of the study was to compare the effects of the pyrethroid insecticide lambda-cyhalothrin (treated at 10, 25, 50, 100, 250 ng a.i./L) on a drainage ditch ecosystem in spring and late summer. Microcosms (water volume approx. 430 L) were established using enclosures in a 50 cm deep experimental ditch sytem containing communities typical of macrophyte-dominated freshwater ecosystems. Effects on macro-invertebrates, zooplankton, phytoplankton, macrophytes and community metabolism were assessed and evaluated using univariate and multivariate statistical techniques. The macroinvertebrate community responded most clearly to treatment, and as anticipated insects and crustaceans were among the most sensitive organisms. Statistical analysis showed that the underlying community structure was significantly different between the spring and summer experiments. However, the most sensitive species (*Chaoborus obscuripes* and *Gammarus pulex*) were abundant in spring as well as in late summer. Both in spring and late summer only slight and transient effects were observed at the community level in the 10 ng/L treatment. Overall, the study did not show substantial differences in the responses of sensitive taxa between spring and late summer treatments, and effects thresholds were similar irrespective of season of treatment.

Introduction

Experiments in microcosms and mesocosms can be performed in higher-tier aquatic risk assessment for pesticides in the European Union when potential concerns are identified in the preliminary risk assessment (SANCO 2002). One recurring point of discussion regarding the use of micro- and mesocosm data in higher-tier risk assessment has been the extent to which data from a single experiment can be extrapolated to potential for effects in the real world where multiple stressors may occur, environmental conditions may vary, biocoenoses may differ, and so on (Giddings *et al.* 2002).

One of the variables in the real world is that spraying of a pesticide may take place in different periods of the season. This may affect the impact of a toxicant on freshwater communities for several reasons. Firstly, environmental conditions, like temperature, light, nutrients, biomass and vegetation structure are seasonal. Changes in these conditions may influence the fate and bioavailability of compounds. Brock *et al.* (1992), for example, showed for chlorpyrifos that the mixing of the compound through the water column was strongly influenced by the vegetation structure in microcosms. For lambda-cyhalothrin, it has been demonstrated that different densities of macrophytes can have considerable influence on the fate and bioavailability of this compound (Hand *et al.* 2001; Leistra *et al.* 2003). Experiments with chlorpyrifos simulating different environmental conditions indicated that at higher water temperatures, in combination with higher light intensities, dissipation rates increased (Van Wijngaarden *et al.,* 2005). Secondly, species assemblages and the developmental stages of populations change with time. Several laboratory studies have suggested that this may affect the impact of a toxicant on freshwater communities (Kindig *et al.* 1983; Swartzman *et al.* 1990; Taub *et al.* 1991). One explanation for this is that, in general, juvenile growth stages are more sensitive to toxicants than older ones (Mayer and Ellersieck 1986; Hutchinson *et al.* 1998). Laboratory studies also indicate that temperature and toxicity are positively correlated for most chemicals: toxicity increases as temperature increases (Mayer and Ellersieck 1986).

In temperate climates in springtime, surface water ecosystems are typically in a developmental stage (i.e., young sensitive organisms, little plant biomass leading potentially to higher bioavailability of the toxicant) and exposed to cool environmental conditions (i.e., less potential for toxicity, lower degradation). In late summer, these systems have matured (i.e., older less sensitive organisms, more plant biomass leading to potentially lower bioavailability toxicant) and are exposed to warmer conditions (i.e., more potential for toxicity, higher degradation). Hence, counteracting factors may simultaneously influence the outcome of a pesticide exposure, and this may make it difficult to predict the relative sensitivity of systems tested under spring conditions compared to those tested later in the year.

The contribution of seasonal factors for determining risks is an important question in ecological risk assessments of pesticides (Campbell *et al.* 1999; Giddings *et al.* 2002). Nevertheless, relatively few microcosm studies with pesticides have investigated the influence of season of application on the response of aquatic ecosystems. In outdoor microcosm studies that focussed on the effects of 10 μg/L of the herbicide atrazine on plankton communites in different periods of the year, it appeared that the clear water phase (June) was the period when the algal communities were most sensitive to restructuring by atrazine, whereas they were the least sensitive during the spring blooms (Bérard *et al.* 1999). In experimental ponds, carbaryl treatment at different stages in the seasonal cycle induced distinct recovery patterns in zooplankton communities. It was suggested that temperature, competitive interactions and population trends were significant factors influencing recovery of the zooplankton (Hanazato and Yasuno, 1990). Both previous studies worked at concentration levels that were well above the threshold concentration for direct effects and indicated differences in community responses related to season. However, a study with pentachlorophenol in lake enclosures, which also included concentrations around the threshold level, indicated that direct effects on planktonic communities varied little with season. It was suggested that season was not a particularly important factor for ecological risk assessment and the determination of ecological threshold levels (Willis *et al.* 2004).

The aim of the present study was to compare the effects of a spring insecticide treatment with the effects of a late-summer treatment with the same compound. We focussed on the questions (1) whether sensitivities of populations and communities were different, specifically at concentration levels near those used as thresholds in regulatory risk assessments and (2) whether above these concentrations, direct and indirect effects and recovery times were different.

Microcosms were established using enclosures in a 50 cm deep experimental ditch system containing natural communities typical of macrophyte-dominated drainage ditches. The pyrethroid insecticide lambda-cyhalothrin was used as the test compound. Lambda-cyhalothrin was chosen as it is highly lipophilic and tends to bind rapidly and strongly to organic materials (Maund *et al.* 1998; Leistra *et al.* 2003). Consequently, seasonal changes in biomass of plants and algae in aquatic ecosystems may affect exposure to this compound. Furthermore, lambda-cyhalothrin is highly toxic to some groups of aquatic organisms, particularly insects and crustaceans (Maund *et al.* 1998; Schroer *et al.* 2004). These taxonomic groups provide a range of uni- and multivoltine species, giving an opportunity to test different life-stages present in the spring and the late summer experiment.

The work described in this paper is part of a series of concurrent experiments done in ditch enclosures to study the fate and effects of lambda-cyhalothrin under varying environmental conditions. Leistra *et al.* (2003) studied the fate of the

compound. Separate enclosures were installed to follow the dynamics lambda-cyhalothrin in detail. Roessink *et al.* (2005) report the effects of lambda-cyhalothrin in enclosures of different trophic states Schroer *et al.* (2004) investigated the toxicity of lambda-cyhalothrin to freshwater invertebrates using data from short-term laboratory toxicity tests and *in situ* bio-assays and population effects observed in the enclosures.

Materials and Methods

Experimental outline

The experiments were carried out in macrophyte-dominated ditches (length: 40 m; width: 2.80 m at water surface) at the Sinderhoeve experimental station, Renkum, The Netherlands. The spring experiment was performed in May-June, and the late summer experiment in August-September of the year 2000.

In the spring experiment, one ditch from the twelve available on the site was selected on the basis of having an evenly-distributed, well-developed macrophyte coverage. In the late-summer experiment, the ditch selected was very similar to that used in the spring experiment (based on vegetation structure and composition). Twelve enclosures (polycarbonate, translucent cylinders: diameter: 1.05 m; height: 0.9 m; water volume: c. 0.43 m^3) were placed in an evenly-distributed row down the centre of the ditch. The cylinders were pushed about 15 cm into the sandy-loam sediment and contained a water column of 0.5 m depth. In both experiments, the enclosures were placed in the ditches three weeks before treatment. In each season, three applications of lambda-cyhalothrin were made at weekly intervals. Treatment started on May 16th for the first experiment, and on August 15th for the second. The formulated product KARATE (100 g lambda-cyhalothrin/L as capsule suspension) was applied at nominal concentrations of 10, 25, 50, 100, and 250 ng a.i./L and each treatment was duplicated. Two enclosures served as controls, and were only treated with water. Treatments were randomly assigned to the enclosures. Treatments were made by pouring a carefully measured volume of treatment solution into the enclosures, after which the water column was gently stirred to mix the compound throughout the water column, but without disturbing the sediment. Methods of application and chemical analysis are further described in Leistra *et al.* (2003).

Fate

Nominal initial treatment concentrations were based on measured concentrations of lambda-cyhalothrin in the treatment solutions and the water volume of the enclosures. Initial measured concentrations were assessed by taking depth-integrated water samples (with a perspex tube diameter: 4 cm; length: 50 cm) at 1 hour after treatment. Two water-column samples were taken and pooled from each enclosure..

Approximately 100 ml of the sampled water was stored in pre-weighed bottles and taken to the laboratory for analysis. In the laboratory, the bottles were weighed and 30 ml of distilled hexane was added. After weighing, the water and hexane were thoroughly mixed on a shaking apparatus for 15 minutes. The hexane layer was isolated in pre-weighed tubes after which the tubes were weighed again. Hexane was evaporated under a flow of pressurised air. The residue was then dissolved in 1 ml of distilled hexane. This was mixed on a vortex and transferred to a GC-vial. Lambda-cyhalothrin was analysed using a HP 5890 gas chromatograph, equipped with an electron capture detector (ECD). For further details see Leistra *et al.* (2003).

Macroinvertebrates

Artificial substrates were used to sample the macroinvertebrate community. These consisted of two litterbags (see *Decomposition*), two multiplates and two pebble baskets. A detailed description of the former two substrates is given in Brock *et al.* (1992). Substrates were collected from each enclosure at intervals of 2 or 3 weeks. At the time of sampling, the artificial substrates were gently retrieved from the enclosures, using a net to prevent the escape of organisms. Pebble baskets and multiplates were first washed in a container to remove invertebrates. Subsequently, the macroinvertebrates retrieved with the net, both substrates and from the litterbags were carefully sorted by hand. Organisms that were alive were identified and counted, after which they were released again into their original enclosures. Data from the artificial substrates and litterbags were pooled for further analysis.

Phyto- and zooplankton

Plankton were sampled at weekly intervals from each enclosure using a perspex tube (length: 0.4 m; volume: 0.8 L). Several subsamples were collected from each enclosure until a 10-L sample had been obtained. Five litres were used for the zooplankton analysis. The 5-L sample was concentrated by means of a 55 μm mesh net (Hydrobios, Kiel, Germany) and was preserved with formalin (end volume: c. 4%). Of the remaining 5 litres, 1 L was collected for chlorophyll-a analysis in order to estimate phytoplankton biomass.

The total number of cladocerans, ostracods, and juvenile and adult copepods was counted under a binocular microscope at a magnification of 25 times. Numbers of rotifers and copepod nauplii were determined by counting a known volume using an inverted microscope (100 – 400 times magnification). Rotifers and cladocerans were identified to the lowest practical taxonomic level. Copepods were divided into calanoids and cyclopoids. Abundances were adjusted to numbers per litre.

Phytoplanktonic chlorophyll-*a* measurements were made by concentrating a 1-L water sample over a glass-fibre filter (Schleicher and Schuell GF_{52}, mesh size: 1.2 μm).

Filters were stored in petri-dishes, wrapped in aluminium foil, and kept in a deep-freezer at a temperature below –20 °C until analysis. Extraction of the pigments was performed using a spectrophotometer (Beckman, DU-64) following the method of Moed and Hallegraeff (1978).

Periphyton

Glass slides (7.6 x 2.6 cm) were used as artificial substrates for sampling the periphyton. The slides were vertically positioned in a frame at a fixed depth of approximately 25 cm below the water surface of each enclosure. The substrates were introduced 15 or 16 days before the first application. Substrates were collected on days –1, 6, 13, 20, 27 and 41.

At sampling, a maximum of 5 slides per enclosure were collected to measure the amount of chlorophyll-*a* as an estimate of periphytic algae biomass. The slides were brushed and washed with tap water to collect the periphyton. The chlorophyll-*a* content of the water-periphyton suspension was processed and analysed as described above for phytoplankton.

Macrophytes

At Day –7 (spring experiment) and at Day – 11 (late summer experiment) the above-sediment macrophyte biomass of two representative plots (0.25 x 0.25 m) in the experimental ditches (but not inside the enclosures) was sampled. At this time, biomass in the area of the ditch outside the enclosures was similar to that inside the enclosures. At the end of both experiments, the complete above-sediment vegetation within the enclosures was harvested. Before drying (24 hours, 105 °C), the plant material was rinsed under tap water to remove loosely attached materials, like sediment particles and macroinvertebrates.

Bioassays

The crustacean *Asellus aquaticus* and the insect *Chaoborus obscuripes* were tested in *in situ* cage experiments. We used these relatively sensitive species (Schroer *et al.* 2004) for comparison of the acute effects of lambda-cyhalothrin between the two experiments and to determine whether potential recovery was different between the two seasons. *Asellus* and *Chaoborus* were collected in the field and kept in aquaria in the laboratory for several weeks before use. A week before the experiments started, the organisms were acclimated to the experimental conditions by transferring them to containers located in one of the experimental ditches.

The bioassay cages used were constructed of stainless steel gauze (mesh size: 0.5 mm; length: 33 cm; diameter: 6 cm; volume: 930 cm^3). In each cage, 25 or 30 adult *A. aquaticus* (mean size (± s.d.): 5.9 (± 1.4) mm in spring and 5.7 (± 1.3) mm in late

summer were introduced. *Populus* leaves (ca. 1 g dry weight) were supplied to these cages to provide sheltering substrates for *Asellus*. Thirty specimens (4th instar) were used per cage in the *C. obscuripes* bioassays, and two cages were introduced into each enclosure.

Two bioassays were performed, one directly after the first application (acute effects bioassay) and a second after the third application (recovery bioassay). The *Asellus* and *Chaoborus* in the first bioassays were introduced at Day –1. After application of the insecticide, the surviving organisms were counted and reintroduced in the bioassay on Days 1, 2, 3, and 6. In the recovery bioassays, tests started on 0, 4 and 8 days after the last application. Effects were scored after 4 or 5 days of exposure. After counting, the surviving organisms were not reintroduced, but fresh test organisms were used for each recovery bioassay.

To promote water exchange between enclosures and cages, cages were gently pulled up and down the water column at regular intervals. Because of the known rapid dissipation of lambda-cyhalothrin from the water, this was done most intensively during the day of application (after 1, 2, 4 and 8 h), thereafter on the days of data collection. The intention of this mixing was to ensure that the insecticide exposure patterns within the cages followed that of the enclosures as closely as possible.

Results of the bioassays were quantified by calculating percentile effect concentrations (EC*x* and LC*x* values for immobility and mortality, respectively). Bioassay results of individual replicates were used for the EC and LC calculations and initial nominal concentrations were used as input for the regression model (Schroer *et al.* 2004).

Decomposition

Decomposition of particulate organic matter (POM) was studied using leaf litter bags made up with *Populus x canadensis* leaves. Before use, the *Populus* leaves had been soaked in water three times for 2 days to remove the more easily soluble humic compounds, and then dried in an oven for 72 hours at 60 °C.

A portion of 2 g dry weight was enclosed in each litter bag, consisting of a glass Petri-dish (diameter: 11.6 cm), closed with a cover of stainless steel wire (mesh size: 0.7 mm), in which 2 holes (diameter: 0.5 cm) were made to allow invertebrates to enter. In each enclosure, two litter bags were placed on the sediment surface for a period of 2 weeks. At the end of each incubation period, the litter bags were gently retrieved from the enclosures and emptied in a white tray to separate POM from adhering sediment particles and macroinvertebrates by rinsing with tap water. The plant material was dried in aluminium foil at a temperature of 105 °C. After 24 h, dry weight was determined. Macroinvertebrates were counted and included in the macroinvertebrate sampling scores (see *Macroinvertebrates*).

Community metabolism

As an indicator of the overall oxygen metabolism of primary producers, dissolved oxygen (DO) was measured. Changes in primary productivity affect pH, alkalinity and electrical conductivity (EC). Since DO, pH, alkalinity and EC are often found to be highly correlated, and indirect treatment effects can be regarded as a stress syndrome (Giddings 1982), these endpoints were monitored on a weekly basis. Measurements were made at a depth of 10 cm in the approximate centre of each enclosure. DO was measured with a WTW Oxi330 portable oxygen meter (Retch, Ochten, The Netherlands). Electrical conductivity was measured with a WTW LF191 conductivity meter (Retch, Ochten, The Netherlands). pH was measured with a WTW PH197 portable pH-meter (Retch, Ochten, The Netherlands). The alkalinity of 100-ml water samples taken at a depth of 10 cm was measured by titration with 0.02 N HCl to pH 4.2.

Data analysis

Prior to analysis, the macroinvertebrate data set and the zooplankton data set were, respectively, $\ln(2x+1)$ and $\ln(10x+1)$ transformed, where x was the abundance value. This was done to down-weight high abundance values and approximate a normal distribution of the data (Van den Brink *et al.* 2000).

No observed effect concentration (NOEC) calculations at parameter or taxon level were derived using the Williams test (ANOVA, $p < 0.05$; Williams 1972). Analyses were made with the Community Analysis computer program (Hommen *et al.* 1994). ECx calculation methods on the results of the bioassays are described by Schroer *et al.* (2004).

Effects of the lambda-cyhalothrin treatments on the community level of zooplankton and macroinvertebrates were analysed by the Principal Response Curves method (PRC), which is based on the Redundancy Analysis ordination technique, the constrained form of Principal Component Analysis (Van den Brink and Ter Braak 1998,1999). For a complete description and discussion of the PRC method, the reader is referred to Van den Brink and Ter Braak (1998, 1999). The PRC analysis was performed using the CANOCO for Windows® software package, Version 4 (Ter Braak and Smilauer 1998). In the CANOCO computer program, redundancy analysis is accompanied by Monte Carlo permutation tests to assess the statistical significance of effects of the explanatory variables on species composition of the samples (Van den Brink *et al.* 1996). The significance of the PRC diagram, in terms of displayed treatment variance, was tested by Monte Carlo permutation of the entire time series in the redundancy analysis from which the PRC is obtained, using an *F*-type test statistic based on the eigen value of the component (Van den Brink and Ter Braak 1999).

Monte Carlo permutation tests were also performed by sampling date, using the ln-transformed treatment levels as the explanatory variable (Van den Brink *et al.* 1996).

This allowed the significance of the treatment regime to be tested for each sampling date. We also determined which treatments differed significantly from the controls, so as to infer the NOEC at the community level ($NOEC_{community}$). The $NOEC_{community}$ calculations were done by applying the Williams test to the sample scores of the first principal component of the principal component analysis of each sampling date in turn (for the rationale of this, see Van den Brink *et al.* 1996).

Monte Carlo permutation tests were also performed for each sampling date to test whether the communities differed significantly between seasons, and whether there was interaction between the factors 'treatment' and 'season'. The model used was

$$y_{d(j)ka} = y_{0k0} + T_d + S_a + (T_d * S_a) + \varepsilon_{d(j)ka}$$

where $y_{d(j)ka}$ is the abundance of species k in treatment d of replicate j of season a, y_{0k0} is the abundance of species k in the reference treatment (control = 0) and reference season (spring = 0). T_d indicates the effect of the treatment, S_a of the season. The T_d*S_a factor denotes the effect of the interaction term of treatment and season. $\varepsilon_{d(j)ka}$ is an unknown error term associated with observation $y_{d(j)ka}$. Within CANOCO for each sampling date separately, 'treatment' was tested by introducing ln-transformed (see Van den Brink *et al.* 1996 for details) treatment levels as explanatory variable and nominal variables denoting 'season' plus its 'interaction with treatment' as covariables. 'Season' was tested for each sampling date by introducing a nominal variable denoting 'season' as explanatory variable and the treatment variable and its 'interaction with season' as covariables. 'Interaction' was tested by entering the 'interaction between season and treatment' as explanatory variables and ln-transformed treatment levels and the nominal variable denoting 'season' individually as covariables.

Results

General description of test systems

The general characteristics of the test systems are summarized in Table 1. Water temperatures were almost similar in spring and late summer. Temperatures tended to increase during the spring experiment, whilst there was a tendency to decrease in the late summer experiment.

The macrophyte stands in the ditches selected were dominated by *Myriophyllum spicatum,* with *Elodea nuttallii* co-dominant in some patches; *Sagittaria sagattifolia* was also common. The latter two species became more dominant in late summer. Mean macrophyte biomass at the beginning of the experiments was almost 2.5 times higher in late summer than in spring (Table 1). The organic matter content of the upper sediment layer was more or less similar for both experiments (Table 1). Dissolved organic carbon in the water column was somewhat higher in spring (Table 1).

The ditches are representive of mesotrophic freshwater bodies. As an indication of nutrient levels in the systems, geometric mean values in the enclosures measured over the time-span of the spring experiment were 0.02 mg/L (NH_4^+) and 0.03 mg/L (NO_3^- + NO_2^-) and for ortho-P, concentrations were 0.04 mg/L (Roessink *et al.*, 2005). Overall, community metabolism was higher in spring since dissolved oxygen levels and pH were relatively high and electrical conductivity and alkalinity levels were relatively low compared to those in late summer (Table 1).

Table 1. Characterization of the enclosures set up in spring and in late summer. Values are from the control enclosures. For water quality endpoints mean values (± 95% confidence interval) over the time span of the experiments are given. DOC: dissolved organic carbon, DO: dissolved oxygen, EC: electrical conductivity, dw: dry weight. Mean macrophyte biomass represents values of the vegetation in the ditches housing the enclosures at start of the experiments.

		Spring	**Summer**
Sediment[1]			
	organic matter (%)	26	23
	0 - 2 cm upper layer		
Water			
	DOC[1] (mg C/L)	9.1	7.9
	DO (mg/L)	9.7 ± 0.9	5.8 ± 0.5
	pH	9.9 ± 0.1	7.4 ± 0.2
	EC (µS/cm)	124 ± 8	174 ± 9
	Alkalinity (meq/L)	1.06 ± 0.10	1.50 ± 0.10
	Temperature(°C)	17.5 ± 1.6	17.9 ± 1.1
Macrophytes			
	biomass (g/m² dw)	104	241

[1)] Values at start of experiments, from Leistra *et al.*, 2003.

Fate

Despite efforts to promote mixing after application, measured concentrations in water column samples taken 1 h after application indicated very high spatial variability (Table 2). The mean over all treatments ± SD was 109 ± 81%. It was therefore considered more appropriate to use the nominal initial concentrations (concentrations in enclosures as calculated from measured concentrations in dose solutions and water volumes in enclosures) as estimators of the initial exposure concentrations. Overall, nominal initial concentrations were near to the intended nominal concentrations (Table 3).

Table 2. Measured concentrations of lambda-cyhalothrin (ng a.i./L) in water samples collected 1 h after application. Data for each replicate (R1 and R2) are given.

Intended	Spring						Summer					
	Treatment						Treatment					
	1		2		3		1		2		3	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
10	--	--	7	17	12	15	10	19	9	61	11	11
25	--	--	32	48	25	28	26	28	25	31	25	26
50	--	--	25	35	--	21	52	52	45	50	44	48
100	--	--	82	88	51	90	91	99	76	82	84	91
250	--	--	161	185	129	176	241	--	214	228	210	222

-- Sample lost.

Table 3. Intended and nominal initial concentrations of lambda-cyhalothrin (ng a.i./L). Nominal initial concentrations are the average and (range) of the three applications per experiment. Nominal initial concentrations were based on measured concentrations of lambda-cyhalothrin in dose solutions and water volumes of enclosures.

Intended	Nominal			
	Spring		Late-summer	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
10	10 (9-11)	9 (6-10)	10 (9-11)	11 (8 –13)
25	26 (23-29)	25 (24-26)	24 (23 – 25)	24 (21 – 30)
50	53 (45 – 59)	50 (43 – 56)	48 (42 – 58)	50 (39 – 59)
100	107 (91 – 134)	112 (106 – 118)	103 (97 – 107)	96 (86 – 107)
250	267 (253 – 293)	277 (252 – 309)	261 (240 – 279)	277 (266 – 284)

The lowest treatment level (nominal 10 ng a.i./L) is of special importance for the response of the very sensitive insect species *Chaoborus obscuripes* (48h-EC50: 2.8 ng a.i./L (Schroer *et al.* 2004)). Variations at this treatment level in actual exposure concentrations might strongly influence the response of this species. Measurements showed that in two out of the three applications, the mean initial concentrations of the 10 ng/L-treatment level were higher in the late summer experiment than in the spring experiment. On the average, the three summer applications were 13% higher than in spring.

Leistra *et al.* (2003) showed that lambda-cyhalothrin dissipated rapidly from the water column. In spring, lambda-cyhalothrin concentrations in the water were 24% (range: 23% – 25%) of the initial nominal concentration by 1 day post-treatment. After 7 days, only 2% (range: 1.8% – 2.1%) of the initial nominal concentration could be detected in the water column.

In late summer, concentrations of lambda-cyhalothrin in the water were reduced to 34% (range: 31%– 37%) of the initial nominal concentrations after 1 day, and had further decreased to 0.6% (range: 0.4% –0.7%) after 7 days (Leistra *et al.* 2003). Rates of dissipation of lambda-cyhalothrin from the water column were considered to be comparable irrespective of the season (Leistra *et al.* 2003).

Macroinvertebrates

Community composition in spring and late summer. A total of 67 macroinvertebrate taxa were identified in the enclosures in the spring experiment. In late summer, this was slightly less with 61 taxa identified. Statistical testing indicated that species composition as a whole in late summer differed significantly from that of spring (Monte Carlo permutation test, $p < 0.05$). In late summer, the snail *Armiger crista,* the flatworm *Polycelis nigra/tenuis*, juvenile *Glossiphonia* (leech) and trichopterans (caddis

flies) were present in relatively low numbers. On the other hand, the molluscs Pisidiidae and *Lymnaea stagnalis*, the flatworm *Dugesia tigrina*, the crustaceans *Asellus aquaticus* and *Proasellus meridianus/coxalis*, and the insects *Cloeon dipterum*, Anisoptera and Zygoptera were present in relatively higher numbers compared to the spring experiment (Fig. 1). The species in the cluster Chironomidae – *Valvata cristata* (Fig. 1) contained in addition to those species that were present in both seasons, also the species that had very low abundance values throughout the year. The potentially most sensitive species, i.e. *C. obscuripes, Gammarus* juveniles and *G. pulex* were relatively abundant in both experiments (species weight (b_k) between -1 and 1, Fig. 1).

Community level response The multivariate analyses indicated that the macroinvertebrates showed significant treatment-related effects compared to the controls in spring as well as in late summer (Table 4). In both experiments, impacts of treatments occurred directly after the first treatment and were most pronounced after these applications (Figs 2 and 3). NOECs were at their lowest values at 7 d post-treatment (respectively < 10 ng a.i./L and 10 ng a.i./L, Table 4).

In spring, short-term effects occurred down to the 10 ng/L-treatment level. At the 250 ng/L-treatment level effects were the most pronounced and lasted throughout the study (Fig. 2). Macroinvertebrate communities in enclosures treated with concentrations up to and including 100 ng a.i./L recovered within the study period (Table 4).

In late summer, effects at the 25 and 50 ng/L-treatment level were transient: the two treatment levels were not significantly different from the controls by the second sampling occasion (Week 3, Table 4). The greatest reductions in macroinvertebrate abudance occurred in the 100 and 250 ng/L-treatments (Fig. 3). Communities exposed to these two treatment regimes did not recover within the study period (Fig. 3, Table 4).

Although treated macroinvertebrate communities showed consistent statistically significant differences compared to the controls, there was no interaction between 'treatment' and 'season' (Table 5). In other words, the sensitivity of the aquatic community to lambda-cyhalothrin treatment was not significantly different between spring and late summer.

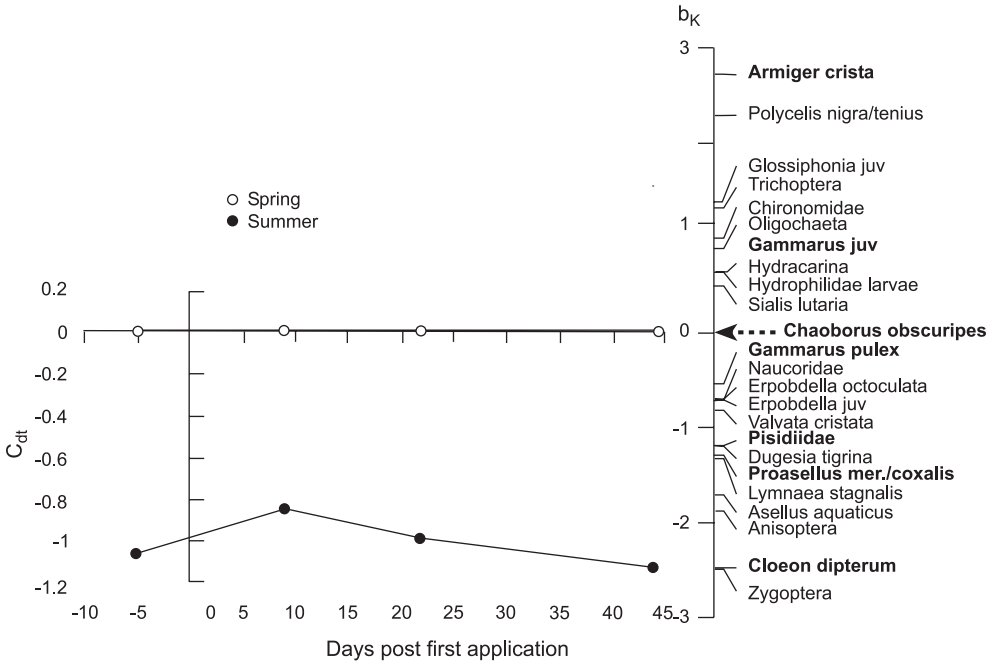

Fig. 1. Principal Response Curves (PRC) indicating the differences in **macroinvertebrate** species composition in spring and late summer in macrophyte-dominated control enclosures. Of all variance 19% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-eight percent of all variance could be attributed to differences between season. Of this variance 61% is displayed on the vertical axis of the PRC diagram. Abundant species are indicated in **bold.** Abundant species were considered the 25% of the species having the highest abundance numbers per sampling date and were present in that list for 3 of the 4 samplings in at least one of both seasons. The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species with a weight (b_k) between 0.5 and -0.5 are not shown (except for *Chaoborus obscuripes*).

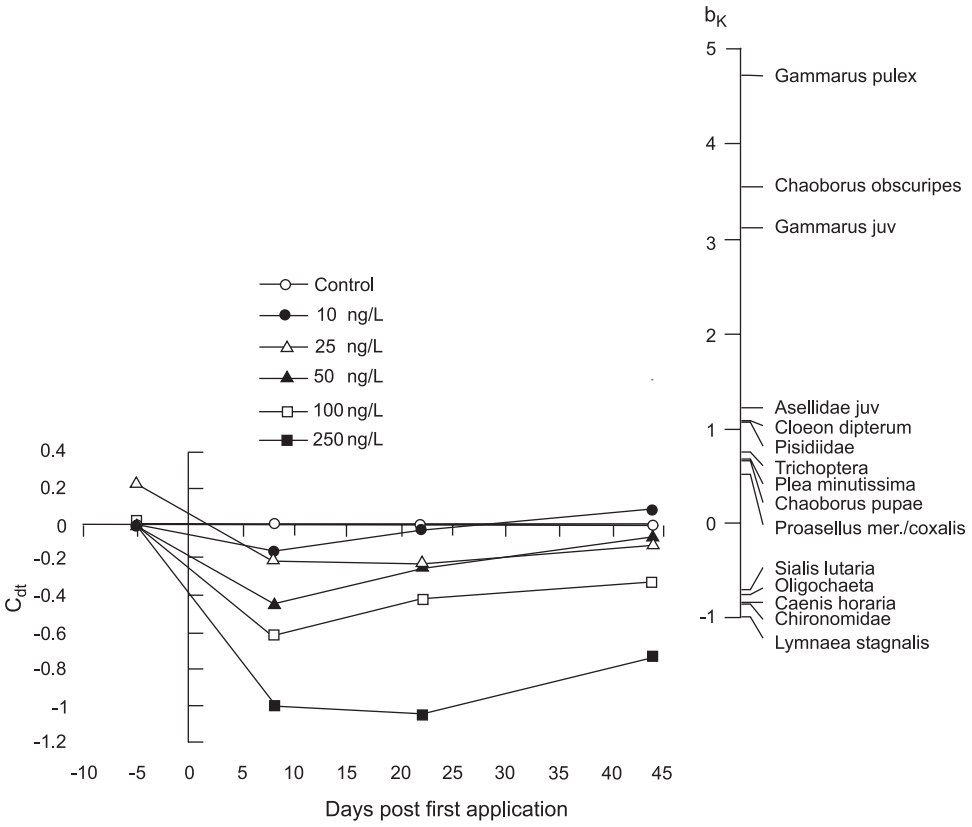

Fig. 2. Principal Response Curves (PRC) indicating effects of the **spring** lambda-cyhalothrin applications on the **macroinvertebrate** communities in ditch enclosures. Of all variance 35% could be attributed to sampling date; this is displayed on the horizontal axis. Thirthy-five percent of all variance could be attributed to treatment. Of this variance 29% is displayed on the vertical axis of the PRC diagram. The PRC diagram shows a significant (p = 0.014) part of the treatment variance.The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species weight (b_k) can be interpreted as the affinity of a taxon to the PRC.

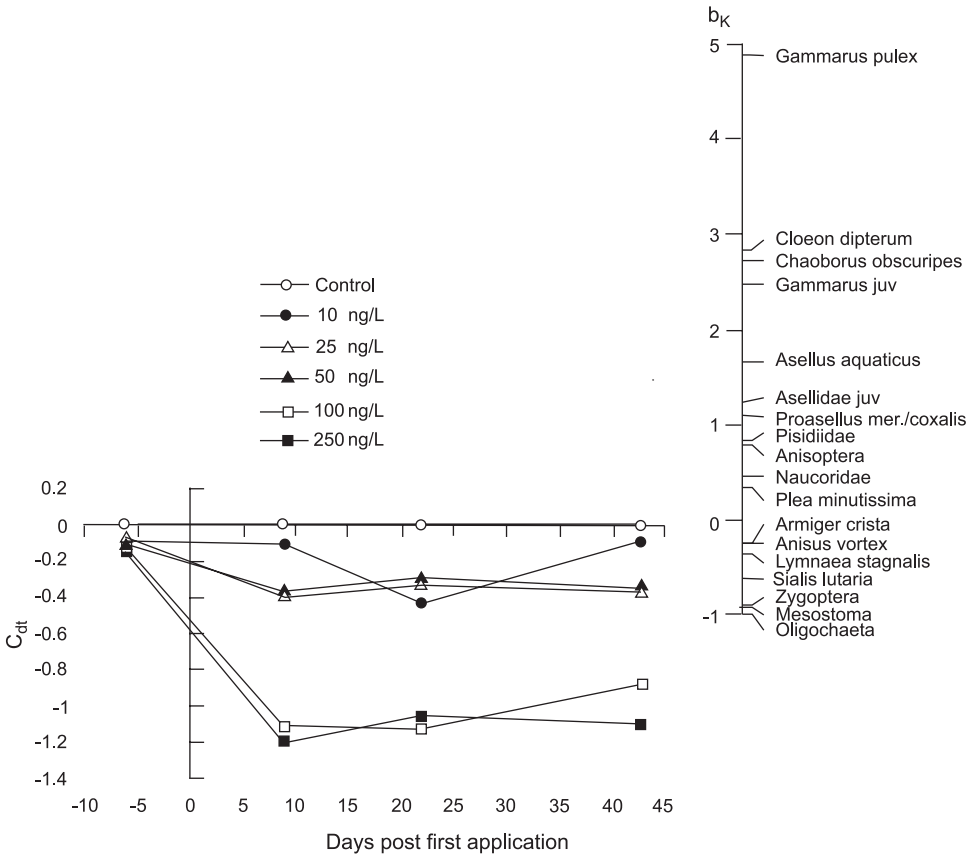

Fig. 3. Principal Response Curves (PRC) indicating effects of the late **summer** lambda-cyhalothrin applications on the **macroinvertebrate** communities in ditch enclosures. Of all variance 29% could be attributed to sampling date; this is displayed on the horizontal axis. Forty percent of all variance could be attributed to treatment. Of this variance 45% is displayed on the vertical axis of the PRC diagram. The PRC diagram shows a significant (p = 0.008) part of the treatment variance. The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species weight (b_k) can be interpreted as the affinity of a taxon to the PRC.

Table 4. Monte Carlo permutation tests on PRC coordinates (*p*-values) for the spring and late summer macroinvertebrate and zooplankton communities, indicating the significance of the treatment regime of lambda-cyhalothrin and $NOEC_{community}$ calculations (William tests, $p < 0.05$) indicating significance of the different treatment levels. NOECs in ng/L.

	Macroinvertebrates			
	Spring		**Summer**	
Week	***p*-value**	**$NOEC_{community}$**	***p*-value**	**$NOEC_{community}$**
-1	> 0.05	250	> 0.05	250
1	< 0.001	< 10	< 0.001	10
3	< 0.001	50	0.006	50
6	0.016	100	< 0.001	50

	Zooplankton			
	Spring		**Summer**	
Week	***p*-value**	**$NOEC_{community}$**	***p*-value**	**$NOEC_{community}$**
-1	> 0.05	≥ 250	> 0.05	≥ 250
1	> 0.05	≥ 250	> 0.05	≥ 250
2	> 0.05	≥ 250	0.025	≥ 250
3	≤ 0.005	25	0.010	≥ 250
4	> 0.05	≥ 250	0.050	≥ 250
5	--	--	> 0.05	≥ 250
6	> 0.05	≥ 250	--	--

Table 5. Results of Monte Carlo permutation tests (*p*-values) on the combined macroinvertebrate and combined zooplankton data sets of the spring and late summer experiments with lambda-cyhalothrin for testing statistical significance of treatment, differences between seasons, and interaction between 'treatment' and 'season'.

	Macroinvertebrates			**Zooplankton**		
Week	**Treatment**	**Season**	**Interaction**	**Treatment**	**Season**	**Interaction**
-1	> 0.05	0.001	> 0.05	> 0.05	0.005	> 0.05
1	0.001	0.001	> 0.05	> 0.05	0.005	> 0.05
2	--	--	--	> 0.05	0.005	> 0.05
3	0.001	0.001	> 0.05	0.005	0.005	> 0.05
4	--	--	--	> 0.05	0.005	> 0.05
5	--	--	--	> 0.05	0.005	> 0.05
6	0.002	0.001	> 0.05	--	--	--

Table 6. No Observed Effect Concentrations (Williams test, $p < 0.05$) per sampling date for macroinvertebrate populations in enclosures exposed to lambda-cyhalothrin in spring and in late summer. Sampling dates are weeks relative to the first applications. Concentrations (ng a.i./L) > NOEC showed significant increases (↑) or reductions (↓). Grey shading indicates responses considered consistent, this is, showing statistical deviations in the same direction for at least two consecutive sampling dates. Number of statistical deviations (Stat. dev.) shows the sum of all NOECs generated per sampling date on the basis of the complete macroinvertebate data set.

		NOEC				
		-1	**1**	**4**	**8**	See.
Spring						
Armiger crista			100(↓)			
Asellidae					100(↓)	
Caenis horaria			100(↑)			
Chaoborus pupae					10(↓)	
Chaoborus obscuripes			10(↓)	10(↓)		Fig. 4
Cloeon dipterum			100(↓)			
Gammarus juv.			100(↓)	100(↓)		Fig. 4
Gammarus pulex			100(↓)	25(↓)	50(↓)	Fig. 4
Haliplidae		<10(↓)				
Lymnaea stagnalis				100(↑)		
Notonecta sp.		<10(↓)				
Sialis lutaria			25(↑)			
Stat. dev.	decrease	2	5	3	3	
	increase	0	2	1	0	
Summer						
Armiger crista		100(↓)	100(↑)			
Asellidae				<10(↓)		
Asellus aquaticus			50(↓)			
Chaoborus obscuripes			<10(↓)	<10(↓)	100(↓)	Fig. 5
Cloeon dipterum			25(↓)		25(↓)	Fig. 5
Gammarus juv.			10(↓)	25(↓)		Fig. 5
Gammarus pulex			50(↓)	50(↓)	50(↓)	Fig. 5
Mesostoma sp.		<10(↓)		100(↑)		
Paraponix clavata				<10(↓)		
Sialis lutaria			50(↑)			
Zygoptera			50(↑)			
Stat. dev.	decrease	2	5	5	3	
	increase	0	3	1	0	

Population level response In spring, consistent responses (i.e., significant responses in the same positive or negative direction for at least two sequential sampling dates) were observed for three taxa, namely *Chaoborus obscuripes*, *Gammarus pulex* and *Gammarus* juveniles (Table 6, Fig. 4). The number of statistical differences was the highest after the first treatments of lambda-cyhalothrin (Table 6). In addition, significant reductions in the post-treatment period on individual sampling dates were observed in Asellidae, *Chaoborus* pupae, and *Cloeon dipterum* (Table 6). *Chaoborus* was the species most affected (NOEC: 10 ng a.i./L) but recovered within the study period (Table 6, Fig. 4). *Gammarus* was affected at the 50 ng/L-treatment and higher (NOEC: 25 ng a.i./L). At the 100 and 250 ng/L-treatment levels, this species had not recovered at the end of the study period (Table 6, Fig. 4).

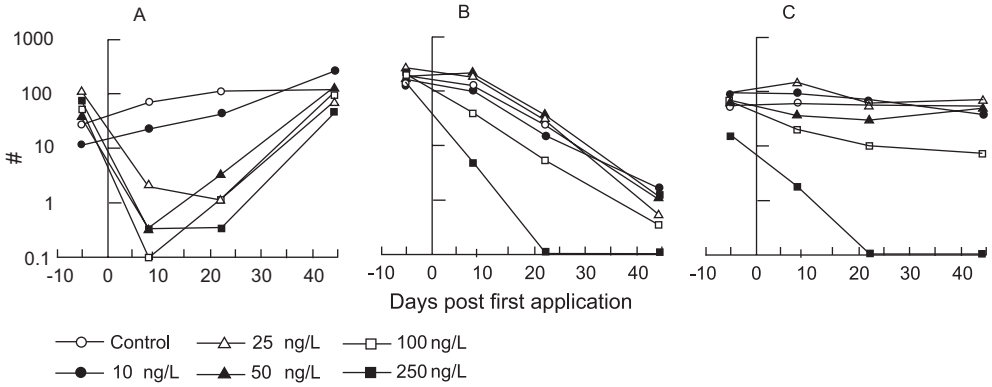

Fig. 4. Dynamics of **macroinvertebrate** species showing consistent responses in the **spring** experiment. Geometric mean numbers of (A) *Chaoborus obscuripes*, (B) *Gammarus* juvenile and (C) *Gammarus pulex*. In the figures, 0.1 denotes absence.

In late summer, responses on at least two sequential sampling dates were again observed for the same three taxa, *C. obscuripes*, *G. pulex* and *Gammarus* juveniles (Table 6, Fig. 5). The number of statistical differences was the highest after the first and second treatments of lambda-cyhalothrin (Table 6). Significant reductions in the post-treatment period on individual sampling dates were observed in Asellidae and *A. aquaticus*, *C. dipterum*, and *Paraponix clavata* (Table 6). Again, *Chaoborus* (Fig. 5) was the species most affected (NOEC: < 10 ng a.i./L). Except for the highest treatment level, the species recovered within the study period on the basis of statistical information (Table 6). Abundance, however, was still much lower (10-fold or more) than controls in the 10 to 100 ng/L-treatment levels at the end of the study (Fig. 5A), implying a high variability of response among replicates. *Gammarus* juveniles (Fig. 5) were affected at the 25 ng/L-treatment and higher (NOEC: 10 ng a.i./L). At the end of the experiment, abundances were not significantly different from the control (Table 6).

Adult *Gammarus* were significantly affected in the 100 and 250 ng/L-treatment levels and had not recovered at the end of the study period (Fig. 5, Table 6).

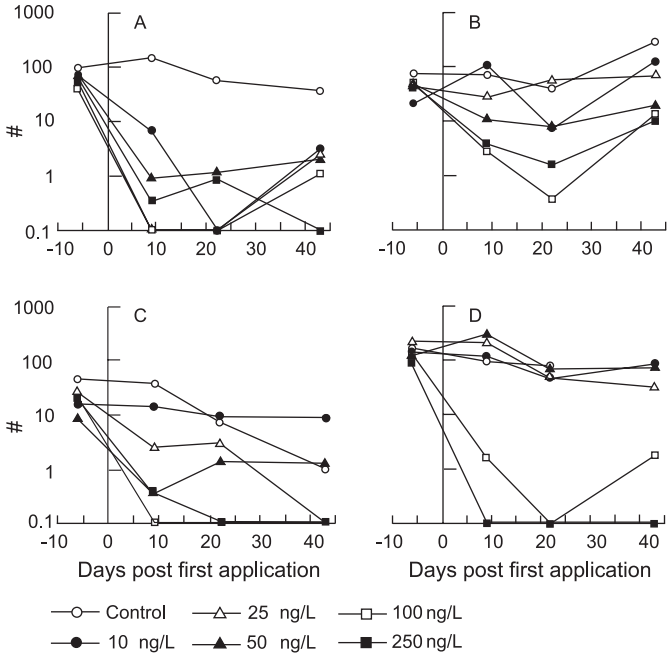

Fig. 5. Dynamics of **macroinvertebrate** species showing consistent responses in the late **summer** experiment. Geometric mean numbers of (A) *Chaoborus obscuripes*, (B) *Cloeon dipterum*, (C) *Gammarus* juvenile and (D) *Gammarus pulex* are shown. In the figures, 0.1 denotes absence.

Bioassays

Exposure to lambda-cyhalothrin affected the survival of *C. obscuripes*. Calculated EC50s indicated that incipient values were reached after two days (Table 7). The EC50 was about 5 ng a.i./L in both experiments (Table 7).

The response pattern of *Asellus aquaticus* was similar to that of *C. obscuripes*, in that lambda-cyhalothrin affected the species acutely and responses stabilised within the first two days. Incipient EC50s were reached at about 70 ng a.i./L in spring and at about 50 ng a.i./L in late summer (Table 7). EC50 values did not appear to differ significantly between seasons considering the overlapping 95%-confidence limits (Table 7).

The second series of bioassays, focusing on recovery, showed that for both *Chaoborus* and *Asellus,* EC50 values in spring as well as in late summer increased with time, indicating potential recovery (Table 8). In the case of *A. aquaticus*, bearing in

mind the 95%-CIs, EC50s did not show consistent differences between spring and late summer (Table 8). For *C. obscuripes*, although 95%-CIs overlap, the EC50 values tended to be lower in the late summer bioassay (Table 8). Only in the last bioassay (8 through 12 days) were EC values considerably lower in late summer (Table 8).

Table 7. EC50 values (ng a.i./L) obtained from *in situ* cage experiments after the first application in enclosures treated with lambda-cyhalothrin. EC50 values are based on nominal concentrations. Lower and upper limits of the 95%-confidence limits are given between brackets.

Test species	**Day**	**EC50**	
		Spring	**Summer**
Chaoborus obscuripes	1	14.4 (10.9-19.0)	9.1 (5.5-15.0)
	2	4.9 (2.5-9.9)	5.0 (2.7-9.3)
	3	4.8 (2.2-10.4)	5.2 (2.6-10.3)
	6	8.0 (a)	4.3 (1.5-12.8)
Asellus aquaticus	1	58.0 (46.7-72.0)	70.4 (54.2-91.4)
	2	71.9 (54.5-95.1)	51.9 (40.9-65.8)
	3	69.4 (52.1-92.4)	50.7 (38.5-66.7)
	6	78.9 (56.8-109.5)	48.9 (37.4-64.0)

a: no 95% confidence limits could be calculated

Table 8. EC50 values (ng a.i./L) obtained from *in situ* cage experiments to study recovery in enclosures treated with lambda-cyhalothrin. Lower and upper limits of the 95%-confidence limits are given between brackets.

Test species	**Day**	**EC50**	
		Spring	**Summer**
Chaoborus obscuripes	0 – 4	9.6 (6.5-14.4)	7.9 (7.7-8.5)
	4 – 8	24.9 (21.0-29.6)	17.1 (13.4-21.8)
	8 – 12	93.0 (79.0-109.4)	28.3 (22.2-36.0)
Asellus aquaticus	0 – 4	57.1 (38.3-85.3)	45.3 (39.2-52.3)
	4 – 8	285.1 (158.6-512.6)	172.5 (144.5-206.0)
	8 – 12a	b	313.9 (228.5-431.3)

a 8 – 13 days in case of late summer experiment.

b no 95% confidence limits could be calculated.

Zooplankton

Community composition in spring and late summer A total of 33 and 35 zooplankton taxa were identified in the enclosures of the spring and late summer experiments, respectively. In both experiments rotifers were the most abundant, followed by cladocerans. The experiments had many taxa in common and several abundant species (e.g. Cyclopoida, nauplii, *Anuraeopsis fissa*) were present in both experiments (Fig. 6). Overall, however, analysis indicated that the late summer zooplankton community structure differed significantly from that in spring (Table 5). The rotifers *Keratella cochlearis*, *Lecane* gr. *lunaris*, *Keratella quadrata*, *Lepadella patella* and *Mytilinia ventralis* were less abundant in late summer than in spring (Fig. 6). The taxa in the cluster *Euchlanis dilatata* – *Trichocerca porcellus* were more abundant in late summer (Fig. 6).

Community response spring and late summer Overall, statistical testing did not provide strong evidence of treatment-related effects of lambda-cyhalothrin on the zooplankton communities in both the spring and the late summer experiment (Figs 7 and 8). In spring, analysis by sampling date yielded an incidental statistically significant deviation for the Week 3 sampling (NOEC: 25 ng a.i./L; Table 4). In late summer, treatment-related deviations were indicated for three sequential sampling dates (Monte Carlo permutation test). The Williams test, however, did not detect a statistically significant concentration-effect relationship for these same sampling dates (Table 4).

No interaction between 'treatment' and 'season' could statistically be detected (Table 7). Zooplankton species composition in late summer differed significantly from that in spring (Monte Carlo permutation test, $p < 0.05$).

Population level response Univariate analysis of the 33 separate zooplankton populations from the spring experiment resulted in consistent statistically significant responses for four of the taxa. Rotifers (group *Anuraeopsis fissa* – *Trichocerca capucina*) generally tended to increase (Table 9). Copepoda (copepod nauplii and cyclopoida) showed consistent reductions (Fig. 9). NOECs were at the 25 ng/L-treatment level (Table 9). As in the macroinvertebrate samples, *Chaoborus obscuripes* was most severely affected. Significant reductions of this species were observed in the lowest treatment level (NOEC: < 10 ng a.i./L). The major reductions in *Chaoborus* populations were observed after the first (Week 1) and second (Week 2) applications of lambda-cyhalothrin (Fig. 9). Thereafter only the highest treatment level showed statistically significant reductions (NOEC: 100 ng a.i./L) (Table 9, Fig.9). Recovery of *Chaoborus* and other species had occurred within the study period (i.e., within 3 weeks after the last treatment) (Table 9).

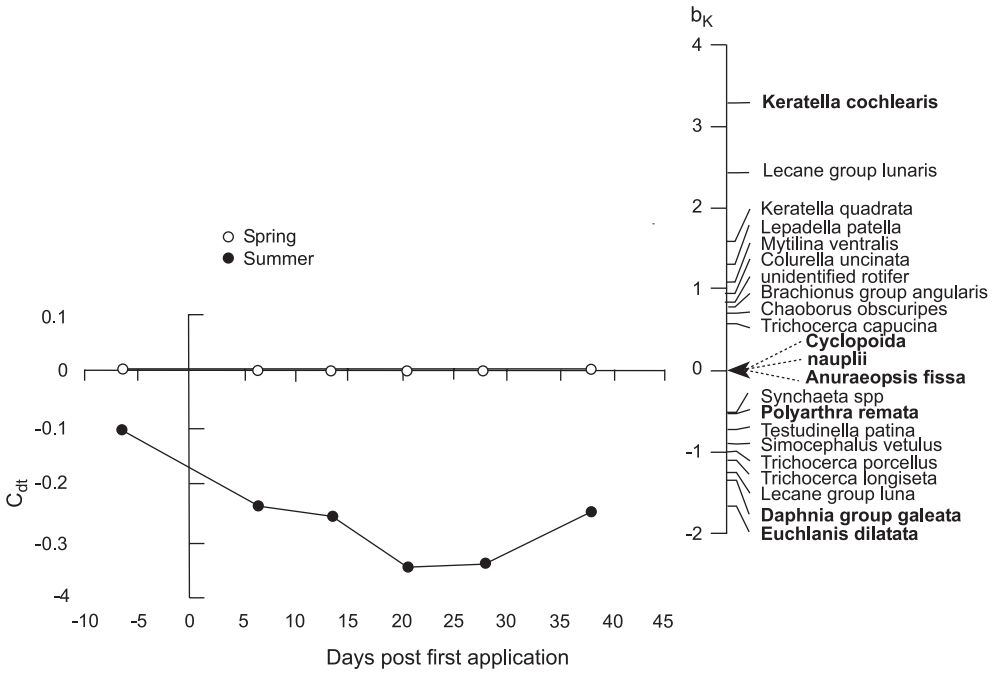

Fig. 6. Principal Response Curves indicating the differences in **zooplankton** species composition in spring and late summer season in macrophyte-dominated control enclosures. Of all variance 23% could be attributed to sampling date; this is displayed on the horizontal axis. Fifty-nine percent of all variance could be attributed to differences between season. Of this variance 65% is displayed on the vertical axis of the PRC diagram. Abundant species are indicated in **bold.** Abundant species were considered the 25% of the species having the highest abundance numbers per sampling date and were present in that list for 3 of the 4 samplings in at least one of both seasons. Species with a weight (b_k) between 0.5 and -0.5 are not shown (except for Cyclopoida, nauplii and *Anuraeopsis fissa*). The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species weight (b_k) can be interpreted as the affinity of a taxon to the PRC.

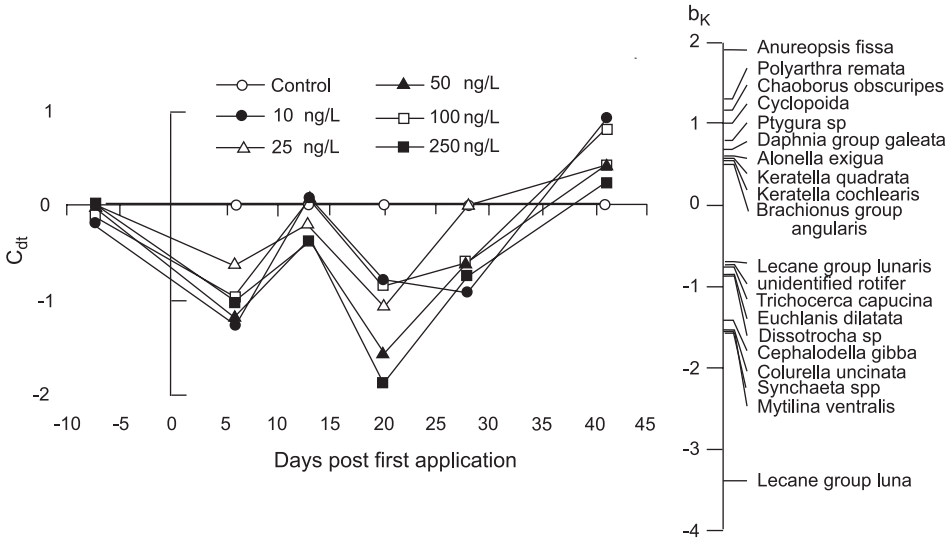

Fig. 7. Principal Response Curves (PRC) indicating effects of **spring** lambda-cyhalothrin applications on the **zooplankton** communities in ditch enclosures. Of all variance 63% could be attributed to sampling date; this is displayed on the horizontal axis. Nineteen percent of all variance could be attributed to treatment. Of this variance 17% is displayed on the vertical axis of the PRC diagram. The PRC diagram shows a moderately significant ($p < 0.075$) part of the treatment variance. The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species weight (b_k) can be interpreted as the affinity of a taxon to the PRC.

In late summer, 2 of the 35 zooplankton populations showed consistent responses (Table 9). Unlike the spring experiment, cladocerans (*Daphnia* gr. *galeata*) showed statistically significant reductions at the 100 and 250 ng/L-treatment levels. Effects at the 100 ng/L-level were observed for one week, after the third treatment (Fig. 10). Again, *C. obscuripes* was the most sensitive species with significant reductions at the 10 ng/L-treatment level. In contrast to the observations in the macroinvertebrate samples, *Chaoborus* had recovered within the study period (compare Tables 6 and 9).

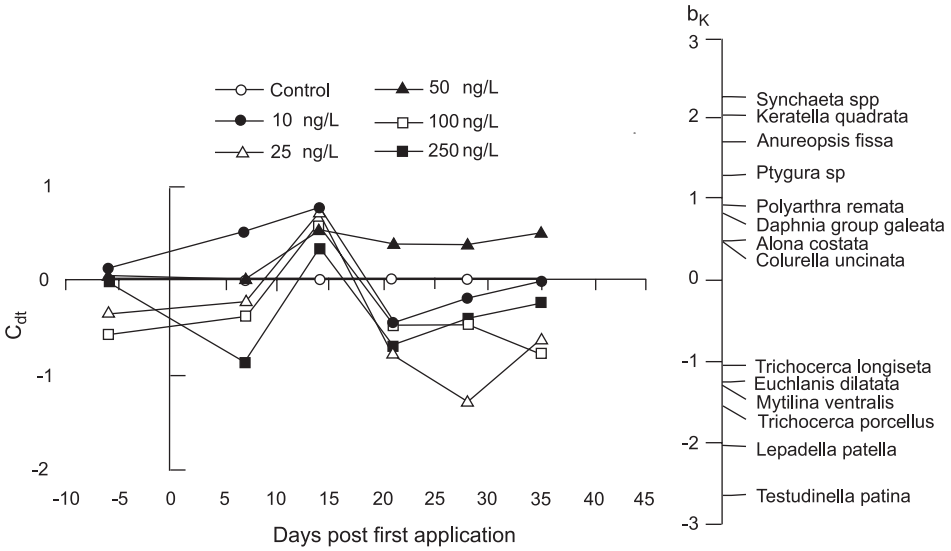

Fig. 8. Principal Response Curves (PRC) indicating effects of the late **summer** lambda-cyhalothrin applications on the **zooplankton** community in ditch enclosures. Of all variance 44% could be attributed to sampling date; this is displayed on the horizontal axis. Twenty-nine percent of all variance could be attributed to treatment. Of this variance 25% is displayed on the vertical axis of the PRC diagram. The PRC diagram shows a moderately significant ($p < 0.070$) part of the treatment variance. The vertical axis represents the differences in community structure between treatment and controls expressed as regression coefficients (C_{dt}) of the PRC model. Species weight (b_k) can be interpreted as the affinity of a taxon to the PRC.

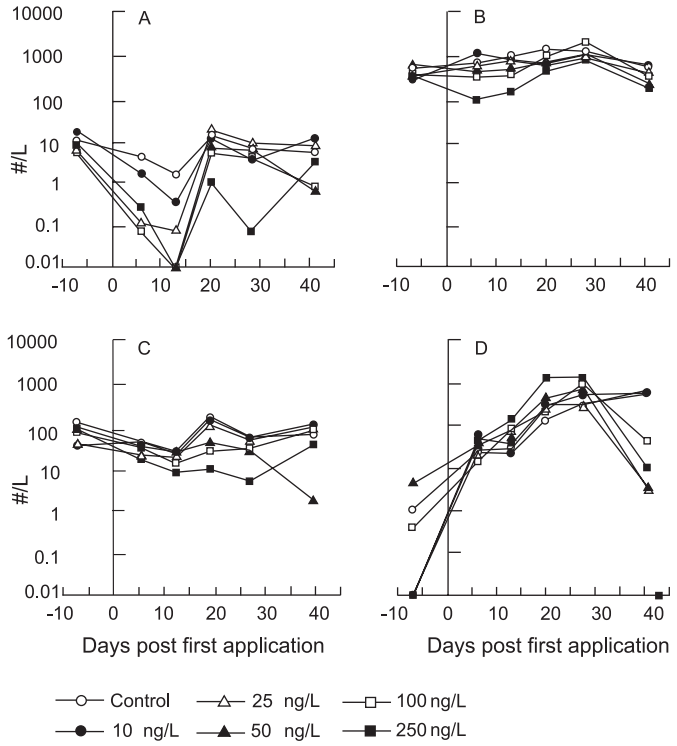

Fig. 9. Dynamics of **zooplankton** species showing consistent responses in the **spring** experiment. Geometric mean numbers of (A) *Chaoborus obscuripes*, (B) nauplii, (C) Cyclopoida, and (D) *Lecane* group *lunaris* are shown. In the figures, 0.01 denotes absence.

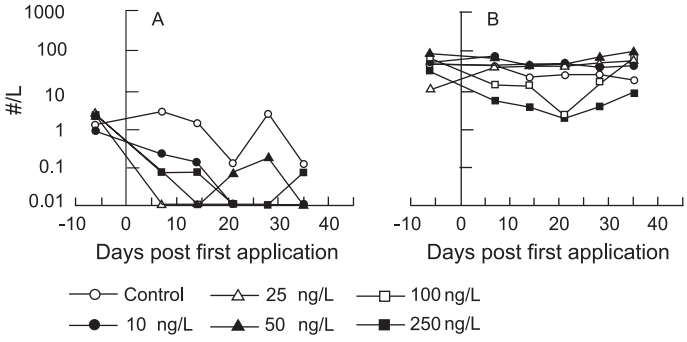

Fig. 10. Dynamics of **zooplankton** species showing consistent responses in the late **summer** experiment. Geometric mean numbers of (A) *Chaoborus obscuripes* and (B) *Daphnia* group *galeata* are shown. In the figures, 0.01 denotes absence.

Table 9. No Observed Effect Concentrations (Williams test, $p < 0.05$) per sampling date for zooplankton populations in enclosures exposed to lambda-cyhalothrin in spring and in late summer. Sampling dates are weeks relative to the first applications. Concentrations (ng a.i./L) > NOEC showed significant increases (↑) or reductions (↓). Grey shading indicates responses considered consistent, this is, showing statistical deviations in the same direction for at least two consecutive sampling dates. Number of statistical deviations (Stat. dev.) shows the sum of all NOECs generated per sampling date on the basis of the complete macroinvertebate data set.

		NOEC							
		-1	**1**	**2**	**3**	**4**	**5**	**6**	See.
Spring									
Anuraeopsis fissa					100(↓)				
Brachionus sp.		<10(↑)							
Brachionus anguilaris						<10(↓)			
Cephalodella gibba			100(↑)						
Colunaris uncinata			100(↑)						
Lecane gr. *lunaris*				100(↑)	<10(↑)				Fig. 9
Lecane gr. *luna*			<10(↑)						
Synchaeta spp						100(↑)			
Trichocerca capucina					50(↑)				
Nauplii			100(↓)	25(↓)	100(↓)				Fig. 9
Cyclopoida					25(↓)	100(↓)			Fig. 9
Daphnia gr. *galeata*						10(↓)			
Ostracoda		<10(↓)							
Chaoborus obscuripes			10(↓)	<10(↓)	100(↓)	100(↓)			Fig. 9
Stat. dev.	decrease	1	2	2	4	4	--	0	
	increase	1	3	1	1	1		0	
Summer									
Synchaeta spp				<10(↑)			<10(↑)		
Nauplii					100(↓)		100(↓)		
Daphnia gr. *galeata*			100(↓)	100(↓)	50(↓)	100(↓)			Fig. 10
Ostracoda						100(↑)			
Chaoborus obscuripes			<10(↓)	<10(↓)		<10(↓)			Fig. 10
Stat. dev.	decrease	0	2	2	2	2	1	--	
	increase	0	0	1	0	1	1	--	

Phytoplankton and periphyton

Neither in spring nor in late summer did chlorophyll-*a* concentrations for either phytoplankton and periphyton show treatment related effects. Only once, in the fourth week of the post-treatment period of the spring experiment, were phytoplankton chlorophyll-*a* concentrations significantly lower than control levels (mean control level: 50 μg/L against 20 – 33 μg/L in the treated systems; Williams test, $p < 0.05$). In comparison, chlorophyll-*a* amounts for both the phytoplankton and periphyton were lower in late summer than in spring. Phytoplankton chlorophyll-*a* concentrations over the entire experimental period in the controls of the spring experiment were 42 ± 11 μg/L compared to 12 ± 5 μg/L (mean ± SD) in late summer. Similarly, mean periphyton chlorophyll-*a* amounts were 32 ± 29 μg/m^2 and 5 ± 2 μg/m^2 in spring and late summer, respectively.

Macrophytes

In spring, mean macrophyte biomass surrounding the enclosures increased with time (104 ± 35 to 138 ± 16 g/m^2 dw), indicating that the vegetation was in a growth phase (Fig. 11A). In late summer, this biomass showed a decrease in time (241 ± 108 to 167 ± 10 g/m^2 dw) and indicated that the vegetation was in its decline phase (Fig. 11A).

In both experiments, vegetation harvested in the enclosures at the end of the experiments did not statistically differ (Williams test, $p > 0.05$) between treatment levels (Fig. 11B).

Decomposition and community metabolism

In spring, no effects on the decomposition of *Populus* leaf litter were measured. In late summer, the remaining biomass of *Populus* leaves was significantly higher in the 100 and/or 250 ng/L-treatment levels than in the controls (Williams test, $p < 0.05$) (Table 10).

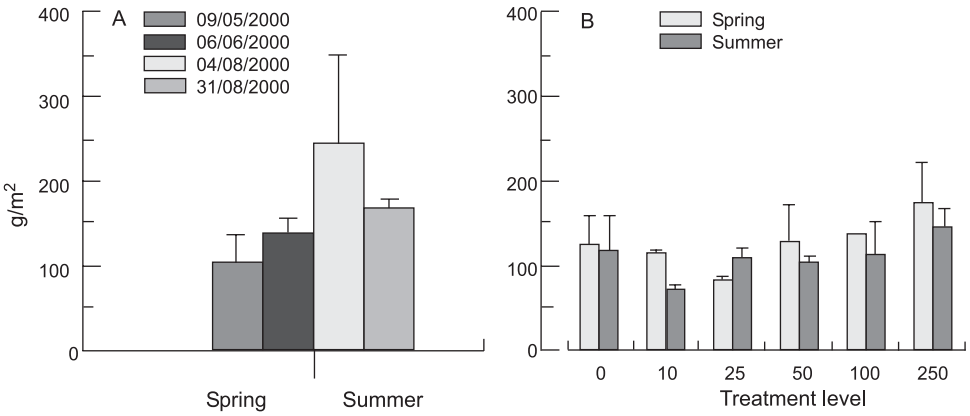

Fig. 11. Mean dry weight of macrophytes (g/m^2) collected in ditches housing the enclosures for the spring and late summer experiment. Macrophytes were collected outside enclosures (A), and mean macrophyte dry weight (g/m^2) collected in enclosures at the end of the experiments (B). No statistically significant differences between treatments and controls were detected (Williams test, $p > 0.05$). Williams tests were done on the enclosure vegetations of the spring and late summer experiments.

Table 10. Remaining dry weight of *Populus* leaves in litterbags. Initial *Populus* dry weight was 2 grams. Duration of experiments was 2 weeks. Mean values (g dw) per treatment level of lambda-cyhalothrin (ng/L) are given. One series of experiments was performed in spring and one in late summer. * Values deviated significantly from control levels (Williams test, $p < 0.05$).

		g dw					
Spring	Week	Controls	10	25	50	100	250
	-3	1.34	1.31	1.18	1.30	1.36	1.38
	-1	1.36	1.32	1.32	1.30	1.36	1.38
	1	1.41	1.36	1.38	1.42	1.45	1.46
	4	1.38	1.37	1.32	1.41	1.41	1.40
Summer	Week	Controls	10	25	50	100	250
	-3	1.23	1.15	1.27	1.09	1.20	1.23
	-1	1.20	1.10	1.23	1.18	1.23	1.24
	1	1.33	1.33	1.40	1.39	1.40*	1.42*
	4	1.33	1.33	1.36	1.34	1.39	1.41*

Table 11. NOECs (Williams test, $p < 0.05$) per sampling date for community metabolism endpoints in enclosures treated with lambda-cyhalothrin (treatments: 0 – 250 ng/L). One experiment was performed in spring and one in late summer. Endpoints were electrical conductivity (EC), dissolved oxygen (DO), pH and alkalinity. Treatments resulted in significant reductions (↓) (Williams test, $p < 0.05$).

	NOEC					
Week	-1	1	2	3	4	5
Spring						
DO[1]						
pH[1]						
EC						25(↓)
Alkalinity				< 10(↓)	100(↓)	25(↓)
Summer[1]						

[1] No statistically significant deviations found.

Discussion

Experimental design

We compared the impact of lambda-cyhalothrin in one experimental ditch in spring and another experimental ditch in late summer. This means that at the level of the ditch, observations were not replicated. Strictly speaking, this implies that differences detected between experiments cannot be attributed solely to 'ditch' or 'season'. However, both ditches were from the same population of twelve similar ditches with shared history, climate and biological communities since their construction in the late 1980s. This makes it reasonable to conclude that any differences in impact between the experiments are a result of the factor 'season' and not of the factor 'ditch'.

Fate

As lambda-cyhalothrin is highly lipophilic (log Kow = 7), the compound tends to bind greatly and rapidly to non-aquatic surfaces such as organic materials and sediment (Hand *et al.* 2001). The high spatial variation in concentrations in the water found shortly after the applications indicated that the distribution of the compound between the different compartments was still in progress. It is well known for pyrethroids that exposure concentrations are difficult to measure in the highly dynamic phase shortly after application. In our study, substantial dissipation of the compound had occurred within the first day of treatment (Leistra *et al.* 2003).The rapid decrease in water concentrations of lambda-cyhalothrin was expected, as this has been reported

previously in many studies with synthetic pyrethroids (e.g., Hill 1989; Hand *et al.* 2001).

The three applications of lambda-cyhalothrin did not lead to an accumulation of the compound in the water phase. Concentrations were very low and/or at the detection limit level by 7 days (Leistra *et al.* 2003), at which point the next application was made. The rapid dissipation of lambda-cyhalothrin indicates that during the study the communities were subjected to repeated, short-term exposures rather than chronic exposures.

Acute effects

As would be expected from laboratory and other field toxicity data (Schroer *et al.* 2004; Maund *et al.* 1998), lambda-cyhalothrin applications resulted in effects down to and including the 10 ng/L-treatment level and specifically occurred within arthropod populations. Observations at the water surface indicated that clear effects, ranging from agitated to dead specimens, had already developed within 10 h after application of lambda-cyhalothrin. This rapid onset of effect coupled with rapid dissipation from the water column is typical for synthetic pyrethroids.

In both experiments, the number of statistical significant effects clearly increased after the treatments started. For the most sensitive group - the arthropods - effects were greatest immediately directly after the first application (Table 6), suggesting that the compound had its major impact on this group after the first applications.

Secondary effects

Although some reductions in the zooplankton were observed at the higher treatment levels, these did not lead to indirect effects in the form of increases of the algae due to a release of grazing pressure. Functional redundancy might have dampened secondary responses because relatively complex natural species assemblages were present. Algal development might also have been repressed because of the dominance of macrophytes in the test sytems. Lambda-cyhalothrin treatments did not or only had minor effects on the community metabolism parameters measured. Later on in the experiment, decomposition was decreased in late summer at the 100 and 250 ng/L-treatment level which may be explained by the reduction of sensitive macro-invertebrate shredders (e.g. *G. pulex*, *A. aquaticus*). It might be that the effect observed in late summer was obscured in spring because availability of suitable organic matter in the detritus layer is larger in spring. Compared to late summer, this could have resulted in less need for the food source in the litter bags. Inherently, the smaller differences between presence and absence of consumption by shredders is harder to detect.

Roessink *et al.* (2005) noted that in spring there was a tendency for higher densities of cladocerans in the enclosures treated with 25, 50 and 100 ng a.i./L

compared to those in the controls and the 250 ng/L-treatment level. This response pattern was explained by the authors as a combination of direct toxic effects and indirect effects. The relatively low abundance of cladocerans in the controls is probably the result of high predation pressure by *Chaoborus*. With increasing treatment levels up to 100 ng a.i./L, predation by *Chaoborus* decreases resulting in higher densities of cladocerans which are less sensitive to lambda-cyhalothrin. This similar inverted U-shaped response also seems to have occured later in the season, as the abundance of cladocerans in the intermediate treatment levels of 10 to 50 ng a.i./L tended to be higher than in the controls and the 100 and 250 ng a.i./L treated enclosures of the late summer experiment (see Fig. 10B as an example).

Comparison of seasons

The communities of the two macrophyte-dominated test systems did not differ very much in macrophyte structure and biomass (Fig. 11B). The amount of algae was lower in the late summer experiment. This phenomenon can be explained by seasonal shifts in algal abundances due to competition on nutrients between algae and the dominating macrophytes (Scheffer 1998). Also, compared to spring, community metabolism was lower in late summer. Despite the statistically significant differences in species composition between both experiments, communities varied little with respect to dominant and sensitive species (e.g., *Chaoborus* and *Gammarus,* Fig. 1).

Summarizing the two experiments into effect classes shows that, except for *Chaoborus,* only slight and transient effects were observed at the lowest treatment level in both experiments (Table 12). In spring, this was expressed at the community level and concerned only some populations, whilst in the late summer only some populations of sensitive macrocrustaceans and insects showed incidental negative responses at the 10 ng/L-treatment level. In combination with the analysis of interactions between 'season' and 'treatment' at the community level, which indicated that there was no significant interaction between these two factors, our study suggests that sensitivity of the macrophyte-dominated system was independent of season. Considering the inconsistent and few incidental responses at the 10 ng/L-treatment level on both the community level as well as on population level, the $NOEC_{community}$ is lower but near to this treatment level in both experiments. At higher concentration levels, the overall picture shows that clear effects tend to be of a shorter duration in spring than in late summer.

The only exception of the general finding of approximately similar threshold levels for both seasons was the response of *Chaoborus obscuripes*. In late summer, this species showed clear effects followed by recovery (Effect Class 3) at the 10 ng/L-treatment level, while it only showed slight effects (Effect Class 2) in spring (Table 12). One difference in effect was that initial reductions were larger in late summer than in spring (compare, e.g., Fig. 4A with Fig. 5A). To find out whether this

difference in effects could be explained by differences in the relative contributions of older and younger cohorts in the populations of *Chaoborus*, the head lengths of specimens caught in the zooplankton control samples of the first sampling post-treatment were measured (following Swift and Federenko, 1975). We observed that the spring population was dominated by younger life-stages whilst the late summer population was dominated by older ones. Assuming that younger life-stages are more sensitive, as is often found (Hutchinson *et al.* 1998; Stark 1999), it appears that differences in cohort structure of the *Chaoborus* populations do not explain the more severe reductions in late summer.

We also investigated whether small differences in exposure concentrations could have had an influence. Mean nominal initial concentrations in spring were 9.5 ng a.i./L compared to 10.5 ng/L in late summer at the lowest treatment level (Table 3). Based on a laboratory concentration – response relationship for *C. obscuripes*, the affected fraction in this concentration range would be 85 – 86% (after Schroer *et al.* 2004). Another possibility causing differences between the responses might be that differences in bioavailability of lambda-cyhalothrin between the experiments might have occurred, for instance, due to the higher phytoplankton densities in spring. The similarity in response of *Chaoborus* in the *in situ* bioassays, however, make this possibility ambiguous also.

The approach used here to categorise 'severity of effects' includes both inherent 'sensitivity' along with the duration of the observed effects. The time span of effects on *Chaoborus* was longer in late summer, when recovery did not occur, or was not complete (compare Figs. 4A and 5A). However, the *in-situ* cage experiments with *Chaoborus* after the last treatment demonstrated that, in both experiments, recovery of the species potentially was possible shortly after the treatments (significant increase of toxicity values already after 8 – 12 d after the last treatment (Table 8)).

The differences in actual recovery can be explained by the different recolonization patterns found for spring and late summer. The number of specimens in the youngest life-stage class found in the zooplankton samples indicate that the colonization rate of *Chaoborus* is much higher in spring than in late summer. In spring we found several tens of new recruits at every sampling date. In late summer these numbers were well below ten individuals (Fig. 12). Recolonization, even at the highest treatment level, started in the week of the last spring application. In late summer, recolonization hardly occurred at all (Fig. 12).

Table 12. Summary of effects observed in the spring and summer experiments in enclosures treated with lambda-cyhalothrin. The numbers in the table follow the effect classes as described by Brock *et al.* (2000). 1 = No effect; 2 = slight effects; 3 = clear short-term effects, full recovery observed (within 4 – 8 weeks); 4 = clear effects, no full recovery observed at the end of the experiment. ↓ = decrease of endpoint; ↑ = increase of endpoint; ↓↑ decrease and increase of endpoint. PRC: Principle Response Curves of either macroinvert(ebrates) or zooplankton. excl. *Chaob*: without *Chaoborus*. *Chaoborus* = *Chaoborus obscuripes*.

	Endpoint	**Treatment levels (ng/L)**				
		10	**25**	**50**	**100**	**250**
Spring	PRC macroinvert	2	2	3	3	4
	Macrocrustaceans	1	2↓	2↓	4↓	4↓
	Insecta (excl. *Chaob*)	1- 2↓	3↓	3↓	3↓	3↓
	Chaoborus	2↓	3↓	3↓	3↓	3↓
	Remaining Macroinvert	1	1	1	1	2↓↑
	PRC zooplankton	1	1	2	2	2
	Microcrustaceans	1	2↓	2↓	2↓	4↓
	Rotifers	2↓↑	2↓↑	2↓↑	2↓↑	2↓; 3↑
	Algae	1	1	1	1	1
	Macrophytes	1	1	1	1	1
	Community metabolism	1	1	1	1	1
Summer	PRC macroinvert	1	2	2	4	4
	Macrocrustaceans	1	2↓	3↓	4↓	4↓
	Insecta (excl. *Chaob*)	1-2↓	1-2↓	4↓	4↓	4↓
	Chaoborus	3↓	3↓	3↓	3↓	4↓
	Remaining Macroinvert	1	1	1	1	2↑
	PRC zooplankton	1	1	1	1	1
	Microcrustaceans	1	1	1	2↓	3↓
	Rotifers	2↑	2↑	2↑	2↑	2↑
	Algae	1	1	1	1	1
	Macrophytes	1	1	1	1	1
	Community metabolism	1	1	1	1	1

This difference in recolonization potential of *Chaoborus* had a considerable impact on the recovery of the complete macroinvertebrate community, as indicated in the PRC analysis (Figs 2 and 3). Together with *Gammarus*, *Chaoborus* dominated the community response curves as they have the highest species weights. *Gammarus*, however, was a constant factor in the sense that it was eradicated at the highest treatment level in both experiments and in neither case could it recover due to the lack of suitable recolonization conditions as this species is obligately aquatic. The dominant varying factor was the difference in abundance of *Chaoborus* and thus this contributed greatly to the overall community response. Lack of recovery of the community in late summer was mainly due to the absence of recolonization by *Chaoborus* later in the season.

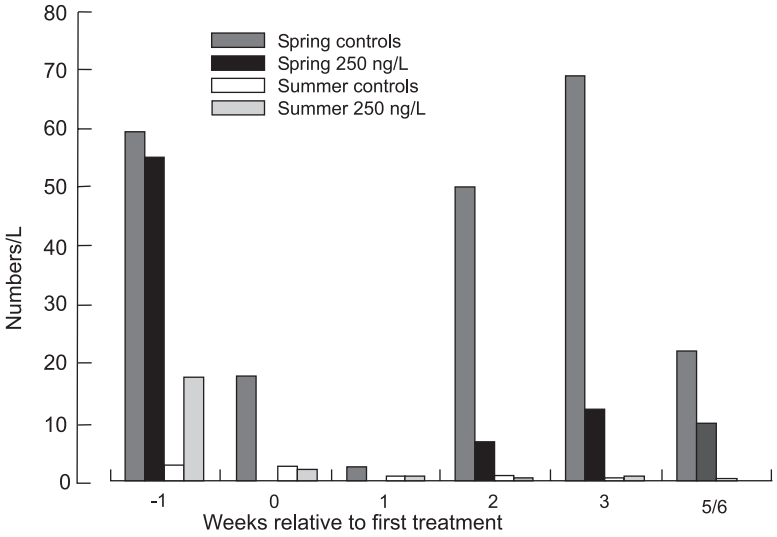

Fig. 12. Mean numbers of specimens within the youngest life-stage class of *Chaoborus obscuripes* in relation with time. Youngest life-stage class contained larvae with head lengths ≤ 0.6 mm. Applications of lambda-cyhalothrin were in Weeks 0 - 2.

Comparison with other studies

The partner study which focussed on differences in effects between macrophyte-dominated and plankton-dominated systems, indicated that the factors 'treatment regime', 'community structure' and the interaction between these two were statistically significant variables. This indicated that, overall, the macrophyte-dominated and the plankton-dominated systems responded differently to the same treatment regime although the overall threshold levels were similar (Roessink *et al.,* 2005). In the plankton-dominated systems only slight and transient effects at the 10 ng/L-treatment level were observed. An indoor microcosm study with a pesticide mixture containing lambda-cyhalothrin, with several applications of the compound gave a $NOEC_{community}$ at the treatment level containing 10 ng lambda-cyhalothrin/L. However, lack of response at the 10 ng/L-treatment level was explained by the very low numbers of *C. obscuripes* in the test systems (Van Wijngaarden *et al.* 2004). Microcosm studies indicate that pronounced effects (Effect Class 3 –5) of lambda-cyhalothrin on sensitive populations can be expected at exposure concentrations of 16 – 25 ng a.i./L and higher (Hill *et al.* 1994, Farmer *et al.* 1995, Roessink *et al.* 2005, present study).

Overall, our study did not provide straight forward evidence of major differences in effects around threshold levels between spring and late summer. At higher concentrations, recovery took more time in late summer. Similar observations are reported for an enclosure experiment with pentachlorophenol (Willis *et al.* 2004).

For temperate regions, the CLASSIC guidance document, which deals with the interpretation of results of aquatic microcosm and mesocosm studies in relation to risk assessment procedures of pesticides, recommends to apply test substances in the period between spring and midsummer (Giddings *et al.* 2002). On the basis of outdoor model ecosystem experiments (Willis *et al.* 2004; this present study) it seems that exposure concentrations around threshold levels for direct effects observed in early season studies are reasonably predictive for effects later in the season. Above these threshold concentrations, however, severity, duration, and type of direct as well as indirect effects may be much more variable during different periods of the year due to seasonal variation in population densities, recovery potential and food web interactions.

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6 Aquatic risk assesment of a realistic exposure to pesticides used in bulb crops: a microcosm study

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Abstract

The fungicide fluazinam, the insecticide lambda-cyhalothrin and the herbicides asulam and metamitron were applied to indoor freshwater microcosms (water volume approximately 0.6 m^3). The treatment regime was based on a realistic application scenario in tulip cultivation. Concentrations of each pesticide were equal to 0%, 0.2%, 0.5%, 2%, and 5% spray drift emission of label-recommended rates. Contribution of compounds to the toxicity of the pesticide package was established by expressing their concentrations as fractions of toxic units. The fate of the compounds in the water, and responses of phytoplankton, zooplankton, periphyton, macroinvertebrates, macrophytes, decomposition, and water quality were followed for 13 weeks. The half-lives of lambda-cyhalothrin, metamitron, and fluazinam were one to two days, that of asulam was > 30 days. No consistent effects could be demonstrated for the 0.2% treatment regime that was therefore considered the no-observed-effect $concentration_{community}$ (NOEC). The macroinvertebrate populations of *Gammarus pulex*, *Asellus aquaticus,* and *Proasellus meridianus* were the most sensitive endpoints, followed by species of copepods and cladocerans. Responses were mainly due to lambda-cyhalothrin. The 0.5% treatment regime resulted in short-term effects. Pronounced effects were observed at the 2% and 5% treatment levels. At the end of the experiment, the macrophyte biomass which consisted of *Elodea nuttallii,* showed a decline at the two highest treatment levels, asulam being the causal factor (NOEC: 0.5% treatment level). Primary production was reduced at the 5% treatment level only. In our experiment, the first-tier risk assessment procedure for individual compounds was adequate for protecting sensitive populations exposed to realistic combinations of pesticides. Spray drift reduction measures seem to be efficient in protecting aquatic ecosystems in agricultural areas.

Keywords- Crop protection scenario; Drift reduction measures; Pesticide package; Risk assessment; Toxic units

Introduction

Pesticides used for crop protection may enter aquatic ecosystems due to any combination of spray drift, leaching, runoff or accidental spills [1]. Consequently, traces of pesticides, or mixtures of them, are not uncommon in aquatic ecosystems in agricultural landscapes [2-4]. This may result in undesirable side-effects on nontarget aquatic biota. For the protection of natural populations, authorities involved in the licensing of pesticides have set certain criteria to reduce adverse effects on aquatic ecosystems [5-6]. However, in current risk-evaluation procedures pesticides are generally evaluated individually, while in agriculture and horticulture it is common practice to apply several pesticides simultaneously and repeatedly, and in differing combinations through time.

Relatively little information is available on the effects of realistic combinations of pesticides on the structure and functioning of shallow freshwater ecosystems [7]. Experiments with mixtures of pesticides are generally restricted to a combination of a few compounds and do not mimic specific pesticide treatment packages [7-10].

In the present experiment, we used a crop-based approach for the selection of pesticides and treatment regime, to gain information on ecological risks of actual emissions of pesticides to surface water. As a reference, we used a spraying regime associated with tulips, since bulb crops are an important agricultural product in The Netherlands and are relatively intensively treated with pesticides [11]. In the flat polder-landscape of The Netherlands, spray drift is considered to be a main entry route for causing peak concentrations of pesticides in bodies of water [12]. Treatment levels were therefore based on spray drift emissions derived from reference tables, with pesticide concentrations resulting from 5% emission as the highest, and 0.2% emission as the lowest treatment level. Based on crop type and current agricultural practice, a maximum of approximately 5% emission of the label-recommended dosage can be assumed to enter neighboring drainage ditches, when no emission-reducing measures are implemented [13,14]. At the other extreme, emission levels can technically be reduced to approximately 0.2% when several emission-reducing measures (buffer zones, special spray nozzles, etc.) are used simultaneously [15,16].

The selected pesticides in the present experiment are often used on tulip crops and comprise the dinitroaniline fungicide fluazinam, which uncouples mitochondrial oxidative phosphorilation [17]; the carbamate herbicide asulam, which inhibits dihydropteroate synthase and induces chlorose [17]; the triazine herbicide metamitron, which inhibits photosynthetic electron transport at the photosystem II receptor site [17] and lambda-cyhalothrin which is a synthetic pyrethroid and is a neurotoxic insecticide [17].

The present research aims to assess the potential ecological impact of realistic pesticide exposure in surface waters bordering bulb fields (crop approach), to evaluate

the protective value of the first-tier risk assessment procedure adopted by the European Union for realistic exposure events to pesticide combinations, and to gain insight into the protection of aquatic communities adjacent to bulb fields by mitigating measures that reduce spray drift emission.

We describe the concentration dynamics in the water phase of the pesticides used in this microcosm study. We further focus on the responses of planktonic and macroinvertebrate communities, and on the effects on macrophytes and community metabolism. Next, we will discuss the results in the light of risk assessment and in relation to spray drift reduction measures.

Materials and Methods

Experimental design

Twelve indoor microcosms were used (length 110 cm, width 110 cm, height 70 cm, water volume ~ 0.6 m^3) (Fig. 1). Each microcosm contained a sediment layer (sandy loam) of 10 cm and a water column of 50 cm. The sediment originated from an uncontaminated freshwater lake near Wijchen, The Netherlands. The water introduced was unchlorinated tap water. Artificial daylight was provided by Philips HPI-T 400 W high-pressure metal halide lamps (Philips, Eindhoven, The Netherlands). A daily photoperiod of 14 h and a temperature of approximately 20 ± 1 ºC was maintained in the climate chamber.

Elodea nuttallii shoots, plankton, and macroinvertebrates, collected from uncontaminated drainage ditches (Sinderhoeve Experimental Station, Renkum and Veenkampen, experimental field site of Wageningen University, Wageningen, The Netherlands) were introduced to develop a macrophyte-dominated freshwater community. During the acclimatization period of two months, the systems were interconnected and the water was circulated so that there would be similarity between the microcosms at the start of the experiment. The circulation of water was stopped fifteen days before the first application. In order to maintain some water movement the microcosms were lightly aerated. To support plant growth, small doses of inorganic nitrogen (0.09 mg N/L) and phosphorus (0.015 mg P/L) were added to each cosm every week. These nutrients were applied as NH_4NO_3 and KH_2PO_4, respectively.

The microcosms were randomly assigned to the different treatment levels. Four microcosms were used as controls. Treatments were performed in duplicate. Due to the accidental application of pesticides in one control cosm, however, three controls remained. Two weeks prior to the first treatment, all biological endpoints were sampled once or twice to establish the pretreatment situation.

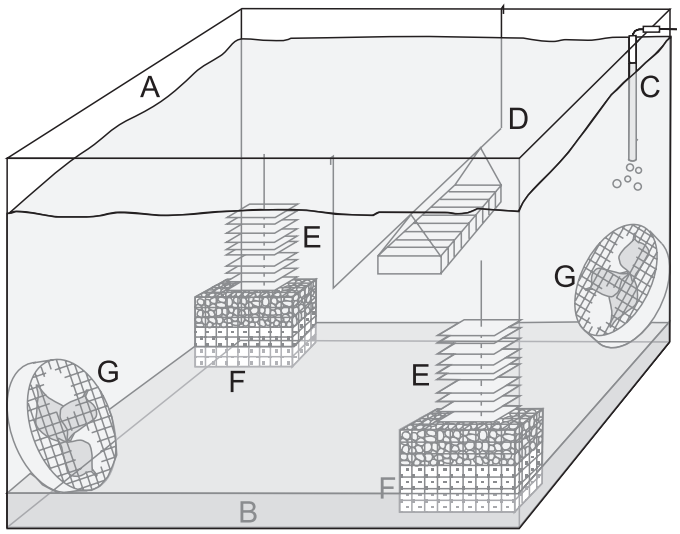

Fig. 1. Lay-out of the microcosms (after [41]). A: glass aquarium (110 x 110 x 70 cm), water depth 50 cm. B: sediment layer of 10 cm thickness. C: aeration point to stimulate water movement at the surface. D: rack with glass slides to study periphyton. E: pebble basket and multiplate serving as artificial substrates for macroinvertebrates. F: pebble basket embedded in the sediment serving a basis for the artificial substrates. G: petri-dish containing leaf material to study decomposition.

Pesticide application and sampling

Concentrations of each pesticide applied to the microcosms corresponded to 0.2%; 0.5%; 2% and 5% spray drift emission of label-recommended rates (Table 1). Target concentrations were calculated by translating the recommended doses for pest control (expressed in g/ha) to g/m^2, which were then multiplied by the respective percentage of spray drift to calculate the amount of pesticide deposited on the water surface (g/m^2). To calculate the resulting water concentration (g/L) per surface unit (m^2), a water depth of 0.3 m was chosen. This depth followed that of the standard drainage ditch scenario used for pesticide registration procedures in the European Union [18].
Fluazinam was applied as the formulated product Shirlan®, a.i. 500 g/L (Zeneca b.v., Ridderkerk, The Netherlands). Asulam was applied as Asulox®, a.i. 400 g/L (Rhône-Poulenc Agro b.v., Etten-Leur, The Netherlands). Metamitron was applied as Goltix®, a.i. 70% (Bayer Agrochemie, Mijdrecht, The Netherlands). Lambda-cyhalothrin was applied as Karate®, a.i. 50 g/L (Zeneca b.v., Ridderkerk, The Netherlands). The pesticides were applied at different frequencies and sequences into the microcosms using the crop culture of tulips as a realistic application scenario. In total seven weekly applications containing different combinations of the compounds were used (Table 2). To simulate the predicted environmental concentration (i.e., compounds

are assumed to mix instantaneously through the body of water resulting in equal concentrations through the entire water column), we poured the dosing solutions evenly over the water surface of the microcosms, and immediately thereafter mixed the water gently using a glass rod. Controls were treated with unchlorinated tap water only (same volume as the treated microcosms).

To follow the concentrations of the pesticides in the water column, water samples were collected from the microcosms shortly before, and at preset time intervals after the application of pesticides. Samples were taken with a perspex tube to obtain depth-integrated samples. The samples were transferred into glass bottles, and transported to the laboratory for analysis.

Table 1. Emission levels, target concentrations and nominal concentrations of pesticides used in the study. Concentratons in μg/L. Nominal concentrations are based on the measured dosage concentrations of each application divided by the water volumes of the microcosms. Nominal concentrations for each pesticide are given as means and (range) of the number of applications given (*n* = number of applications)

		Emission level			
		0.2%-level	0.5%-level	2%-level	5%-level
Fluazinam [*n*=5]	Target	0.27	0.67	2.7	6.7
	Nominal	0.34 (0.21-0.64)	0.61(0.52-0.69)	2.5 (2.2-2.8)	6.3 (5.8-6.8)
Asulam [*n*=3]	Target	0.54	1.34	5.4	13.4
	Nominal	0.63 (0.61-0.65)	1.86 (1.51-2.53)	6.0 (5.9-6.0)	14.7 (14.5-15.1)
Metamitron [*n*=2]	Target	0.47	1.17	4.7	11.7
	Nominal	0.55 (0.53-0.56)	1.32 (1.25-1.39)	5.2 (5.0-5.3)	12.6 (12.2-13.0)
λ-Cyhalothrin [*n*=5]	Target	0.01	0.025	0.1	0.25
	Nominal	0.01 (0.008-0.012)	0.024 (0.021-0.027)	0.09 (0.09-0.11)	0.25 (0.23-0.27)

Chemical analysis

Fluazinam and metamitron were extracted from water samples by solid phase extraction (C_{18}). The solid phase extraction columns were preconditioned with methanol (5 ml) and distilled water (5 ml) respectively, before extraction of the water samples. Both fluazinam and metamitron were eluted from the columns with acetonitril (2 x 0.5 ml) into volumetric tubes. Samples were diluted with distilled water to a proper end-volume, before analysis on a high-performance liquid chromatography system (Table 3). Recovery efficiencies for fluazinam and metamitron were 72.7% ± 10.4% (*n* = 30) and 91.0% ± 4.6% (*n* = 16), respectively. Concentration calculations were based on external standard samples. In the case of fluazinam, concentrations were corrected for recovery since the concentration was relatively low.

Table 2. Application scheme and summary of physico-chemical and biological endpoints investigated in the microcosms

	Weeks relative to first application															
Application	-2	-1	**0**	**1**	**2**	**3**	**4**	**5**	**6**	7	8	9	10	11	12	13
Fluazinam			x	x	x		x		x							
Asulam				x	x				x							
Metamitron					x				x							
λ-Cyhalothrin					x	x	x	x	x							
Physico chemical																
Temperature	x	x	x	x	x	x	x	x	x	x	x	x	x			x
Dissolved oxygen	x	x	x	x	x	x	x	x	x	x	x	x	x			x
pH	x	x	x	x	x	x	x	x	x	x	x	x	x			x
Electrical conductivity	x	x	x	x	x	x	x	x	x	x	x	x	x			x
Alkalinity	x	x	x	x	x	x	x	x	x	x	x	x	x			x
NH_4^+-N	x		x		x		x		x		x		x			x
NO_3^{2-}-N	x		x		x		x		x		x		x			x
ortho-P	x		x		x		x		x		x		x			x
Biological																
Phytoplankton																
Species omposition	x	x			x		x		x		x		x			x
Chlorophyll-a	x	x			x		x		x		x		x			x
Periphyton																
Chlorophyll-a	x		x		x		x		x		x		x			
Zooplankton																
Species composition	x	x			x	x		x		x			x			x
Macroinvertebrates																
Species composition		x				x				x						x
Macrophytes																
Biomass																x
Decomposition																
Poplar-leaves		x				x				x						x

Table 3. Parameters for the high-performance liquid chromatography (HPLC) analysis of the pesticides fluazinam, metamitron, and asulam. (Waters products: Etten-Leur, The Netherlands; Perkin-Elmer ISS autosampler: Überlingen, Germany)

	Fluazinam	**Metamitron**	**Asulam**
HPLC model	Waters M590 + Perkin-Elmer ISS 100 autosampler + Waters LC90 ultraviolet detector		
Injection volume	100 µL		
Column	Waters Novapak® C_{18} (4.6 x 150 mm, 4 µm)		Waters Xterra™ MS C_{18} (4.6 x 150 mm, 3.8 µm)
Guard column	Waters Novapak® C_{18} (3.9 x 20 mm, 4 µm)		Waters Xterra™ MS C_{18} (3.9 x 20 mm, 3.8 µm)
Mobile phase	acetonitril 75% water 25% acetic acid 0.1%	acetonitril 25% water 75%	methanol 15% water 75% buffer pH=3 10%
Flow	1 ml/min	1 ml/min	0.75 ml/min
Oven temperature	40 °C		
Wavelength	260 nm	310 nm	270 nm
Retention time	4.5 min	3.8 min	6.8 min
Detection limit	0.05 µg/L		

For the analysis of asulam, a known volume of the water sample was transferred to a flask and 15 ml of a buffer solution (pH = 3) was added. The OASIS™ HLB solid phase extraction columns (Etten-Leur, The Netherlands) were preconditioned with 3 ml methanol and 3 ml of distilled water, respectively. The water samples were extracted with these solid phase extraction columns and asulam was eluted from the column with methanol (2 x 0.5 ml). The eluate was collected in volumetric tubes and the samples diluted with water to an end-volume of 5 ml, before analysis on a high-performance liquid chromatography system (Table 3). The recovery efficiency for asulam was 96.6% ± 4.3% (n = 17). Asulam concentrations were not corrected for recovery.

For the analysis of lambda-cyhalothrin, a known volume of water was transferred to a flask and 35 ml of distilled petroleum-ether was added. The flasks were closed with a lid provided with an aluminium inlay and shaken thoroughly for at least fifteen minutes. The organic layer was concentrated by evaporation of the petroleum-ether on a rotavapor and the residue dissolved in 1.5 ml hexane. The samples were transferred into gc-vials for analysis on a gas chromatograph. The compound was detected with an HP5890 gas chromatograph equipped with an HP 7673 autosampler (Hewlett-Packard, Avondale, PA, USA) and an electron capture detector. Concentration calculations were based on external standard samples. Chromatographic conditions were set at injection volume 3 µL; column CP Sil5 CB (25 m x 0.53 mm); film thickness 5 µm; injection temperature 250 °C; mobile phase helium 8 ml/min (11 psi); oven temperature 265 °C (isothermal); detector temperature 325 °C; make-up gas N_2 52 ml/min; retention time 9.7 min. The detection limit of lambda-

cyhalothrin in freshwater was 0.005 μg/L. The recovery efficiency for lambda-cyhalothrin was 89.0% ± 8.7% (n = 11). Lambda-cyhalothrin concentrations were not corrected for recovery.

We followed the course of the concentration in detail for the 0.5% and 5% treatment levels. The other two treatment levels were checked by taking samples of the dosage concentrations and water samples directly before, and one hour after applications. Nominal concentrations were determined by analysis of the dosage concentrations, and then dividing them by the water volumes of the microcosms. Instantaneous mixing of the pesticides was assumed.

We calculated half-life times ($t_{1/2}$) for each of the chemicals and used them for ranking dissipation rates from the water. The course of the dissipation was approximated by first-order kinetics. Half-lives were calculated according to the Organisation for Economic Cooperation and Development Guideline 308 [19]. They were calculated for each application period separately, and based on the measurements above detection limit.

Physico-chemical variables

Temperature, dissolved oxygen (DO), pH, electrical conductivity, and alkalinity were measured weekly in the morning, and at the end of the photo-period, commencing two weeks before the first application (Table 2). Dissolved oxygen was measured at a depth of 10 cm (WTW Oxi 196 oxygen meter, Weilheim, Germany). Electrical conductivity and pH were measured using a WTW conductivity meter and a Metrohm Herisau pH meter, respectively. Alkalinity was analysed in 100 ml samples taken from a depth of 10 cm (titration with 0.05 N HCl until a pH of 4.2 was reached).

Nutrients were measured biweekly from depth-integrated water samples (Table 2). For this purpose, subsamples from at least five locations well distributed over each microcosm were collected. A portion of the pooled subsamples was filtered through pre-washed glass-fibre filters (Whatman GF/C 1.2 μm, Maidstone, UK). Some of the filtered water was transferred to 100-ml iodated polyethylene bottles and stored at –20 °C. At the end of the experiment the defrosted samples were colometrically analyzed for ammonium, nitrate and orthophosphate using a Skalar 5100 Autoanalyser (Breda, The Netherlands).

Plankton and periphyton

Zooplankton and phytoplankton were regularly sampled from each microcosm for qualitative and quantitative analysis (Table 2). For both planktonic groups, depth-integrated water samples were randomly collected in each microcosm on each sampling day by using a perspex tube (0.4 m long, 0.8 L in volume). Several subsamples were collected until an 8 L sample had been obtained. These samples were

passed through a 40-µm mesh net to collect the plankton. The plankton was preserved with formalin (c. 4% end volume).

Phytoplankton species composition was studied by counting the number of cells of a known volume. Taxa and number of cells were based on 40 counting fields of an object glass under a microscope (magnification 400 x). In the case of colony forming and filamentous algae, the number of colonies/filaments was counted. Identification took place to the lowest practical taxonomic level. For confirmation of the identification of the preserved plankton, living algae in some of the samples were identified before conservation.

To determine the amount of chlorophyll-*a* of the phytoplankton, another two litres of water was collected on the same sampling dates as for the phytoplankton species composition (Table 2). The water was filtered through 1.2 µm pore size glass fibre filters (Schleiger & Schuell GF_{52}, Dassel, Germany). The filters were kept in the dark, stored in aluminium foil, and frozen (-18 °C) until extraction took place [20]. Extraction of chlorophyll-*a* was performed using the method described by Moed and Hallegraeff [21].

Periphyton was sampled from glass slides on a biweekly basis (Table 2). The slides were positioned in a frame at a fixed depth of approximately 10 cm below the water surface, and incubated for four to five weeks. On each sampling day, five slides were collected for chlorophyll-*a* analysis. The slides were brushed visually clean and the periphyton removed was collected in tap water. The chlorophyll-*a* content of the water-periphyton solution was processed and analysed using the same method as for phytoplankton.

To study the species composition of the zooplankton, the total number of cladocerans, ostracods, and copepods was counted using a binocular microscope at a magnification of x25. Using an inverted microscope, the numbers of rotifers and nauplii were determined by counting a known volume. Rotifers and cladocerans were identified to the lowest practical taxonomic level. Copepods were identified to suborder, and a distinction made between nauplii and more mature stages. Ostracods were not identified any further.

Macroinvertebrates

Macroinvertebrates were regularly (Table 2) sampled from each microcosm by means of litterbags (see *Decompostion* section) and artificial substrates. In each microcosm, two multiplates and two pebble baskets served as artificial sample substrates (Fig. 1). The colonisation period was two weeks, with the exception of the last sampling date, when it was three weeks.

On each sampling day, the artificial substrates were gently retrieved from each system, using a net in order to prevent the escape of swimming invertebrates. Pebble baskets were first washed in a container to remove any invertebrates from the

substrate. Subsequently, the macroinvertebrates present on multiplates and in the pebble baskets were collected manually. Living organisms were identified and counted. These organisms were then released into their original microcosms. Identification of the macroinvertebrates was usually to lowest practical taxonomic level. Before analysing the data, collected numbers from the artificial substrates and litterbags of each microcosm were pooled per sampling date.

Macrophytes

Two months before the first application, 25 shoots of *Elodea nuttallii* were introduced to each microcosm. The microcosms developed into clear water systems with vegetation solely consisting of this plant species. At the end of the experiment the total above-ground biomass of the macrophytes was removed from all microcosms. The plant material was carefully washed to remove periphyton, macroinvertebrates, and sediment particles. The plant material was dried in an oven (105 °C; 24 h) to determine the dry weight of *Elodea.*

Decomposition

Decomposition of particulate organic matter was studied by means of a litterbag technique (Table 2). The particulate organic matter consisted of *Populus x canadensis* leaves. The poplar leaves were leached three times for two days to remove the more easily soluble humic compounds. After the leaves had been dried in an oven for 72 h at 60 °C, they were stored before usage. Two grams dry weight of *Populus* leaves without petioles were enclosed in each litterbag. The litterbags consisted of a glass petri dish (diameter 11.6 cm) closed with a cover of stainless steel wire-mesh (mesh size 0.7 x 0.7 mm), in which two holes (diameter 0.5 cm) had been punched to allow the passage of most invertebrates. Two replicates were introduced into each microcosm on the sediment surface in an almost upright position (Fig. 1). The incubation period usually lasted two weeks, but was three weeks in the last period. At the end of each two or three week decay period, the litterbags were washed gently in the overlying water of each microcosm to remove adhering sediment particles. The contents of the two litterbags from each microcosm were transferred to a white tray to separate macroinvertebrates from the remaining poplar leaves. This plant material was subsequently transferred to aluminium foil to determine its dry weight (24 h at 105 °C). The macroinvertebrates were released into their original microcosms after identification and counting.

Toxic units

Based on standard acute toxicity data (Table 4) and target exposure concentrations (Table 1), we could estimate the most critical compound or pesticide package by

expressing concentrations as toxic units (TU) and applying the concept of concentration addition [22]. To estimate the potential toxicity for sensitive invertebrates, TU was scaled to the toxicity of the four pesticides for *Daphnia magna*:

$$y\,\mathrm{TU} = \Sigma(C_i/\mathrm{EC50}_i) \quad (1)$$

where C_i denotes the actual concentration of compound i and $\mathrm{EC50}_i$ denotes the geometric mean 48h-median effective concentration (EC50) of compound i for *D. magna* (Table 4), and y denotes the resulting fraction of TU. The endpoint of the EC50 could be immobility or mortality.

Potential toxicity for phytoplankton and periphyton was scaled to the toxicity of the four compounds for algae also using Equation 1. Here, however, $\mathrm{EC50}_i$ denotes the geometric mean EC50 for one of the standard test algae (Table 4).

The potential toxicity of the pesticide package to the macrophyte *Elodea nuttallii* was calculated by using the acute EC50s of the herbicides asulam and metamitron for the macrophyte *Lemna gibba*; no *Lemna* toxicity data were available for either of the other compounds (Table 4).

The TU for fish were not calculated as they were not part of the study. However, the fish data are included in Table 4 to complete the picture of potentially sensitive taxonomical groups, and so contributes to the ecotoxicological profile of the four studied compounds.

A priori, direct effects on arthropods were expected, since at the highest treatment level the package was calculated to be 0.76 TU for *Daphnia*, with lambda-cyhalothrin as the main contributor (Table 5). We expected slight effects at the most on algae since at the highest treatment level, the peak concentration of the package was not more than 0.06 TU for algae (Table 5). Initially, potential toxicity of the package was estimated to be approximately 0.1 TU for macrophytes at the highest treatment level (Table 5). However, since asulam increased in concentration during the experiment (Fig. 2), a maximum of 0.25 $\mathrm{TU}_{\mathrm{lemna}}$ was reached after the third treatment. Therefore, in the long run, some effects on *Elodea* could not be excluded.

Table 4. Geometric means of toxicity data of species representative of primary producers and invertebrates for the pesticides used in the experiment. Geometric mean median effective concentrations (EC50s) (μg/L) are based on toxicity data for standard laboratory test species commonly used in the first-tier risk assessment procedure for the administration of pesticides. Data were from the ECOTOX data base (www.epa.gov./ecotox/), from the RIVM data base (Rijksinstituut voor Volksgezondheid en Milieu, Bilthoven, The Netherlands) (see De Zwart [42]) and from data kindly provided by industry (Syngenta, Jealotts Hill, UK; Bayer AG, Monheim, Germany). *Daphnia magna*: water flea; *Oncorhynchus mykiss*: rainbow trout; *Selenastrum capricornutum*: green alga; *Chlorella pyrenoidosa*: green alga; *Lemna gibba*: duckweed

Pesticide	Taxon	Geom-EC50	Exposure time
Fluazinam	*Daphnia magna*	132	48 h
	Oncorhynchus mykiss	110	96 h
	Selenastrum capricornutum	160	96 h
	Lemna sp.	--	--
λ-Cyhalothrin	*Daphnia magna*	0.35	48 h
	Oncorhynchus mykiss	0.32	96 h
	Selenastrum capricornutum	>1000	96 h
	Lemna sp.	--	--
Asulam	*Daphnia magna*	32000	48 h
	Oncorhynchus mykiss	> 5000000	96 h
	Chlorella pyrenoidosa	6000	48 – 72 h
	Lemna gibba[a]	140	14 d
Metamitron	*Daphnia magna*	129164	48 h
	Oncorhynchus mykiss	326000	96 h
	Selenastrum capricornutum	852	72 h
	Lemna gibba	1500	14 d

[a] Personal communication, F.M.W. de Jong, RIVM, The Netherlands.

Table 5. Application rates expressed as toxic units (TU) for the highest treatment level (5% emission). Toxic units based on median effective concentrations (EC50s) for *Daphnia magna* as representative of zooplankton and macroinvertebrates, EC50s for algae as representative of phytoplankton, and EC50s for *Lemna gibba* as representative of macrophytes. Used EC50 values are given in Table 4. --: no data

TU	**Fluazinam**	**Asulam**	**Metamitron**	**λ-Cyhalothrin**	**Mixture**
Algae	0.04	0.002	0.014	< 0.00025	0.06
D. magna	0.05	0.0004	0.0001	0.71	0.76
L. gibba	--	0.096	0.008	--	≥ 0.104

Data analysis

Prior to univariate and multivariate analyses of the dynamics of zooplankton, phyto-plankton, periphyton, and macroinvertebrates abundance values were respectively

$\ln(10x + 1)$, $\ln(0.001x + 1)$, $\ln(1x + 1)$ and $\ln(2x + 1)$ transformed, where x stands for the abundance value. This was done to approximate a normal distribution and to down-weigh high abundance values (for rationale, see Van den Brink, et al. [23]).

At the taxon level, NOEC calculations were made using the Williams test (analysis of variance, $p \leq 0.05$) [24]). Analyses were made with the Community Analysis computer program (Hommen, Technical University, Aachen, Germany) [25].

The community level effects of the pesticide treatments for zooplankton, phytoplankton and macroinvertebrates were analysed by the principal response curve (PRC) method, which is based on the redundancy analysis ordination technique, the constrained form of principal component analysis [26]. The PRC method is a multivariate technique specially developed for the analysis of data generated in community response studies based on an experimental design. The PRC results in a diagram showing the sampling weeks on the horizontal axis and the first Principal Component of the treatment effects on the vertical axis (see Fig. 10 as an example). This yields a diagram showing the deviations in time of the treatments compared to the control. The species weights (b_k), shown on the right side of the diagram, can be interpreted as a correlation of each species with the response given in the diagram. Thus, *Gammarus pulex*, which has the highest weight, is indicated to have decreased most at the higher treatment levels. The negative weight of *Stylaria lacustris* indicates that its numbers have increased at higher treatment levels.

The results of the PRC analysis can also be evaluated in terms of the fractions of variance explained by the factors time and treatment, and further indicates which fraction of the variance explained by treatment is shown in the PRC diagram.

For a complete description and discussion of the PRC method, the reader is referred to Van den Brink and Ter Braak [26-28]. The PRC analysis was performed using the CANOCO for Windows® software package, Version 4 [29].

In the CANOCO computer program, redundancy analysis is accompanied by Monte Carlo permutation tests to assess the statistical significance of effects of the explanatory variables on species composition of the samples [30]. The significance of the PRC diagram, in terms of displayed treatment variance, was tested by Monte Carlo permutation of entire time series in the redundancy analysis from which the PRC is obtained, using an *F*-type test statistic based on the eigen value of the component [26].

Although the first principal component extracts the maximum amount of information from the multivariate treatment effects, it does not necessarily describe the effects of all treatments on all taxa in sufficient detail. Further components can be extracted from the residual variation. The PRC diagrams based on the second, third and higher components display treatment effects that are not captured in earlier components. The significance of the second PRC diagram was tested using Monte Carlo permutation tests and showed that the second and higher components were not significant and were therefore not considered further.

Monte Carlo permutation tests were also performed per sampling date, using the ln-transformed treatment levels as the explanatory variable [30]. This allowed the significance of the treatment regime to be tested for each sampling date. Besides this overall significance of the treatment regime, we also determined which treatments differed significantly from the controls, so as to infer the NOEC at the community level ($NOEC_{community}$). The $NOEC_{community}$ calculations were done by applying the Williams test to the sample scores of the first principal component of the principal component analysis of each sampling date in turn (for the rationale of this, see Van den Brink, et al. [30]).

We used the combination of multivariate (PRC) and univariate (Williams test) techniques for the following reasons. By means of the PRC method one can identify the treatment levels which affected the community and, simultaneously, indicate the taxa of which the responses affiliated most to the treatment regime. Analysis of the dynamics on the taxon level by means of the Williams test helps to further identify the number of taxa affected. The graphical interpretation of the univariate analysis is needed to decide whether the statistically significant deviations can be considered consistent effects and whether these deviations make any sense in relation to (variation in) abundance numbers.

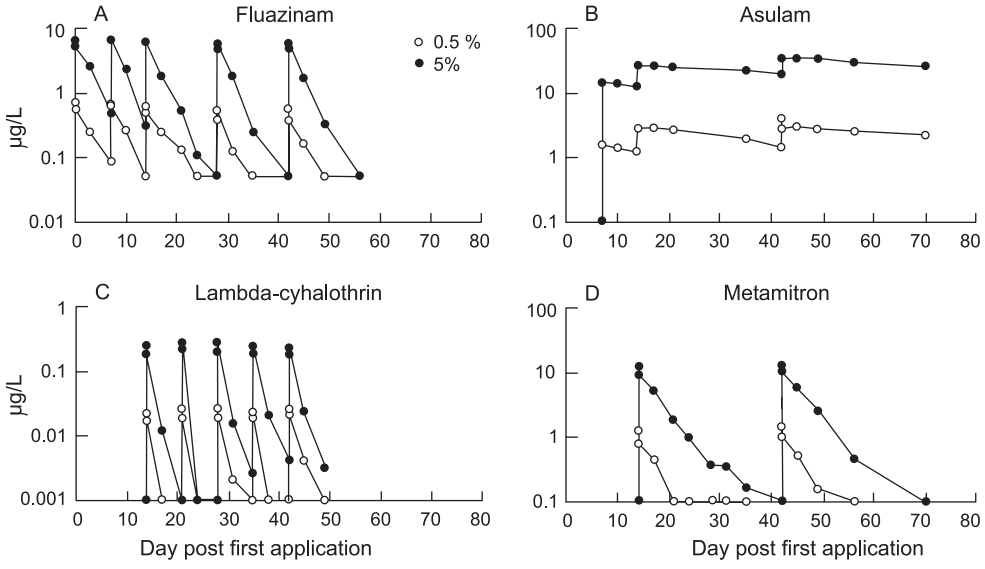

Fig. 2. Trend of concentrations of pesticides used in the pesticide package standing model for a crop protection programme in tulip crop culturing. Concentrations of fluazinam (A), asulam (B), lambda-cyhalothrin (C) and metamitron (D) are those of the 0.5% and 5% treatment levels.

Results

Exposure concentrations

Nominal concentrations based on the doses applied, were generally close to the target concentrations (Table 1). For individual microcosms, concentrations between different applications generally varied within 10%. In the case of fluazinam and asulam, on single occasions dosages were higher than intended resulting in relatively high mean nominal concentrations and wide ranges for the 0.2% and 0.5% treatment levels, respectively (Table 1). The multiple applications were clearly reflected in the saw-tooth pattern of the dynamics in exposure concentrations (Fig. 2). For fluazinam, lambda-cyhalothrin, and metamitron, concentrations were below, or around their detection limit at the next application. Asulam, however, accumulated in time and reached a value of more than twice its initial concentration (Fig. 2).

For the purpose of comparing the behavior of the four pesticides, $t_{1/2}$ in water was calculated for the different application periods assuming first-order kinetics (Table 6). The half-lives of fluazinam, metamitron, and lambda-cyhalothrin were shown to be relatively short. On average, $t_{1/2}$ was 2.0, 2.7 and 1.0 days for fluazinam, metamitron, and lambda-cyhalothrin, respectively (Table 6). No $t_{1/2}$ could be calculated for the 0.5% treatment level of lambda-cyhalothrin, because concentrations were already below the detection limit after the one hour sampling. The average $t_{1/2}$ for asulam was found to be 34 and 62 days for the lower and highest application levels, respectively (Table 6).

When expressed as TUs, peak concentrations reached about 0.09 TU for algae at the highest treatment level (Fig. 3). The fluazinam treatments provided most of the toxic stress for the phytoplankton. Contributions of the other three compounds were of minor (metamitron, short exposure to ≈ 0.06 TU) to negligible significance (Fig. 3). For zooplankton and macroinvertebrates, concentrations peaked at 0.7 to 0.8 TU at the 5% treatment level (Fig. 4). Lambda-cyhalothrin contributed most of the toxic stress for these invertebrates. Contributions of the other compounds were of minor (fluazinam) or negligible (asulam, metamitron) significance (Fig. 4). Macrophytes were exposed to maximum concentrations of about 0.25 TU (Fig. 5). Contribution of the other compounds were negligible (metamitron) or unclear (fluazinam, lambda-cyhalothrin; no toxicity data available [Table 4]) (Fig.4).

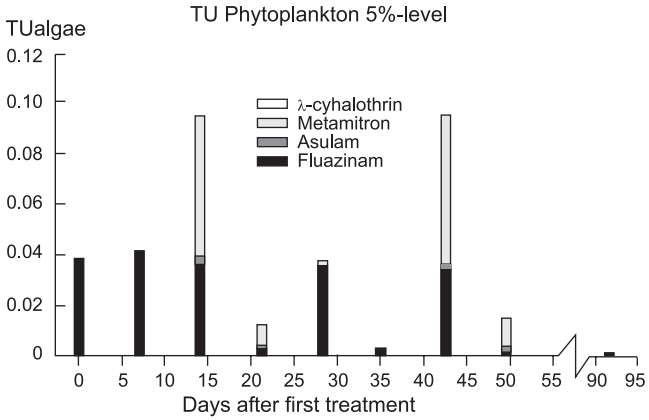

Fig. 3. Toxicity of the pesticide package for the phytoplankton at the highest treatment level expressed as toxic units (TU_{algae}) and the contribution of the individual pesticides to the toxicity. The highest treatment level represents concentrations equal to 5% spray drift emission of label-recommended rates of each pesticide used.

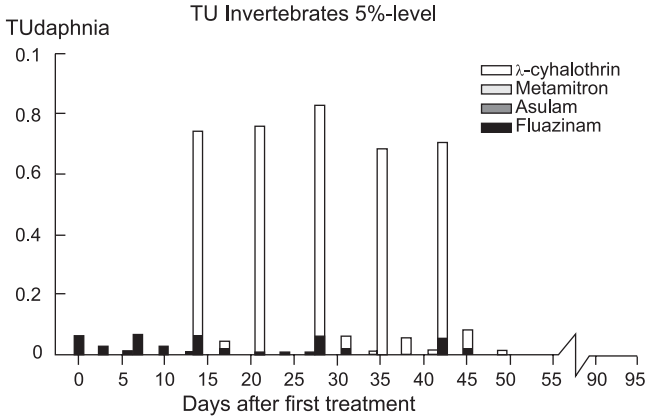

Fig. 4. Toxicity of the pesticide package for the zooplankton and macroinvertebrates at the highest treatment level expressed as toxic units ($TU_{daphnia}$) and the contribution of the individual pesticides to the toxicity. The highest treatment level represents concentrations equal to 5% spray drift emission of label-recommended rates of each pesticide used.

Table 6. Calculated half-lives ($t_{1/2}$, in days) for the compounds in the pesticide package for the water phase of the microcosms. Results were based on 0.5%- and 5%-treatment levels

Application	Fluazinam		Metamitron		λ-Cyhalothrin		Asulam	
	0.5%	5%	0.5%	5%	0.5%	5%	0.5%	5%
1	2.3	1.9	2.3	2.9	-	0.7	23	38
2	1.8	1.5	2.3	3.1	-	-	31	60
3	3.3	1.9			-	1.0	49	89
4	2.2	1.6			-	1.2		
5	2.1	1.7			-	1.1		
Mean	2.3	1.7	2.3	3.0	-	1.0	34	62
Range	1.8 - 3.3	1.5 - 1.9	-	2.9 – 3.1	-	0.7 - 1.2	23 - 49	38 - 89

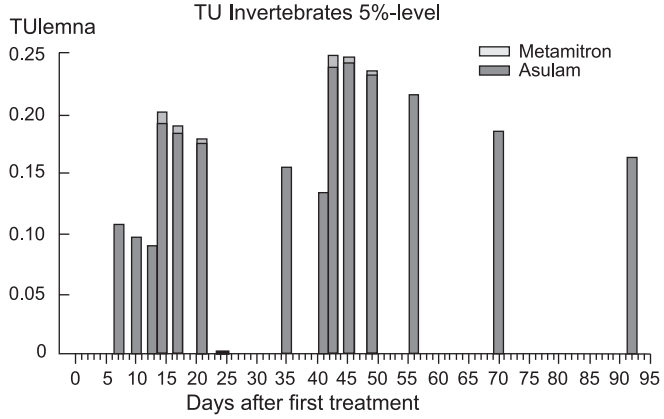

Fig. 5. Toxicity of the pesticide package for macrophytes at the highest treatment level expressed as TU_{lemna}. TU_{lemna} was based on EC50s for the herbicides asulam and metamitron only.

Zooplankton

A total number of 34 zooplankton taxa were identified. Rotifers formed the majority of taxa (20) and dominated abundance numbers. In abundance, rotifers were followed by Cladocera and Copepoda. Ostracoda were present in relatively low numbers.

Of the total variance, 27% was allocated to the treatment regime by the PRC analysis (Fig. 6). This amount is on the low side, compared to other microcosm studies using the same experimental set-up [7,10,23]. The PRC diagram for the zooplankton only shows clear treatment effects at the 5% treatment level compared to the controls (Fig. 6). At this level, reductions were significant from week 3 to 13 inclusive (Table 7). Statistical testing indicated a lowest $NOEC_{zooplankton}$ at the 0.2% treatment level after the first treatment of lambda-cyhalothrin (week 3) (Table 7),

while for most sampling dates thereafter a $NOEC_{zooplankton}$ at the 2% treatment level was calculated.

Particularly Copepoda (nauplii, Cyclopoidea) show high positive weights in the PRC diagram (Fig. 6; see species weights [b_k]). Representatives of Rotifera show both positive (e.g., *Lepadella patella* and *Trichocerca longiseta*) and negative (e.g., *Keratella quadrata*) weights. A similar phenomenon is observed for Cladocera, of which *Simocephalus vetulus* and *Chydorus sphaericus* have positive weights, and *Daphnia* gr. *galeata* a negative one.

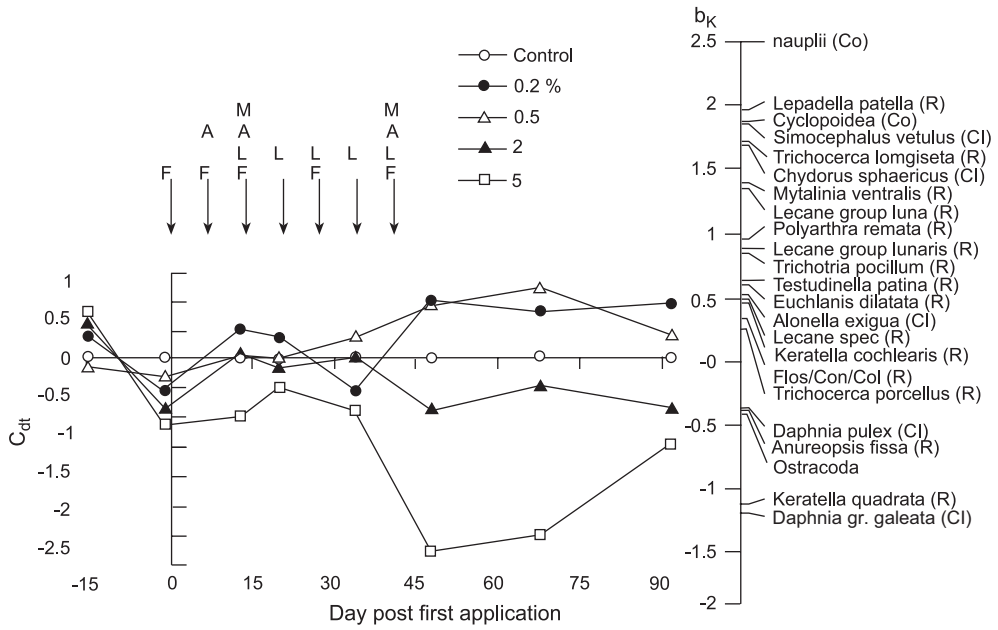

Fig. 6. Principal Response Curves (PRC) with species weights (b_k) for the zooplankton data set, indicating the effects of applications of a pesticide package containing fluazinam (F), asulam (A), metamitron (M) and lambda-cyhalothrin (L). Of all variance, 44% could be attributed to sampling date and is displayed on the horizontal axis. Differences between replicates accounted for 29% of all variance. Twenty-seven percent of all variance could be attributed to the treatment regime. Of this variance 22% is displayed on the vertical axis. The vertical axis represents the differences in community structure between treatments and the controls expressed as regression coefficients (c_{dt}) of the PRC model. The species weight (b_k) can be interpreted as the affinity of the taxon to the PRC (see Van den Brink and Ter Braak [26] for more information). Codes behind species names indicate whether species belong to the Rotifera (R), Copepoda (Co) or Cladocera (Cl).

Table 7. Results of the Monte Carlo permutation test (*p*-value) and no-observed-effect concentrations (NOEC) on the community level (Williams test, $p \leq 0.05$) for the different treatment levels of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. ND: no data.

Week	Zooplankton		Phytoplankton	Macroinvertebrates	
	***p*-value**	**$NOEC_{zooplankton}$**	***p*-value**	***p*-value**	**$NOEC_{macroinvert}$**
-2	> 0.05	> 5%	> 0.05	ND	ND
-1	> 0.05	> 5%	> 0.05	> 0.05	> 5%
2	> 0.05	> 5%	> 0.05	ND	ND
3	< 0.005	0.2%	> 0.05	< 0.005	0.2%
4	< 0.005	2%	> 0.05	ND	ND
6	< 0.005	2%	> 0.05	< 0.005	0.5%
9	0.020	2%	> 0.05	ND	ND
13	0.015	0.5%	> 0.05	< 0.005	0.5%

Treatment-related responses for individual taxa were statistically analyzed and resulted in NOECs (Williams test, $p \leq 0.05$) for seventeen zooplankton taxa (Table 8). For two of the 34 identified taxa, isolated NOECs of less than the 0.2% treatment level were calculated. These taxa (*Lecane* gr. *lunaris*, *Keratella cochlearis*) however, occurred in irregular and/or low numbers (Table 8). Negative effects were most explicit on day 20, when six of a total of 20 statistically significant reductions occurred (Table 8). This time point was the first sampling date after the first application of the insecticide lambda-cyhalothrin (Fig. 6). At the 0.5% and 2% treatment levels statistical deviations were mostly isolated cases (Table 8). Significant, longer lasting reductions in numbers were observed at the 5% treatment level only, and occurred in the copepods (Cyclopoidea [Fig. 7E], nauplii [Fig. 7F]) and the cladoceran *C. sphaericus* (Fig. 7A). Rotifers generally tended to increase with time (Table 8, e.g., *K. quadrata* (Fig. 7G)).

Phytoplankton and periphyton

A total of 37 phytoplankton taxa were identified. Bacillariophyceae, Chlorophyta, and Cyanophyta were dominant groups, while *Anabaena* sp., *Botryococcus braunii*, *Cocconeis placentula*, *Oedogonium* sp., and *Rhopalodia gibba* were the dominant species.

The PRC analysis reveals a trend of an increase in the abundance of phytoplankton species, since only positive values are indicated in the PRC diagram. A clear treatment-response relationship could not be demonstrated, as the increase was greatest at the intermediate treatment levels of 0.5 and 2% (Fig. 8). The shown increase in numbers was not significant on any of the sampling dates (Table 8). At population level, only *B. braunii, Anabaena* sp., *Oedogonium* sp., and *R. gibba,* showed transient increases (Fig. 9). The statistical deviations were mostly isolated cases, and usually occurred on day 27 (three out of a total of five cases; Table 8).

Table 8. No-observed-effect concentrations (NOEC) for zooplankton and phytoplankton populations after applications of pesticide mixtures containing fluazinam, asulam, metamitron, and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% spray drift emission of label-recommended rates. The NOECs (Williams test, $p \leq 0.05$) were based on geometric mean abundance numbers per treatment level. Abundance levels of the populations during the experiment are indicated by the order of magnitude in which they occurred (e.g., 10^0-10^1: numbers ranged from one fold to tenfold). ↓: populations were significantly reduced at concentrations above NOEC. ↑: populations were significantly increased at concentrations above NOEC.

Taxon	**Abundance** (geometric mean numbers/L)	**Days post-treatment**						**Note**
		13	**20**	**34**	**48**	**68**	**93**	
ZOOPLANKTON								
Cladocera								
Alona rectangula[a]	10^0-10^1			0.2%↑				
Chydorus sphaericus[b]	10^0-10^1		2%↓	2%↓	2%↓	2%↓		Fig. 7A
Daphnia gr. *galeata*[c]	10^{-1}-10^1		0.5%↓			2%↑	0.5%↑	Fig. 7B
Daphnia pulex[c,d]	0 –10^{-1}						2%↑	
Graptoleberis testudinaria[a]	10^0-10^1						0.2%↓	Fig. 7C
Macrothrix laticornis[a,d]	0 –10^{-1}				2%↓			
Simocephalus vetulus[c]	10^0-10^1		0.5%↓			0.5%↓		Fig. 7D
Copepoda								
Cyclopoidea[b]	10^0-10^1		0.5%↓	2%↓	2%↓	2%↓		Fig. 7E
Nauplii[b]	10^1-10^2		2%↓	2%↓	2%↓			Fig. 7F
Rotifera								
Keratella cochlearis[a,d]	10^{-1}-10^0	< 0.2%↓						
Keratella quadrata[b]	10^0-10^2				2%↑	2%↑		Fig. 7G
Lecane gr. *lunaris*[a]	10^0-10^2				< 0.2%↑			
Lecane gr. *luna*[a]	10^{-1}-10^1			2%↑				
Lecane quadridentata[b]	10^{-1}-10^1		2%↑	2%↑	0.5%↑			

Continue Table 8

Taxon	Abundance (geometric mean numbers/L)	Days post-treatment 13	20	34	48	68	93	Note
Lepadella patella[a]	10^{-1}-10^{1}			0.2%↑				
Polyarthra remata[f]	10^{1}-10^{3}			0.5%↑		2%↓		
Trichocerca longiseta[c,g]	10^{-1}-10^{1}		2%↓		0.5%↓			Fig. 7H
No. of reductions		1	6	3	5	4	1	
No. of increases		0	1	5	3	2	2	
PHYTOPLANKTON		**Days post-treatment 13**	**27**	**41**	**56**	**69**	**93**	
Anabaena sp.[a]	10^{0}-10^{1}		0.2%↑					Fig. 9A
Botryococcus braunii[a]	10^{0}-10^{1}						2%↑	
Oedogonium sp.[b]	10^{-1}-10^{0}		0.2%↑	0.2%↑				Fig. 9B
Rhopalodia gibba[a]	10^{0}-10^{1}		< 0.2%↑					Fig 9C
No. of reductions		0	0	0	0	0	0	
No. of increases		0	3	1	0	0	1	

[a] one isolated significant deviation. [b]consistent response. [c]significant reduction at day 20, significant increase at end of experiment. [d]very low abundance and high variation within cosms (in time) and between cosms (in space). [e]isolated significant deviations. [f]isolated inconsistent deviations. [g]abundance in 5%-treatment systematically lower than in other cosms.

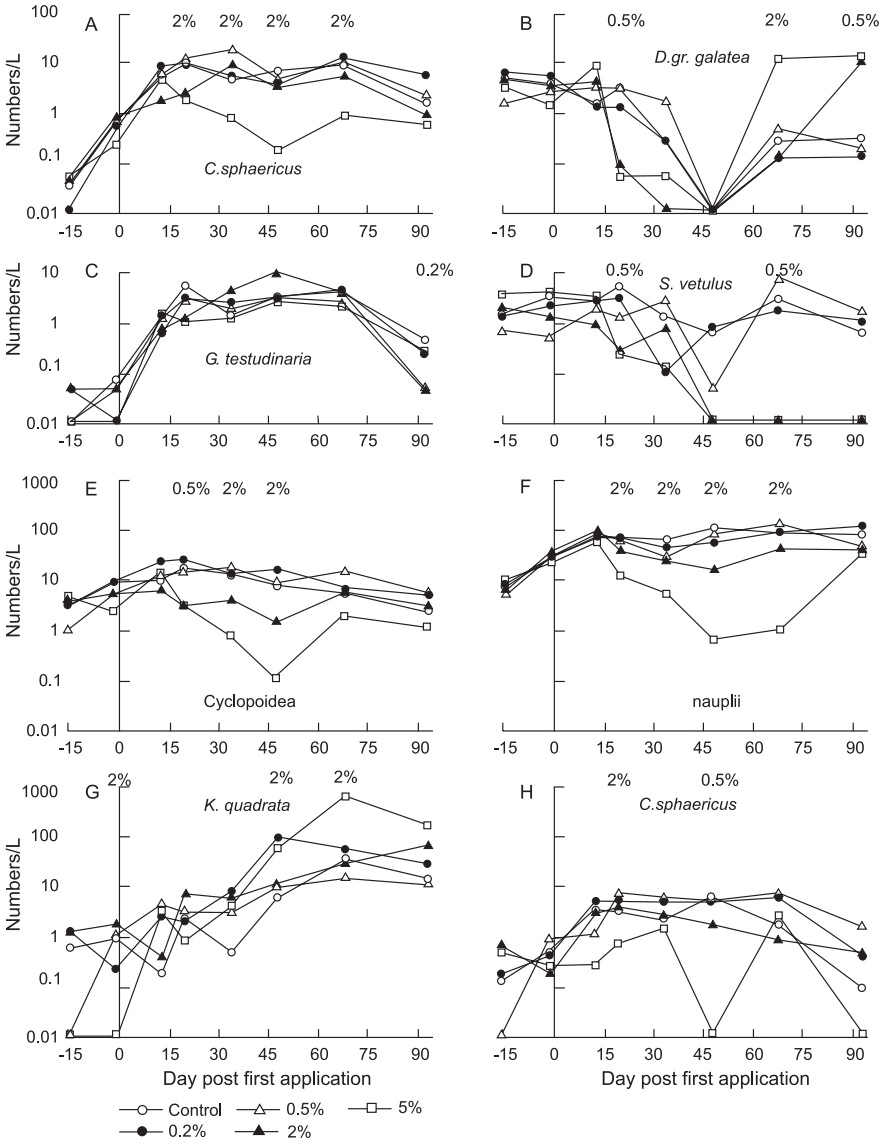

Fig. 7. Dynamics of zooplankton populations most important in the Principle Response Curve (PRC) analysis and/or showing treatment-related responses after applications of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% of spray drift emission of label-recommended rates. Numbers per litre are geometric mean abundance numbers of *Chydorus sphaericus* (A), *Daphnia* gr. *galeata* (B), *Graptoleberis testudinaria* (C), *Simocephalus vetulus* (D), Cyclopoidea (E), nauplii (F), *Keratella quadrata* (G), and *Trichocerca longiseta* (H). Percentage values at the top of the graphs indicate the time points at which significant deviations from the controls were measured. Percentage values are NOECs (Williams test, $p \leq 0.05$).

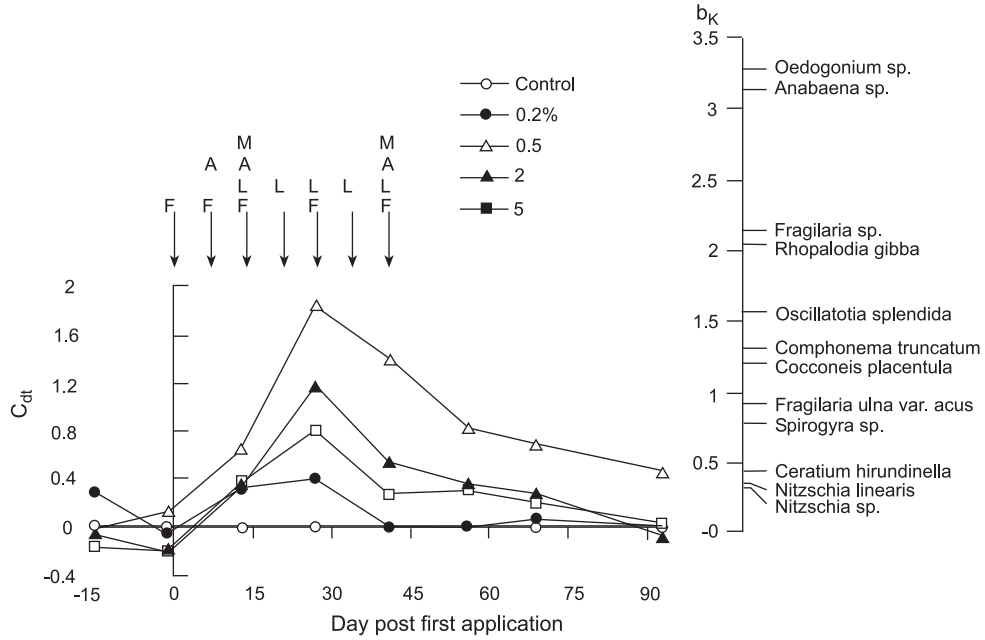

Fig. 8. Principal Response Curves (PRC) with species weights (b_k) for the phytoplankton data set, indicating the effects of applications of a pesticide package containing fluazinam (F), asulam (A), metamitron (M) and lambda-cyhalothrin (L). Of all variance, 22% could be attributed to sampling date and is displayed on the horizontal axis. Differences between replicates accounted for 41% of all variance. Thirty-seven percent of all variance could be attributed to the treatment regime. Of this variance 46% is displayed on the vertical axis. The vertical axis represents the differences in community structure between treatments and the controls expressed as regression coefficients (c_{dt}) of the PRC model. The species weight can be interpreted as the affinity of the taxon to the PRC (see Van den Brink and Ter Braak [26] for more information).

Concentrations of chlorophyll-*a* of the phytoplankton in the controls were 1.9 ± 0.75 μg/L (mean ± standard deviation) during the experiment. At the end (days 56 and 69), chlorophyll-*a* concentrations were significantly higher for the 2% and 5% treatment levels (mean concentrations 4.2 and 4.8 μg/L, respectively). Periphyton, measured as chlorophyll-*a*, did not show any significant response during the experiment. Mean levels were 0.11 ± 0.1 μg/cm².

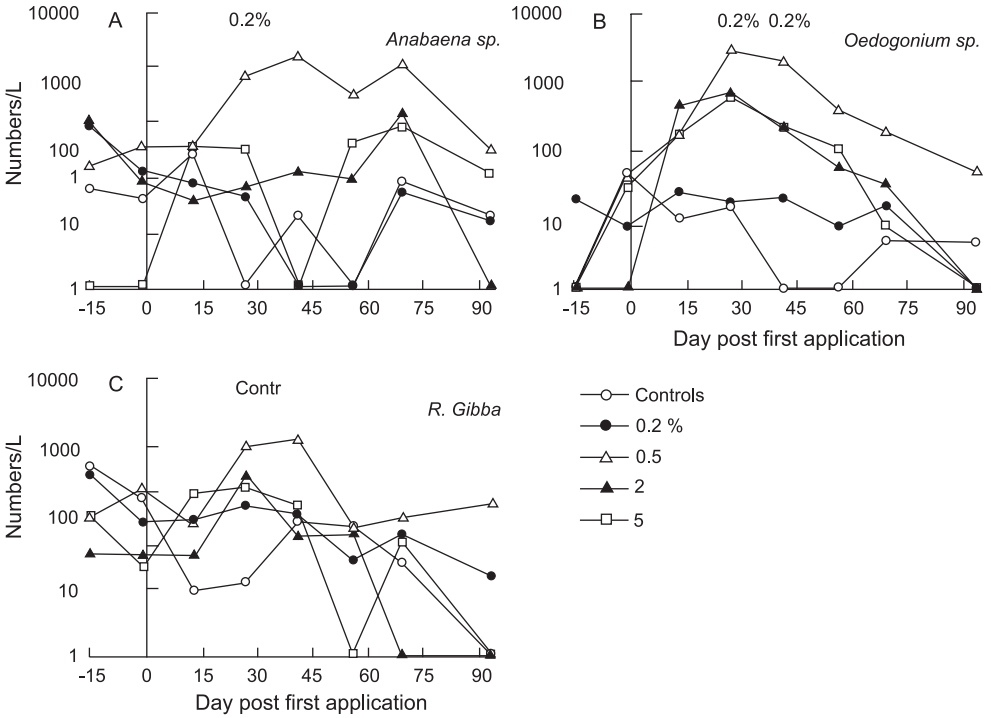

Fig. 9. Dynamics of the most important phytoplankton populations in the Principle Response Curve (PRC) analysis and/or showing treatment-related responses after applications of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% of spray drift emission of label-recommended rates. Numbers per litre are geometric mean abundance numbers of *Anabaena* sp. (A), *Oedogonium* sp. (B), and *Rhopalodia gibba* (C). Percentage values at the top of the graphs indicate the time points at which significant deviations from the controls were measured. Percentage values are NOECs (Williams test, $p \leq 0.05$).

Macroinvertebrates

A total of 77 macroinvertebrate taxa were identified over the experimental period. The microcosms were dominated by oligochaetes, turbellarians, snails, crustaceans (mainly *Gammarus pulex*, *Asellus aquaticus* and their juveniles), and mayflies (*Cloeon dipterum*). Besides mayflies, midges of the family Chironomidae were relatively numerous insects at the start of the experiment. The midge *Corynoneura scutellata* agg. occurred frequently and in high numbers at the end of the experiment. The number of taxa declined slightly during the experiment.

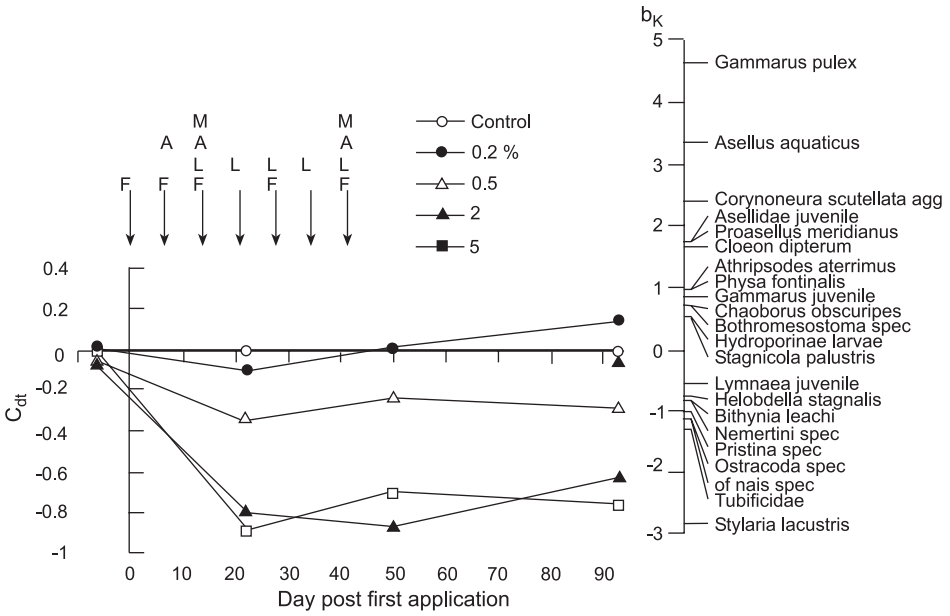

Fig. 10. Principal Response Curves (PRC) indicating effects of the pesticide applications on the macroinvertebrate community. Treatment levels were equal to 0.2, 0.5, 2 and 5% of spray drift emission of label-recommended rates of a pesticide package containing fluazinam (F), asulam (A), metamitron (M) and lambda-cyhalothrin (L). Of all variance 29% could be attributed to sampling date; this is displayed on the horizontal axis. Thirty-seven percent of all variance could be attributed to treatment. Of this variance 40% is displayed on the vertical axis of the PRC diagram. The vertical axis represents the differences in community structure between treatments and the controls expressed as regression coefficients (c_{dt}) of the PRC model. The species weight can be interpreted as the affinity of the taxon to the PRC (see Van den Brink and Ter Braak [26] for more information).The PRC diagram shows a significant difference ($p < 0.005$) of the treatment variance.

By the PRC analysis, 37% of the total variance was attributed to the treatment regime. Twenty-nine percent of the total variance in the macroinvertebrate dataset is explained by time. The PRC diagram (Fig. 10) shows that the macroinvertebrate communities of the two highest treatment levels clearly deviated from the controls and the lowest treatment level after the start of treatment, while the 0.5% treatment regime shows an intermediate position. The impact of the treatment became clearly visible after the first simultaneous application of all pesticides (day 14) and remains visible throughout the rest of the experimental period with no signs of recovery. After the second application of all pesticides on day 42, no further deterioration of the macroinvertebrate communities was observed (Fig. 10). At the 2% and 5% treatment levels, the changes in the macroinvertebrate community (mainly reductions, see Figs. 10 and 11) were

significant from week 3 to 13 inclusive (Table 7). Statistical testing indicated a lowest $NOEC_{macroinvertebrates}$ at the 0.2% treatment level in week 3 after the first treatment (Table 7). Species with high positive weights shown in the PRC diagram (Fig. 10) comprise the crustaceans *G. pulex* (Fig. 11A), *A. aquaticus* (Fig. 11B), *P. meridianus* (Fig. 11C) and juvenile Asellidae (Fig. 11D), and the juvenile stadia of the insects *C. scutellata* agg. (Fig. 11E), and *C. dipterum* (Fig. 11F). These taxa declined dramatically, or even became extinct, at the highest treatment levels. Species with (high) negative weights in the PRC diagram, and thus showing a tendency of increase in time, comprised, among others, the oligochaetes *Stylaria lacustris*, cf *Nais* sp. and Tubificidae and the leech *Helobdella stagnalis* (Fig. 11G).

Statistical analysis of treatment-related responses for individual macroinvertebrate populations resulted in NOECs for 18 taxa (Table 9). Negative effects were most explicit at the end of the application period (day 49) when 9 of a total of 18 statistically significant reductions occurred (Table 9). For 4 of the 77 taxa, isolated NOECs of less than the 0.2% treatment level were calculated (Table 9). These taxa (Asellidae juveniles, *Athripsodes aterrimus*, *Chaoborus obscuripes*, *Bithynia tentaculata*), however, occurred in irregular and/or very low numbers and are therefore not considered any further. Consistent responses, i.e., statistically significant deviations pointing in the same direction on at least two consecutive sampling dates, were observed for 7 taxa (Table 9). Longer lasting reductions were observed at the 2% and 5% treatment levels and occurred within crustaceans (*G. pulex* [Fig. 11A], *A. aquaticus* [Fig. 11B], *P. meridianus* [Fig. 11C]) and within insects (*C. scutellata* agg. [Fig. 11E], *C. dipterum* [Fig. 11F]). Oligochaetes, triclads, hirudinids and gastropods generally showed inconsistent responses. In time, they either showed isolated reductions or increases in abundance numbers (Table 9).

The lowest consistent NOECs (< 0.2% - 0.2% treatment level) were calculated for crustaceans (*G. pulex*, *A. aquaticus* and *P. meridianus*) and the insect *C. scutellata* agg. (Table 9). The NOECs based on an increase, were found for *H. stagnalis* and Chironomidae sp. (Fig. 11G, H).

Decomposition

The residual dry weights of *Populus* leaves amounted to approximately 60% of the initial dry weight during the decay periods of two weeks (days -6, 22, and 50), and to approximately 50% during the decay period of three weeks (day 92). A treatment-related decrease in decomposition is visible after commencing application of pesticides (Fig. 12), but only on day 50 could a NOEC of 0.2% treatment level be calculated (Fig. 12).

Table 9. No-observed-effect concentrations (NOECs) for macroinvertebrate populations after applications of pesticide mixtures containing fluazinam, asulam, metamitron, and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% spray drift emission of label-recommended rates. The NOECs (Williams test, $p \leq 0.05$) were based on geometric mean abundance numbers per treatment level. Abundance levels of the populations during the experiment are indicated by the order of magnitude in which they occurred (e.g., 10^0-10^1: numbers ranged from one fold to tenfold). ↓: end points were significantly reduced at concentrations above NOEC. ↑: end points were significantly increased at concentrations above NOEC.

Taxon	**Abundance**	**Days post-treatment**			**Note**
		21	**49**	**92**	
Crustacea					
Gammarus pulex[a]	10^0-10^1	0.5%↓	0.5%↓	0.2%↓	Fig. 11A
Asellus aquaticus[a]	10^0-10^1	0.2%↓	0.2%↓	0.2%↓	Fig. 11B
Proasellus meridianus[a,b]	10^{-1}-10^1	0.2%↓	< 0.2%↓		Fig. 11C
Asellidae juveniles[c]	10^{-1}-10^0		< 0.2%↓		Fig. 11D
Insecta					
Corynoneura scutellata agg.[a]	10^{-1}-10^1	< 0.2%↓	0.5%↓	0.5%↓	Fig. 11E
Cloeon dipterum[a]	10^{-1}-10^1	2%↓	0.5%↓		Fig. 11F
Athripsodes aterrimus[c]	10^{-1}-10^0		< 0.2%↓		
Chaoborus obscuripes[d]	10^{-1}-10^0	< 0.2%↓			
Chironomidae spec.[a,b]	10^{-1}-10^0	2%↑	2%↑		Fig. 11H
Oligochaeta					
Stylaria lacustris[d]	10^0-10^1	0.5%↑			
Dero sp.[b]	10^{-1}-10^1			0.5%↓	
cf *Nais* sp.[c]	10^0-10^1		0.5%↑		
Tubificidae sp.[b,c]	0 –10^0			2%↑	
Tricladida					
Polycelis tenuis[c]	10^{-1}-10^1		2%↓		
Hirudinea					
Erpobdella octoculata[b,c]	10^{-1} –10^0			0.5%↓	
Helobdella stagnalis[a,b]	0 - 10^{-1}		2%↑	2%↑	Fig. 11G
Gastropoda					
Gyraulus albus[d]	10^{-1} –10^0		2%↑		
Bithynia tentaculata[d]	0 - 10^{-1}		< 0.2%↓		
No. of reductions		6	9	5	
No. of increases		2	4	2	

[a] consistent response.
[b] low abundance numbers.
[c] one isolated significant deviation, but low abundancy.
[d] one isolated significant deviation. Infrequently occurring numbers within cosms (in time) and between cosms (in space).
[e] one isolated significant deviation.

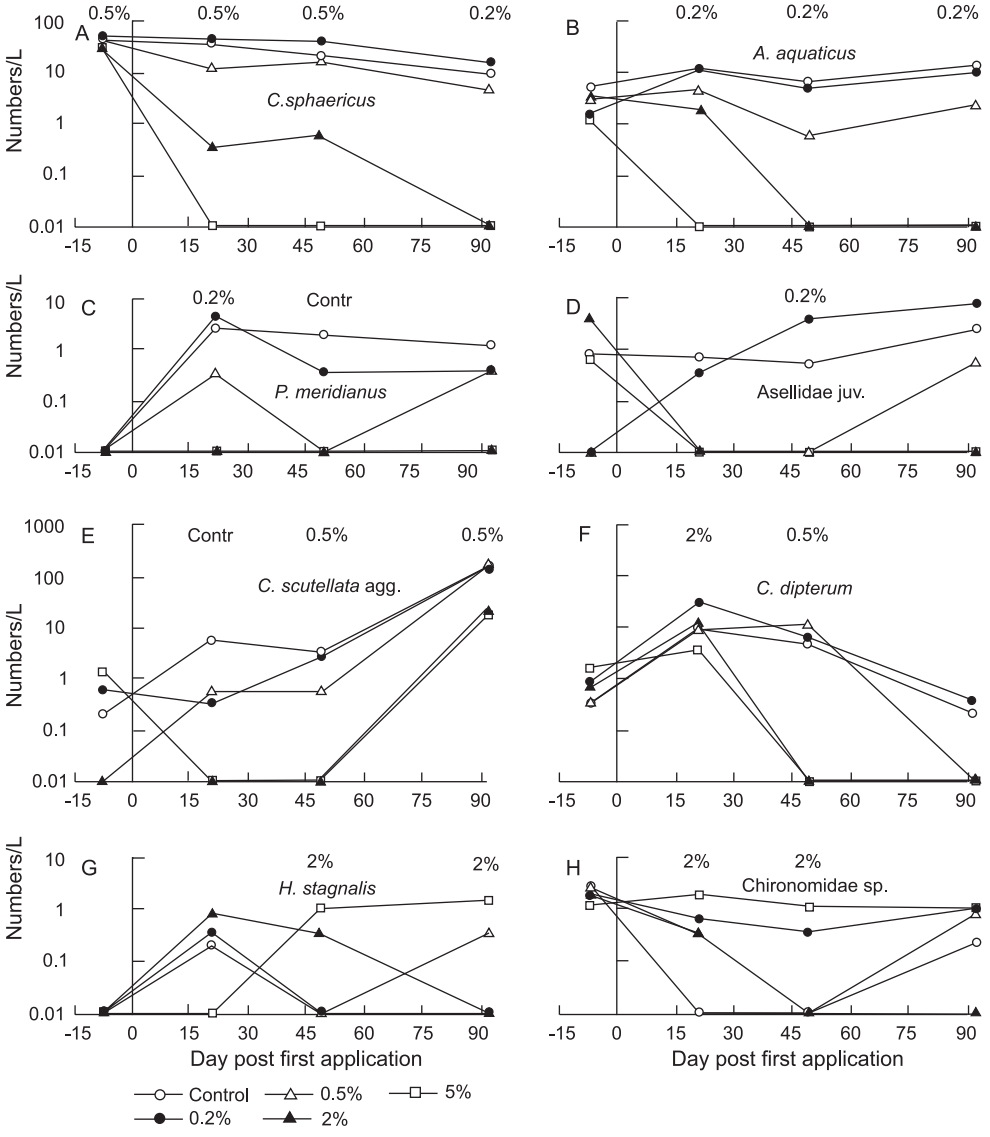

Fig. 11. Dynamics (numbers) of macroinvertebrate taxa, for which a NOEC was calculated and/or were most important in the Principle Response Curve (PRC) analysis (Fig. 10). Geometric mean numbers of *Gammarus pulex* (A), *Asellus aquaticus* (B), *Proasellus meridianus* (C), Asellidae juvenile (D), *Corynoneura scutellata* agg. (E), *Cloeon dipterum* (F), *Helobdella stagnalis* (G) and Chironomidae sp. (H) are shown. Treatment levels were equal to 0.2, 0.5, 2 and 5% of spray drift emission of label-recommended rates of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. In the Figures, 0.01 denotes absence. Percentage values at the top of the graphs indicate time points at which significant deviations from the controls were measured. Percentage values are NOECs (Williams test $p \leq 0.05$).

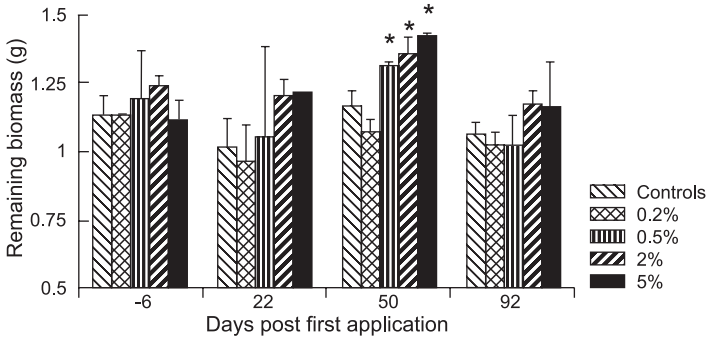

Fig. 12. Results of decomposition experiments with *Populus* leaves (start: 2 g dry weight). The x-axis denotes the day on which the litter bags were retrieved from the microcosms. The first three experiments lasted two weeks, the last one three weeks. Standard deviations were calculated from three control replicates and two replicates for the treatments. Treatment levels were equal to 0.2, 0.5, 2 and 5% of spray drift emission of label-recommended rates of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. Results of the Day 50-experiment deviated significantly (*) from the controls (NOEC: 0.2%-treatment level, Williams test ($p \leq 0.05$).

Macrophytes

A treatment-related decline in the final biomass of *Elodea nuttallii* is clearly visible (Fig. 13). At the end of the experiment, the mean biomass of *Elodea* in the two highest treatment levels were respectively 72% and 60% of that of the controls. A NOEC at the 0.5% treatment level was calculated using the Williams test ($p < 0.05$).

Physico-chemical variables

Over the time-span of the experiment, DO in the microcosms ranged between 8.7 and 10.0 mg/L. Ranges in pH (between 8.5 and 9.3) and alkalinity (~ 0.4 meq/L) were relatively small over this time period. Electrical conductivity increased slightly in all microcosms from 310 μS/cm at the start of the experiment to approximately 330 μS/cm at the end of the experiment. For pH, a NOEC of the 0.5% to 2% treatment level was calculated on three consecutive sampling dates (Table 10). A similar NOEC level was calculated for alkalinity on two consecutive dates (Table 10). On the last sampling date, for all variables in the DO-pH-alkalinity-conductivity syndrome, a NOEC of the 0.5% to 2% treatment level was calculated (for DO and pH based on a decrease; for electrical conductivity and alkalinity based on an increase [Table 10]), indicating a decrease in primary production at the highest treatment level.

Concentrations of nutrients in the microcosms were low during the entire experiment (ammonium: < 0.02 mg N/L; nitrate: < 0.04 mg N/L; orthophosphate: <

0.10 mg P/L). No statistical differences for nutrients were found between controls and treated microcosms.

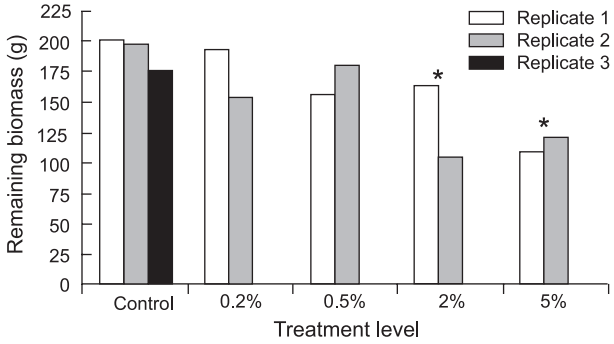

Fig. 13. Macrophyte biomass (*Elodea nuttallii*) retrieved from the microcosms on the final sampling date for each replicate of the treatment levels. Treatment levels were equal to 0.2, 0.5, 2 and 5% of spray drift emission of label-recommended rates of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin. Significant deviations (Williams test, $p \leq 0.05$) compared to the controls are indicated by an asterix.

Table 10. No-observed-effect concentrations (NOEC) for physico-chemical endpoints (DO: dissolved oxygen [mg/L]; pH; EC: electrical conductivity [µS/cm] and alkalinity [meq/L]) after applications of pesticide mixtures containing fluazinam, asulam, metamitron, and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% spray drift emission of label-recommended rates. The NOECs (Williams test, $p \leq 0.05$) were based on mean values per treatment level. ↓: end points were significantly reduced at concentrations above NOEC. ↑: end points were significantly increased at concentrations above NOEC

Physico-chemical endpoints	**Weeks post treatment**										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**13**
DO											2%↓
pH									2%↓	2%↓	0.5%↓
EC											2%↑
Alkalinity			2%↓	0.5%↓			2%↓				2%↑

Discussion

Scenario approach

In the real world, frequency, sequence and choice of pesticides to be applied, depend on many factors. For example, the selection of pesticides will be determined by anticipated or actually occurring pests. Further, decisions whether or not to spray, may depend on the economic damage a specific pest is expected to cause. In many cases, farmers have a choice of different products to reach their objectives, and dosages of pesticides depend on the active ingredient and the pests, diseases and weeds that are to be controlled. Additionally, timing and numbers of applications may depend on the weather and other environmental conditions that could be favourable for the targeted pests.

Because all these factors result in a high variability of actual spraying histories, we chose our scenario on a general application pattern. We consider our scenario to be realistic. It was based on expert judgements by colleagues at the research institute Applied Plant Research, Sector flower bulbs (Praktijkonderzoek Plant & Omgeving, Lisse, The Netherlands) and on their knowledge of use-patterns for controlling the main pests occurring in the tulip crop in recent years. In our scenario we took toxic mechanisms as selection criteria (i.e., use-patterns of insecticides, herbicides and fungicides). We placed less emphasis on the choice of specific compounds because in practice they are interchangeable.

The study was performed in indoor microcosms. The prevailing laboratory conditions hindered certain recovery processes that may play an important role in less isolated field ecosystems [31]. Under field conditions, for example, some environmental breakdown processes, like sunlight induced photolysis, may cause a faster dissipation of pesticides. In addition, compared to interconnected drainage ditches in the field, exposure regimes in isolated systems are relatively strict since here water transport and dilution of pesticides do not occur. Furthermore, recolonization of populations which depend on flying life-stages is hardly possible in indoor microcosms. In pond-like outdoor test systems, and even more so in interconnected water systems, immigration possibilities for populations are more favorable. Therefore, with respect to the duration and level of exposure of organisms to the pesticides, as well as to the restricted possibilities for recovery, we consider our study to be relatively strict for Dutch agricultural waterbodies.

Exposure concentrations

Except for asulam, the dissipation of pesticides from the water of the microcosms was rapid and concentrations were generally back to detection-limit levels before the next application (Fig. 2). The half-life values ($t_{1/2}$) found for metamitron were rather similar to those found in outdoor experiments. In these studies, the DT50 (the time needed

within which the concentration of a test substance is reduced by 50% in the water layer) was 0.7 to 1.9 d [32, 33]). Also for fluazinam the rapid dissipation we found was more or less similar to that found in outdoor microcosms (DT50: 0.9 – 1.1 d [33]). The half-life of asulam in the water phase exceeded 30 days, resulting in increasing concentrations of asulam with time (Fig. 2). The calculated $t_{1/2}$ of asulam increased with applications (Table 6), which was probably due to decreasing sorption of asulam to the sediment and/or other solid phases.

Over the respective sampling periods, dynamics of the compounds approximated to first-order kinetics (Fig. 2). Therefore, the use of $t_{1/2}$ as an estimation of dissipation rates for the four compounds was considered to be acceptable. However, especially in the case of the highly lipophilic and instable lambda-cyhalothrin, where processes of adsorption to organic material and degradation processes play a major role in the fate of the pesticide [34], our approach is only indicative. In any case, dissipation of lambda-cyhalothrin seemed to be the most rapid. This observation complies with the reported DT50 of 5 to 11 hours in water-sediment systems [34] and to the DT50 of less than one day found in field studies [33, 35].

The relatively fast dissipation of fluazinam, metamitron, and lambda-cyhalothrin from the water of the microcosms suggests that in the field, only acute toxic stress on aquatic ecosystems is to be expected from these three pesticides but accumulation of the herbicide asulam may also lead to chronic stress to the community.

Effects and NOECcommunity

To get an overview of the impact of the treatments on the various endpoints studied, we summarised observed effects into effect classes (Table 11). Most sensitive were macroinvertebrate taxa belonging to the endpoint categories Macrocrustaceans and Insects (*scuttelata* agg., *dipterum*), followed by the zooplanktonic Microcrustaceans (*sphaericus*, Cyclopoidea, nauplii). When focussing on the most sensitive endpoints, long-lasting effects occurred at the 2% and 5% treatment levels. Clear negative effects followed by recovery were observed at the 0.5% treatment level (Table 11).

At the 0.2% treatment level some incidental reductions were observed (*cochlearis* [Table 8], Asellidae juv., *obscuripes*, *aterrimus* [Table 9]). In all these cases, however, the species were infrequently present and/or occurred in very low abundance numbers (Tables 8 and 9). Because of this low information density, observed responses of these species were considered less valid. Only in the case of *C. scuttelata* agg. a NOEC of less than the 0.2% treatment level at the first sampling date made part of a consistent response pattern (Table 9). However, also in the case of this species numbers were very low in all microcosms on the first samplig date. Graphical interpretation of the data does not support a treatment related reduction at the 0.2% treatment level. Moreover, at the next higher treatment level (0.5%) abundance numbers showed a trend of increase and thus did not indicate toxic effects on the 0.2% and 0.5%

treatment levels (Fig. 11E). Hence, overall, we consider the 0.2% treatment level as the NOEC for the community. This is also confirmed by the results of the Monte Carlo permutation tests and the multivariate analysis of the total data sets of the zooplankton, phytoplankton and macroinvertebrate communities.

Table 11. Summary of effects observed in microcosms treated with pesticide mixtures containing fluazinam, asulam, metamitron, and lambda-cyhalothrin. Treatment levels of each pesticide were equal to 0.2%; 0.5%; 2% and 5% spray drift emission of label-recommended rates. The numbers in the table refer to effect classes adapted after [36, 37]. I= no effect; II= slight effect; III= clear short-term effect, full recovery observed; IV= clear effects, trend of recovery observed; V= clear long-term or delayed effects. ↓: decrease of endpoint; ↑: increase of endpoint; ↓↑: both decrease and increase of endpoints occurred. PRC = principal response curve

Endpoint catecory	**Treatment level**			
	0.2%	**0.5%**	**2%**	**5%**
Macrocrustaceans	I	II - IV↓	V↓	V↓
Insects	I-II↓	III↓	IV↓	IV↓
Other macroinvertebrates	I	I	I	II-IV↓↑
PRC macroinvertebrates	I	II-IV	V	V
Microcrustaceans	I	I	II↓	IV-V↓
Rotifers	I	I	II↑	IV-V↑
PRC zooplankton	I	II	II	IV-V
Algae	I	III↑	III↑	III↑
PRC phytoplankton	I	I	I	I
Macrophytes	I	I	IV-V↓	IV-V↓
Community metabolism	I	II↓	II↓↑	II↓↑

Toxicity of the pesticide package

For the zooplankton and macroinvertebrates, pesticide stress expressed as TU for *Daphnia,* rose to 0.7 to 0.8 TU after fourteen days at the 5% treatment level (Fig. 4). Adverse effects are very likely to occur on sensitive arthropods at this toxic level, as clear effects can be expected at $TU_{daphnia} \geq 0.1$ [36]. Hence, reductions in the abundance of zooplankton and macroinvertebrate populations at the 5% and 2% treatment levels (the latter representing 2/5 of 0.7 – 0.8 TU ≈ 0.3 $TU_{daphnia}$) were in line with our expectations. At the 0.5% level, adverse effects on some macroinvertebrates were observed. Concentrations of this pesticide treatment regime remained below 0.1 $TU_{daphnia}$. When synthetic pyrethroids and repeated applications are involved, concentrations in the range of 0.01 to 0.1 $TU_{daphnia}$ may or may not result in effects on arthropods [36]. Thus, the responses at the 0.5% treatment level did not deviate from this general pattern.

Looking at the importance of the separate pesticides to the total sum of TU for the package, it is clear that the synthetic pyrethroid insecticide lambda-cyhalothrin had

the major contribution to the toxic stress for the zooplankton and macroinvertebrates (Fig. 4). For algae, exposures peaked at about 0.09 TU and fluazinam treatments potentially provided most of the toxic risk (Fig. 3).

The accuracy of our method for toxic stress estimation is highly dependent on the amount of toxicity data available for the standard test species chosen. The contribution of asulam to TU_{algae} was based on a single EC50 for *Chlorella pyrenoidosa* (6000 μg/L, Table 4). Because of the lack of more data for this compound, we could not evaluate whether the sensitivity of *Chlorella* is in line with that of the more frequently used standard test alga *Selenastrum*. Nevertheless, in practice, the estimated low acute toxicity of maximal 0.09 TU for algae, agrees with the absence of clear direct effects in the phytoplankton. This level of toxic stress lies in the range of the ecological no-effect threshold level of 0.1 TU [37].

The calculation of TU for the macrophyte *Elodea nuttallii* is open to debate. It comprises only the TU of the two herbicides for *Lemna*. First of all, the question is whether this floating species is a good representative of submerged vascular plants since exposure routes for these two types of plant are different. Second, the TU_{lemna} calculation lacks the contribution of the fungicide fluazinam and the insecticide lambda-cyhalothrin. However, the exposure of fluazinam and lambda-cyhalothrin occurred only for short periods of time considering the fast dissipation rates of these compounds. Hence, the main long-term stressor for the macrophyte *Elodea nuttallii* is likely to be asulam.

Our study indicated that TUs can be used to estimate potential ecotoxicological risks of mixtures of pesticides. This is in line with the empirical rule-of-thumb that concentration addition can be applied to calculate the toxicity of pesticide mixtures [22, 38]. The method of TU calculation gives a good indication of the primary effects of a mixture on aquatic biota, and can therefore be a helpful tool in the initial risk assessment when only laboratory toxicity data are available. At the ecosystem level, however, where recovery and secondary effects are an important evaluation factor, mesocosm studies are still very meaningful in providing more insight into risks at community and ecosystem levels.

Ecological effect chain

We summarised the effects at the two highest treatment levels as observed in our study (Fig. 14). Based on its contribution to the TUs, none of the effects found could be attributed to the herbicide metamitron (Table 5) and the role of the fungicide fluazinam most probably is small or obscure (micro-organisms, rotifers, macrophytes). Application of the herbicide asulam most likely caused a reduction of the macrophyte *Elodea nuttallii*, but negative effects on other primary producers (phytoplankton and periphyton) were minimal. On basis of available toxicity data, direct negative effects of the insecticide lambda-cyhalothrin were expected on arthropod populations. Indeed,

within several sensitive groups of macroinvertebrates, such as Isopoda, Amphipoda, and some insects, some were decimated or even eradicated. For zooplankton, the application of lambda-cyhalothrin had a strong negative effect on Copepoda, whereas for Cladocera and Rotifera only some of the taxa decreased in numbers, while others increased. Especially in the case of the rotifers, it is unclear whether this group is indeed sensitive.

All other effects observed during the experiment most probably are of an indirect nature. The reduction in decomposition (of *Populus* leaves) is most likely caused by the decrease in the number of shredders and more particularly by the extinction of *G. pulex*. However, it cannot be ruled out that the fungicide fluazinam contributed to the reduced decomposition. It may have caused changes in the species composition of microbial communities or in microbial activity. The decrease of *E. nuttallii* contributed to several secondary effects. The DO-pH (decrease)-alkalinity-conductivity (increase)-syndrome changed in its characteristic way as a result of less photosynthetically active biomass. The decrease of *Elodea* allowed an increase of phytoplankton due to decreased competition for nutrients, but phytoplankton biomass in the absolute sense remained very low. Also, release of grazing pressure by a decrease in zooplankton enhanced growth of phytoplankton. Some less sensitive (to lambda-cyhalothrin) zooplankters such as *Daphnia* and *Keratella* increased, at least temporarily, as a result of reduced competition and increased phytoplankton biomass. The abundance of periphyton did not show any direct or indirect effects of the treatment. Probably the direct effect of lambda-cyhalothrin on the mayfly *Cloeon dipterum*, which should have resulted in an increase of periphyton, was compensated for by increased grazing pressure on the vegetation by the surface dwellers *S. lacustris* and cf *Nais* sp.

Protection by first-tier risk assessment procedure

The licensing of pesticides is based on criteria for individual compounds (e.g., [5,6]). In a first-tier, maximum permissible concentrations for aquatic ecosystems are established by applying safety factors based on the outcome of a set of standardised laboratory toxicity tests (e.g., [5, 6]). Additional toxic stress occurrences are likely because it is common agricultural practice to use various combinations of pesticides with similar and/or non-similar modes of action, which, moreover, can be applied simultaneously and/or repeatedly during the growing season.

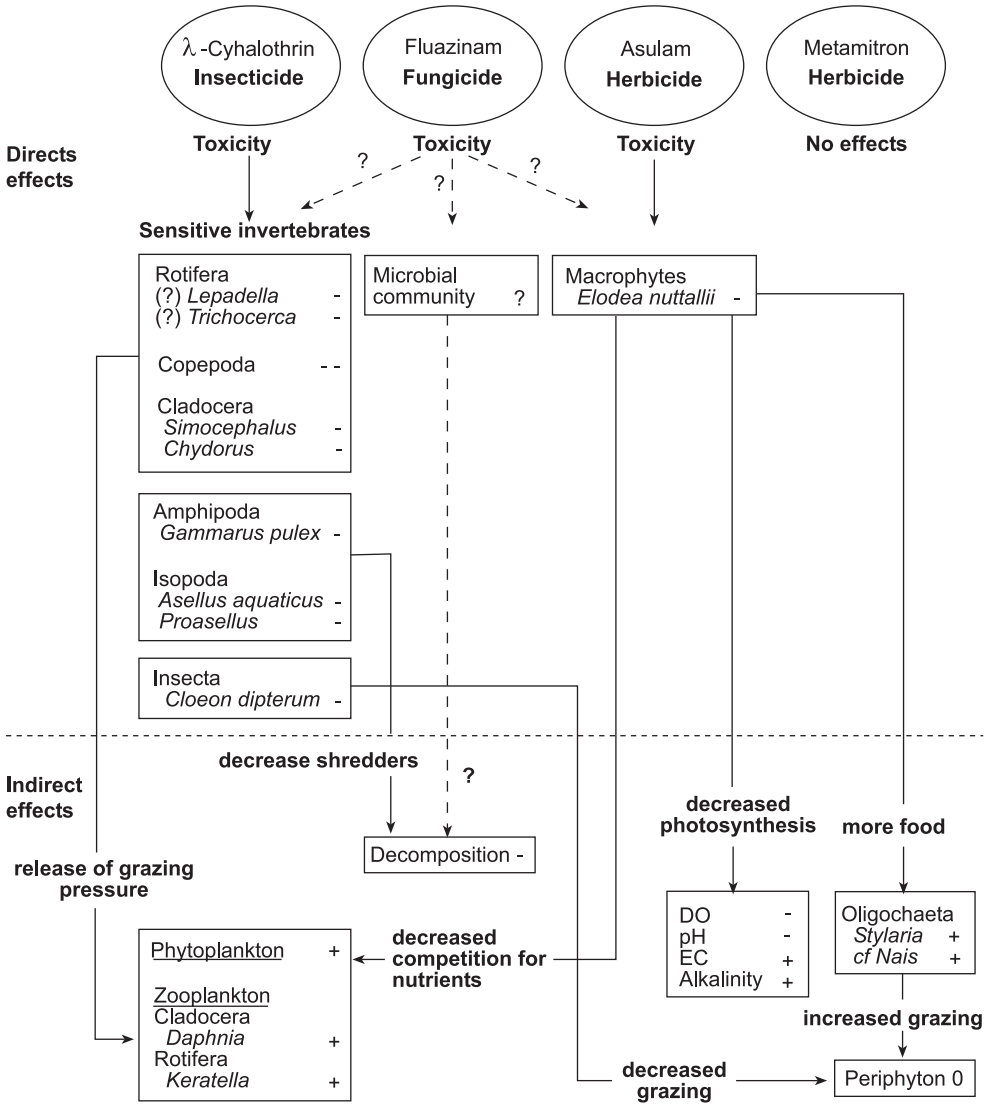

Fig. 14. Schematic overview of the nature and route of effects of high doses of a pesticide package containing fluazinam, asulam, metamitron and lambda-cyhalothrin on the ecosystems of macrophyte-dominated microcosms (-, decrease; +, increase; 0, no response). DO: dissolved oxygen; EC: electrical conductivity.

In the present study, concentrations at the highest treatment level of metamitron were already clearly below the first-tier level as derived from the Uniform Principles of the European Union (Table 12). Therefore, this compound was not a primary candidate to be suspected of having caused effects on the flora and fauna in the microcosms. Fluazinam, asulam and lambda-cyhalothrin, however, exceeded the Uniform Principles first-tier concentrations at certain treatment levels. Target concentrations of fluazinam at the 2% and 5% treatment levels (i.e., 2.7 and 6.7 μg/L, respectively) were above first-tier concentrations (Table 12). At the 5% treatment level, asulam concentrations accumulated to 34 μg/L after the third application (Table 12). At the 2% treatment level, this compound reached a concentration of 13.1 μg/L, which is almost the first-tier concentration of 14 μg/L (Table 12). However, in the case of long-term exposures, as was clearly the situation for asulam, first-tier concentrations better can be derived by taking a safety factor of 10 x the chronic NOEC of the most sensitive standard species. Since a suitable NOEC for this compound was not available, a safety factor of 100 x the acute EC50 of the most sensitive standard species may be used to derive a suitable first-tier concentration. Hence, in the case of asulam we might consider 1.4 μg/L (acute EC50 for *Lemna* = 140 μg/L x 0.01) as a suitable first-tier concentration for long-term exposure. In that case, the concentrations of the 0.5% treatment level and higher were above the 1.4 μg/L concentration level (Fig. 2). When considering lambda-cyhalothrin, this compound, from its first application onwards, was far above the first-tier concentration. Even at the 0.2% treatment level (0.01 μg /L) it was a factor of three above the Uniform Principles first-tier threshold level (Table 12). Three of the four compounds could therefore be considered potentially toxic even at the lower treatment levels. Nevertheless, under our experimental conditions, a $NOEC_{community}$ for the treatment scenario was established at the 0.2% treatment level. Hence, the currently used lower-tier risk assessment procedure, based on single applications of individual compounds, and with its set safety margins, was adequate for protecting the microcosm communities exposed to a combination of realistic pesticide mixtures and multiple applications.

In our study *C. obscuripes*, which is the most sensitive species we know of for lambda-cyhalothrin [34], was not present in high densities. More recent field studies with this compound indicated short-term effects only on *C. obscuripes* at concentrations equivalent to the 0.2% treatment level [39]. Since this concentration level is above the first-tier acceptable concentration, these effects do not disprove the adequacy of the safety margins as set by the Uniform Principles.

Table 12. First-tier maximum permissible concentrations (MPC_{UP}) for the compounds used in a pesticide application scenario simulating tulip crop culturing. Maximum permissible concentrations are based on geometric mean median effective concentrations (EC50s) (Table 4) and on first-tier safety factors conforming to the Uniform Principles of the European Union [16]. Target concentrations (Targ. Concn.) are for the highest treatment level (5% spray drift emmission of label-recommended rates). Peak concentrations (Peak concn.) are the highest concentrations measured during the study; time of occurrence is indicated by day *x*

Compound	MPC_{UP} (µg/L)			Targ. concn. (5%)	Peak concn. (5%)
	Daphnia	Algae	*Lemna*		
	0.01 x EC50	0.1 x EC50	0.1 x EC50		
Fluazinam	1.32	16	--	6.7	6.8 (day 7)
Asulam	320	600	14	13.4	34 (day 45)
Metamitron	129	85	150	11.7	13 (day 42)
λ-Cyhalothrin	0.0035	> 100	--	0.250	0.267(day 28)

Reduction of spray drift emission

Applying spray drift reduction measures is one of the ways in which contamination of aquatic ecosystems can be minimised. These measures may comprise the use of spray-free buffer-zones, label restrictions, adapted application techniques, such as drift reducing nozzles and shielded bed-sprayers, and so forth [15, 16, 40]. To test whether these sometimes expensive measures indeed result in a better water quality in practice, we chose the test range to represent a situation where no spray drift reduction measures (the 5% treatment level) were taken into account as the one extreme, and at the other extreme, we chose a situation where several technically feasible reduction measures were used (the 0.2% treatment level).

It was clear that without taking any measures, the package (essentially lambda-cyhalothrin and asulam) caused severe effects on the communities of the microcosms. Based on our observations, reduction of emission levels should indeed result in reductions of the number of taxa affected, and it would lead to shorter periods of effect duration (Figs. 6 and 10). The 0.2% treatment level caused no effects on the community level and, at the most, some transient effects on few populations. Hence, our study indicates that drift reduction measures can be an efficient tool for protecting aquatic ecosystems in agricultural areas.

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Part III Review and synthesis

7 Threshold levels for effects of insecticides in freshwater ecosystems: a review

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Abstract

A literature review of freshwater (model) ecosystem studies with neurotoxic insecticides was performed to assess ecological threshold levels, to compare these levels with the first tier approach within European Union (EU) administration procedures, and to evaluate the ecological consequences of exceeding these thresholds. Studies published between 1980 and 2001 were reviewed. Most studies covered organophosphates and synthetic pyrethroids in lentic waters. The most sensitive taxa were representatives of crustaceans, insects and fish. Based on toxic units, threshold values were equivalent for compounds with a similar mode of action. This also accounted for the nature and magnitude of direct effects at higher concentrations. Although laboratory single species toxicity tests may not allow predictions on precise ecological effects, some generalisations on effects and recovery can be made with respect to acute standard laboratory EC_{50} data. The $NOEC_{ecosystem}$ usually is a factor of 10 or more higher than first tier acceptable concentrations, particularly in the case of single applications and acetylcholinesterase inhibitors. Acceptable concentrations, as set by the EU first tier approach, appear to be protective. Recovery of sensitive endpoints usually occurs within two months of the (last) application when peak concentrations remain lower than (0.1-1) x EC_{50} of the most sensitive standard test species. The consistency of response patterns found in model ecosystem studies can be useful when estimating the ecological risks of pesticides. The use of an effect classification system was also helpful in evaluating effects.

Keywords: organophosphorus insecticides; carbamates; synthetic pyrethroids; freshwater ecosystems; risk evaluation

Introduction

From their introduction, the use of pesticides has increased tremendously since the time when they were successfully deployed in strategies to increase crop productivity. The quantity of pesticides sold world wide to the agricultural sector had reached over 1.3 million metric tons of active ingredients by 1995 (FAO, www.fao.org/statistical databases/mean of production/pesticide trade/). Of this amount, 295 thousand metric tons (about 23 % of the 1995 total sales) was attributable to insecticides.

In many situations, aquatic ecosystems form highly integrated parts of agricultural areas because they provide water and drainage facilities. With the pesticide application techniques in use for crop protection, it is inevitable that fractions of applied insecticides will enter aquatic ecosystems. Entry routes of pesticides into adjacent bodies of water resulting from normal agricultural usage include spray drift, runoff, and leaching (e.g., Ganzelmeier et al., 1995; Capri and Trevisan, 1998; Van de Zande et al., 2000). Programmes and studies focusing on the detection of pesticides in an aquatic environment report traces of these toxicants in various bodies of water (Wan, 1989; Thoma and Nicolson, 1989; Frank et al., 1990; Teunissen-Ordelman and Schrap, 1996; Lahr and Banister, 1997; Liess and Schulz, 1999; Leonard et al., 2000). Hence, it is demonstrated that non-target species living in water catchments of agricultural areas are potentially at risk when they have similar toxicant receptors as the target organisms. Pesticide admission and regulatory authorities have therefore been set up to control and reduce the undesirable impacts of pesticide usage on the environment.

Essentially, risk assessment is done by comparing concentrations expected, or found, in the environment with concentrations of that pesticide considered acceptable by regulatory authorities. In many countries (e.g., EU countries and the US) a tiered approach for aquatic risk assessments is being applied. The concept of this approach is that when passing through the tiers, the estimates of exposure and effects become more accurate as uncertainty is reduced through the acquisition of more data. The lower tiers are more conservative while higher ones are more realistic (Campbell et al., 1999; Solomon, 2001).

In the first tier risk assessment, criteria are based on the toxicity data of a small set of standard test species generated in the laboratory which are then multiplied by a safety factor (US-EPA, 1998; EU, 1997). This method is sometimes considered to be very strict and has been the subject of debate (e.g., Maund et al., 1998; Giesy et al., 1999). Issues concerning the adequacy of the first tier risk assessment is one of the reasons calling for higher tier risk evaluation, as this type of evaluations consider the outcome of ecotoxicological studies under more realistic exposure conditions in combination with greater ecological realism.

Microcosm and mesocosm studies form an important part of the research that has been done to validate first tier water quality criteria for pesticides and/or to assess

their 'regulatory acceptable concentration' in surface waters. These studies have been done with various active ingredients and under a wide range of conditions. Major differences in conditions between studies are location (e.g., climatological or biogeographical regions) and types of natural and experimental ecosystems used (e.g., plankton or macrophyte-dominated systems, experimental ponds or streams). The relatively large amount of data generated by these freshwater ecosystems provide the opportunity to detect whether there are predictable concentration-effect relationships and/or other generalities in effect patterns between studies.

The present review focuses on the ecological impact of neurotoxic insecticides. We considered two groups: acetylcholinesterase inhibitors (organophosphates and carbamates) and synthetic pyrethroids.

Data presented here are based mainly on experiments in freshwater model ecosystems since descriptive hydrobiological field research into the effects of insecticides is scarce. Following the terminology used by the European Workshop on Freshwater Field Tests (EWOFFT), these systems are also called microcosms (tanks/ponds with a water volume < 15 m^3 or experimental streams < 15 m in length) or mesocosms (systems > 15 m^3 or > 15 m, respectively) (Crossland et al., 1994). An advantage of experimental ecosystems is that they can be replicated, and several concentrations of a pollutant can be tested simultaneously. The pros and cons of working with model freshwater ecosystems are discussed by Brock et al. (1995a), ECETOC (1997), and Caquet et al. (2000).

Objectives of the present literature review are: (a) to list ecological threshold values (e.g., $NOEC_{eco}$ and $LOEC_{eco}$) for individual insecticides as established experimentally by means of freshwater model ecosystems or adequate field studies, (b) to compare $NOEC_{eco}$s with established first tier water quality criteria for insecticides in surface water and (c) to asses the ecological consequences of exceeding the first tier water quality criteria.

We consider $NOEC_{eco}$ to be the highest tested concentration at which no, or hardly any, effects on the structure and functioning of the studied (model) ecosystem are observed. The $LOEC_{eco}$ is the lowest tested concentration at which significant treatment-related effects occur.

Methods

Literature reviewed

The literature database available at our Institute served as a basis for the study. This database has been built up over the years and kept up-to-date by means of the literature bulletins 'Chemical Abstracts' and 'Current Contents'. The existing database was checked for possible gaps through a specific literature search, using the program 'Winspirs' (version 4.0). This program was used to search the databases of 'Agris

Current' (from 1980 onwards), 'Biological Abstracts' (from December 1989 onwards), and 'CAB-Abstracts' (from 1980 onwards). Publications up to and including June 2001 were included in this search. Furthermore, we included recent studies done by our own research group, (Roessink et al., 2005; Van Wijngaarden et al., 2005a en 2005b).

Criteria for the selection of suitable microcosm and mesocosm studies

The yielded ecotoxicological studies were screened on the following criteria:

1. Test systems used represent a realistic freshwater community (organisms of various trophic levels are present).
2. Description of the experimental set-up is adequate and unambiguous
3. Exposure concentrations relevant to the study are reported or can be derived (at least nominal concentrations are known).
4. Investigated endpoints are sensitive to the substance in that direct effects on these endpoints are related to the working mechanisms of insecticides. Arthropods and fish are especially considered to be sensitive endpoints for insecticides (Hill et al., 1994a; Graney et al., 1994; this paper).
5. The effects are evaluated statistically and show an unambiguous concentration-effect relationship or, observed effects are in agreement with a concentration-effect relationship from additional studies.
6. To establish a $NOEC_{eco}$, at least the lowest test concentration within the study should not show a consistent effect attributable to the treatment. A concentration above the $NOEC_{eco}$ should show a significant treatment-related effect ($LOEC_{eco}$).
7. To enable a comparison of field concentrations with target concentrations for registration procedures, toxicity data of standard test organisms (at least for *Daphnia* or fish) should be known.
8. The results of the study were published in 1980 or later.

Subsequently, selected studies were classified according to the exposure regime (single application, multiple applications, or continuous exposure), type of test system (stagnant or running water), and working mechanism of the insecticides.

Comparison between insecticides

To enable comparison of studies using different insecticides, the reported field concentrations were normalised by dividing them by the 48h-EC_{50} of the aquatic standard test species *Daphnia magna* or by the 96h-LC_{50} of a standard test fish (the most sensitive species was used). The unit of the resulting variable is defined as TU_{mso} (= Toxic Unit based on the most sensitive standard test organism). In the case of EC_{50}s for *Daphnia magna*, the effect parameter could also be mortality.

Publications by Crommentuijn et al. (1997), Mayer and Ellersieck (1986), the AQUIRE database (www.epa.gov/ecotox/), and references in the papers about the evaluated microcosm and mesocosm studies have been used as a source of information for the toxicity data. If several EC_{50}s were available for the same standard test organism, the geometric mean of these values was calculated and referred to as 'gm-EC_{50}' (Table 1). When gm-EC_{50}s were available, they were used to calculate TU_{mso}. The toxicity data showed that *Daphnia magna* was usually the most sensitive standard test organism for the evaluated insecticides (Table 1). For some pyrethroids, *Daphnia* as well as fish are a representative sensitive standard test species.

Table 1. Toxicity data (µg/L) of the most sensitive standard test species used to calculate toxic units (TU_{mso}). First tier acceptable concentrations (NEC) are derived from the Uniform Principles criteria and based on the toxicity data in this table. *D. magna*: *Daphnia magna*. crust: crustacean. *P. promelas*: *Pimephalus promelas*. *O. mykiss*: *Oncorhynchus mykiss*. *L.macrochirus*: *Lepomis macrochirus*.

Compound	Toxicity	Species	References	First tier NEC
Azinphos-methyl	gm-L(E)C_{50} = 2.0 (48 h)	*D. magna* (crust)	1,2,3	0.02
Bendiocarb	gm-L(E)C_{50} = 74 (48 h)	*D. magna* (crust)	4	0.74
Carbaryl	EC_{50} = 5.6 (48 h)	*D. magna* (crust)	2	0.056
Carbofuran	gm-L(E)C_{50} = 33.2 (48 h)	*D. magna* (crust)	5,6	0.33
Chlorpyrifos	gm-L(E)C_{50} = 1.3 (48 h)	*D. magna* (crust)	7,8	0.013
Cyfluthrin	gm-L(E)C_{50} = 0.15 (48 h)	*D. magna* (crust)	9	0.0015
Cypermethrin	gm-L(E)C_{50} = 0.68 (96 h)	*O. mykiss* (fish)	10, 11	0.0068
Deltamethrin	gm-L(E)C_{50} = 0.04 (48 h)	*D. magna* (crust)	12, 13	0.0004
Diazinon	gm-L(E)C_{50} = 1.0 (48 h)	*D. magna* (crust)	1, 14	0.01
Esfenvalerate	gm-L(E)C_{50} = 0.25 (96 h)	*P. promelas* (fish)	15	0.0025
Fenitrothion	gm-L(E)C_{50} = 11 (48 h)	*D. magna* (crust)	16, 17	0.11
Fenvalerate	gm-L(E)C_{50} = 0.82 (96 h)	*O. mykiss* (fish)	2	0.008
Lambda-cyhalothrin	LC_{50} = 0.21 (96 h)	*L. macrochirus* (fish)	2	0.0021
Parathion	gm-L(E)C_{50} = 1.1 (48 h)	*D.magna* (crust)	1	0.0011
Parathion-methyl	gm-L(E)C_{50} = 1.4 (48 h)	*D.magna* (crust)	1, 2, 19	0.014
Permethrin	gm-L(E)C_{50} = 0.65 (48 h)	*D.magna* (crust)	2, 9, 11	0.0065
Phorate	gm-L(E)C_{50} = 1.5 (48 h)	*D.magna* (crust)	18	0.015
Tralomethrin	LC_{50} = 0.15 (48 h)	*D.magna* (crust)	9	0.0015

1: Dortland (1980), 2: Mayer and Ellersieck (1986), 3: Giddings et al. (1994), 4: Visser and Linders (1990),5: Trotter et al. (1991), 6: Jansma and Linders (1993), 7: Kersting and Van Wijngaarden (1992), 8: McCarthy (1977) in Barron and Woodburn (1995), 9: Mokry and Hoagland (1990), 10: Stephenson (1982), 11: Crommentuijn et al. (1997), 12: Xiu et al. (1989), 13: Day (1991), 14: AQUIRE database (www.epa.gov/ecotox/), 15: Stay and Jarvinen (1995), 16: Sanders et al. (1983), 17: LeBlanc (1984), 18: Fairchild et al. (1992a), 19: Oikari et al. (1992).

Criteria for effect classification

Reported endpoints were assigned to one of eight endpoint categories: (a) 'Microcrustaceans' (including Cladocera, Copepoda, Ostracoda), (b) 'Macrocrustaceans' (including Amphipoda, Isopoda, Anostraca), (c) 'Insects', (d) 'Fish', (e) 'Rotifers', (f) 'Other macroinvertebrates', (g) 'Algae & macrophytes', and (h) 'Community metabolism'. Within each category, the most sensitive endpoint was decisive for classification into an effect class (worst case approach). The categories 'a' to 'f' represent structural endpoints, while category 'h' represents functional responses. Structural endpoints concern densities (numbers) and biomass of populations. Functional endpoints in most cases concern oxygen balance, water chemistry, and decomposition of particulate matter. Effects reported on these endpoints were classified into five classes based on the following criteria:

Class 1: 'effect not demonstrated'
- no effects observed as a result of treatment (primarily, statistical significance plays an important role for this criterion) and/or,
- observed differences between treatment and controls show no clear causal relationship. Causality in this context is judged through the use of guidelines similar to those developed for identifying causative agents of disease (Koch, 1942; Hill, 1965).

Class 2: 'slight effect'
- effects only observed on individual samplings, especially shortly after treatment, and/or
- short-term and/or quantitatively restricted response of sensitive endpoints.

Class 3: 'pronounced short-term effect'
- clear response of sensitive endpoints, but full recovery within eight weeks after (the last) application, and
- effects observed on some subsequent sampling dates, and
- effects reported on several sensitive species; temporary effects on less sensitive species and/or endpoints.

Class 4: 'pronounced effect in short-term study'
- clear effects observed, but the study is too short to demonstrate complete recovery within eight weeks after (the last) application of the insecticide for the endpoint concerned.

Class 5: 'pronounced long-term effect'
- clear response on various subsequent sampling dates, and recovery time of sensitive endpoints is longer than eight weeks after the last application, and

- effects reported on many sensitive species and/or endpoints; elimination of sensitive species; effects on less sensitive species endpoints and/or other similar descriptions.

A recovery period of eight weeks was applied in the classification to decide whether effects were short-term or longer-term. In relation to the life-cycles of macro-invertebrates, fish and macrophytes, it is common practice in microcosm and mesocosm studies to sample these groups of organisms on a biweekly or monthly basis. Consequently, the typical sampling intervals for macroinvertebrates may not establish actual times of recovery, but will be adequate for determining if effects are persisting beyond the short-term eight week time frame. For short-cyclic organisms, such as phytoplankton and zooplankton, sampling frequencies are generally on a weekly basis. For this group of organisms there are enough observation points to establish the time of actual recovery within this time window.

Effects were reported in the literature in a variety of ways, and generally did not fit exactly into our effect criteria scheme. The process of assigning reported effects to one of the effect classes therefore normally consisted of evaluating both quantitative and qualitative information, and judging on a case-by-case basis into which combination of criteria this information fitted best. If in doubt, the information was evaluated by more than one expert to obtain a consensus answer.

Data analysis

The probability of effects occurring in microcosm and mesocosm studies was calculated by analysing the combined data set of the most sensitive endpoints of both the acetylcholinesterase inhibitors and pyrethroids using logistic regression. For this purpose, the effect classes were reclassified to a nominal variable: a 'no-effect class' (0) and an 'effect class' (1). The 'effect class' contained the former Classes 3, 4 and 5. 'No-effect class' analyses were performed using two definitions; one containing only the data of Effect Class 1, and the other containing the data of Effect Classes 1 and 2. The following logistic model was used for these calculations:

$$y = \frac{1}{1 + e^{-b(\mathrm{Ln}(x)-a)}}$$

in which y is the response variable (effect/no effect), x is the concentration expressed in TU_{mso}; a is the concentration at which an effect has been reported for 50% of the studies, and b is the slope of the sigmoid curve at this concentration. Results of these analyses were expressed as Field Effect Concentrations (FEC) at 5, 50 and 95 percentages of probability. In other words, the model yielded fitted concentrations (expressed in TU_{mso}) for which it predicted that for 5, 50 and 95% of the studies,

effects will occur. The calculations were performed using the GENSTAT statistical program (Payne and Lane, 1993).

Comparison of ecological threshold values with registration criteria

We compared the ecological threshold values ($NOEC_{eco}$s) obtained from microcosm and mesocosm studies with the acceptable concentrations established by the first tier registration criteria applied in the European Union. According to EU Uniform Principles (EU, 1997), in the first tier of the risk assessment, the peak concentration of a pesticide in surface water as calculated from reference tables for spray drift and/or fate models (Ganzelmeier et al., 1995; FOCUS, 2001), should not be higher than 0.01 x the acute EC_{50} for the standard test species of fish or *Daphnia* and 0.1 x the EC_{50} for standard test algae. In addition, the time weighted average exposure concentration should not be higher than 0.1 x the chronic NOEC of *Daphnia* (21 days) and fish (28 days) with long-term exposure. A higher concentration, however, may be considered acceptable if it can be demonstrated by using higher tier tests that the real risk to aquatic organisms is less than predicted by the first tier criteria ('unless clauses').

We established first tier acceptable concentrations on the basis of acute toxicity data for the standard test organisms mentioned in OECD protocols (OECD, 1993). This is established by dividing the gm-EC_{50} of the most sensitive species by a factor of 100 (Table 1). We used acute toxicity data because: (a) adequate chronic toxicity data for the substances studied in microcosm and mesocosm experiments are in many cases not available in the open literature whereas acute toxicity data are; (b) in microcosm and mesocosm studies, only nominal or measured peak concentrations of the studied pesticide are usually reported; and (c) the compounds studied have relatively low environmental persistence making comparison of short-term exposures to acute toxicity data the most relevant.

Available information

Summaries were first made of the selected studies. Concise versions of these are given in Brock et al. (2000b).

Acetylcholinesterase inhibitors

Organophosphorous and carbamate insecticides inhibit the activity of the enzyme acetylcholinesterase. Inhibition of this enzyme results in the accumulation of acetylcholine at choline receptors and consequently in the disturbance of nerve impulses (Klaassen et al., 1986).

Microcosm and mesocosm experiments were only conducted on a small number of the 64 organophosphates listed by Tomlin (2000). After testing against the selection

criteria, 26 studies remained. They yielded adequate information on ecological risks of seven active ingredients (Table 2). The selected studies were mainly conducted on chlorpyrifos (twelve studies), fenitrothion (five studies), and azinphos-methyl (four studies). Five microcosm and mesocosm studies provided adequate information on the active ingredients bendiocarb, carbaryl, and carbofuran [three out of the twenty acetylcholinesterase inhibiting carbamates listed (Tomlin, 2000)]. The study locations were quite diverse, and done under climatological conditions ranging from temperate to subtropical and tropical (Table 2).

Synthetic pyrethroids

Pyrethroids also affect the functioning of the nervous system. Their primary mode of action is by interference with ion channels in the nerve axon, resulting in hyperactivity of the nervous system with a subsequent lack of control of normal function (Clark and Brooks, 1998).

Eighteen microcosm and mesocosm studies of eight active ingredients - out of the 39 listed pyrethroids – (Tomlin, 2000), yielded adequate information after testing against our selection criteria. The studies were performed predominantly in North America and Europe under various climatological conditions (Table 3).

Application method and pesticide behaviour

Most studies were conducted using formulated materials (Tables 2 and 3). Exposure of aquatic organisms to insecticides, and observed effects during microcosm and mesocosm studies, are strongly related to the method of application and the environmental behaviour of these substances. Pollution of watercourses by insecticides may be the result of spray drift. Most studies focusing on acute risks simulated this entry route and applied the insecticide by spraying the water surface. In studies with a chronic exposure regime, insecticides are usually directly mixed into the water column.

Table 2. Experiments with acetylcholinesterase inhibitors included in this report. Test form: active ingredients (a.i.) were applied as a formulated product (F), or as a.i. in acetone (S), or as a.i. without a solvent (A). -: not reported. S-stag = single application in a stagnant system; S-stream = single application in a running system; M-stag = multiple applications in a stagnant system; M-stream = multiple applications in a running system; L-stag = prolonged constant exposure in a stagnant system; L-stream = prolonged constant exposure in a running system.

Active ingredient	Test form	Experiment	Location	Authors
Organophosphorous insecticides				
Azinphos-methyl	F	S-stag	USA (lab)	Stay & Jarvinen 1995
--	F	S-stag	USA (Minnesota)	Tanner & Knuth 1995
--	F	M-stag	USA (Kansas)	Giddings et al. 1994
--	F	S-stag	USA (Minnesota)	Knuth et al. 1992
Chlorpyrifos	F	S-stream	Australia	Pusey et al. 1994
--	F	L-stag	NL (lab)	Van den Brink et al. 1995
--	F	L-stream	Australia	Ward et al. 1995
--	F	S-stag	USA (Kansas)	Biever et al. 1994
--	F	M-stag	USA (Kansas)	Giddings et al 1997
--	F	S-stag	NL	Van Wijngaarden et al. 1996; Van den Brink et al. 1996; Kersting & Van den Brink 1997
--	F	S-stag	NL (lab)	Brock et al. 1992 a,b; 1993
--	F	S-stag	NL (lab)	Van Donk et al. 1995; Brock et al 1995b; Cuppen et al 1995
--	F	S-stag	USA (Minnesota)	Siefert et al. 1989; Brazner et al. 1989; Brazner & Kline 1990
--	-	S-stag	USA (lab)	Stay et al. 1989
--	F	S-stag	Canada	Hughes et al. 1980
--	F	S-stag	Canada (Manitoba)	Zrum et al. 2000
--	F	S-stag	NL (lab)	Van Wijngaarden et al. 2005b
Diazinon	A	M-stag	USA (Kansas)	Giddings et al. 1996
Fenitrothion	F	S-stag	Senegal	Lahr & Diallo 1993
--	F	M-stag	Canada	Fairchild & Eidt 1993
-- (*)	F	S-stream	UK	Morrison & Wells 1981
-- (*)	F	S-stream	Canada	Poirier & Surgeoner 1988
-- (*)	F	S-stream	Japan	Yasuno et al. 1981
Parathion-ethyl	A	L-stag	NL	Dortland 1980
Parathion-methyl	S	S-stag	UK	Crossland 1984; Crossland & Bennett 1984
--	S	S-stag	UK	Crossland 1988
Phorate	F	S-stag	USA (S. Dakota)	Dieter et al. 1996
Carbamates				
Bendiocarb	F	S-stag	Senegal	Lahr et al. 1995
Carbaryl	F	S-stag	USA (Ohio)	Havens 1994; 1995
--	F	S-stream	Canada (Maine)	Courtemanch & Gibbs 1980
Carbofuran	F	S-stag	Canada (Alberta)	Wayland 1991
--	F	S-stag	Canada (Alberta)	Wayland & Boag 1995

(*) studies do not meet all criteria but yield information on low exposure concentrations.

Table 3. Experiments with synthetic pyrethroids included in this report. Test form: active ingredients (a.i.) were applied as a formulated product (F), or as a.i. in acetone (S), or as a.i. without a solvent (A). -: not reported. S-stag = single application in a stagnant system; S-stream = single application in a running system; M-stag = multiple applications in a stagnant system; M-stream = multiple applications in a running system; L-stag = prolonged constant exposure in a stagnant system; L-stream = prolonged constant exposure in a running system.

Active ingredient	Test form	Experiment	Location	Authors
Cyfluthrin	F	M-stag	USA (Texas)	Johnson et al. 1994; Morris et al. 1994
Cypermethrin	F	M-stag	UK	
--	-	M-stag	UK	
--	F	M-stag1	USA (N. Carolina)	Hill 1985
--	F	M-stag2	USA (N. Carolina)	Hill 1985
Deltamethrin	F	S-stag	Senegal	Lahr et al. 1995
--	F	S-stag	Canada	Morill & Neal 1990
Esfenvalerate	F	M-stag	USA (Alabama)	Webber et al. 1992
--	S	M-stag	USA (Missouri)	Fairchild et al. 1992b
--	F	M-stag	USA (Minnesota)	Lozano et al. 1992; Tanner & Knuth 1996
--	A	S-stag	USA (lab)	Stay & Jarvinen 1995
--	F	S-stag	Denmark	Samsøe-Petersen et al. 2001
Fenvalerate	F	S-stag	Canada (Ontario)	Day et al. 1987
--	F	L-stream	USA (Iowa)	Breneman & Pontasch 1994
Lambda-cyhalothrin	-	M-stag	UK	Farmer et al. 1995
--	F	M-stag	USA (N. Carolina)	Hill et al. 1994b
--	F	M-stag	NL	Roessink et al., 2005
--	F	M-stag	NL	Van Wijngaarden et al., 2005a
Permethrin	S	S-stag	Canada (Ontario)	Kaushik et al. 1985
Tralomethrin	F	M-stag	USA (Texas)	Mayasich et al. 1994

In the studies with organophosphates and carbamates, active ingredients were almost always applied in dissolved form via the aqueous phase (spray drift or direct mixing in the water column). In most studies with pyrethroids, active ingredients were also applied by spraying onto, or injecting below, the water surface. In one study with the organophosphorous compound chlorpyrifos (Giddings et al., 1997) and three studies with pyrethroids [lambda-cyhalothrin (Hill et al., 1994b); tralomethrin (Mayasich et al., 1994); cyfluthrin (Johnson et al., 1994)], drift as well as runoff applications were performed in the same test system. In the case of runoff applications, the compound is brought into the systems bound to soil material. In the three pyrethroid studies specifically, it was not always clear whether the observed effects were caused by the drift or by the runoff application. This is to do with the fact that reported measured concentrations do not always tally, because of the high disappearance rate of pyrethroids from the water and variation in the first sampling instance after spraying

(less than 1 hour to 24 hours). We therefore evaluated effects in these studies on the nominal concentration caused by drift application(s) only. In all cases, this is a worst-case approach since the observed effects may in part also be attributed to exposure via the runoff-emission route. The contaminated soil material of the runoff applications rapidly disappears from the water column by sedimentation, and bio-availability of the soil-bound pyrethroids is also lower (Hill, 1985,1989; Maund et al., 1997; 1998). These factors are likely to mitigate the contribution of a runoff application to the effects of a combined spray and runoff application.

Particularly in drift simulating applications to stagnant waters, clear concentration gradients of insecticides can be found in the first hours post-treatment (Muir et al., 1992; Fairchild and Eidt, 1993; Crum and Brock, 1994; Farmer et al., 1995; Van Wijngaarden et al., 1996; Samsøe-Petersen et al., 2001). Shortly after drift applications, most of the active ingredient is then found in the superficial water layer. Also the influence of the type of formulation and/or additives on the dissipation mechanisms may play a role. Oil-based formulations are much more likely to retain high concentrations in superficial water layers than emulsifiable concentrate formulations which will dissipate more quickly throughout the water column.

Hence, superficially, initial concentrations may be considerably higher than the intended nominal concentrations. Simultaneously, exposure concentrations in subsurface water are then considerably lower than nominal concentrations. This implies that species, although they may be equally sensitive in the laboratory, may respond very differently in the field when they occupy different spatial niches in their natural environments. This is shown from a study with lambda-cyhalothrin (Hill et al., 1994b) in which surface bugs (Gerridae and Veliidae) reacted more sensitively than water bugs and beetles such as Notonectidae and Haliplidae.

In time, insecticides usually get mixed in the water column and often a considerable amount dissipates from the water. This disappearance, especially during the first days after application, is not only caused by physicochemical degradation but also by the distribution of the active ingredient over different environmental compartments such as sediment, organic and inorganic particulate material, aquatic plants (e.g., Hill, 1989; Brock et al., 1993; Crum and Brock, 1994, Samsøe-Petersen et al., 2001; Hand et al., 2001) and volatilisation from the water (e.g., Larkin and Tjeerdma, 2000).

Initial half-life values of dissolved organophosphates and carbamates in the water of stagnant (model) ecosystems are in the order of less than one to ten days (Crossland and Bennett, 1984; Hanazato and Yasuno, 1990; Lahr and Diallo, 1993; Crum and Brock, 1994; Tanner and Knuth, 1995; Wayland and Boag, 1995; Giddings et al., 1996). In the case of pyrethroids, initial half-life in the water columns are in the order of less than one hour to three days (Stephenson et al., 1986; Heinis and Knuth, 1992; Fairchild et al., 1992b; Johnson et al., 1994; Farmer et al., 1995; Hand et al.,

2001; Roessink et al., 2005). Reported half-life of sediment-adsorbed pesticides is generally much longer (days to weeks) in the above-mentioned studies.

These spatio-temporal processes indicate that nominal concentrations cannot be directly converted into actual exposure concentrations for aquatic organisms in the field. The observed initial stratification of insecticides in the water column makes it likely that benthic organisms and those present in internal refugia, such as dense vegetations, are initially exposed to lower concentrations than organisms having niches and/or home ranges close to the water surface. In fact, spatio-temporal distribution of non-persistent insecticides forms a major issue in the discussion related to refinements of ecotoxicological risk assessments (Giesy et al., 1999; Hendley et al., 2001; Maund et al., 2001; Travis and Hendley 2001).

Nevertheless, we have taken nominal concentration as a reference for describing the effects resulting from peak exposures because: (a) the applied nominal dose is given in almost all studies, (b) measured initial concentrations are not always comparable and/or reliable due to large differences in the first sampling instance after treatment (hours to days) in relation to the relatively high initial disappearance rate of most insecticides, (c) in registration policies the short-term exposure as a result of drift is calculated by assuming instantaneous mixing of the dose over the water column.

Effects on sensitive endpoints

Effects reported

A distinction between direct and indirect effects is frequently made in the reported effects of insecticides in microcosm and mesocosm experiments. However, a decrease in population density of a species after application of an insecticide cannot, in advance, be considered as a direct effect; it could also be the result of an indirect effect due to shifts in species interactions.

Reductions in population densities at relatively low insecticide concentrations are found especially in populations of crustaceans (cluster Amphipoda – Ostracoda/Anostraca in Tables 4 and 5), insects (cluster Trichoptera – Coleoptera) and fish (Pisces). Negative effects in these groups were observed below 1 TU_{mso} after single applications of acetylcholinesterase inhibitors (Table 4) and below 0.1 TU_{mso} after repeated applications of pyrethroids (Table 5). Reductions in numbers of Rotifera, Mollusca, Annelida and Turbellaria are only observed at relatively high exposure concentrations and in a limited number of studies. Negative effects on plants are only reported at exposure concentrations higher than 1-10 TU_{mso}.

When laboratory toxicity tests have been conducted with species that are found in microcosm and mesocosm experiments, the sensitivities among these species to insecticide exposures have been shown to be similar in both test systems (Dortland, 1980; Crossland,1984; Van Wijngaarden et al., 1996; Lahr, 1998; Maund et al., 1998; Van den Brink et al., 2002). In addition, responses found in the evaluated studies for

specific taxonomic groups correspond well with those found in laboratory single-species toxicity tests with indigenous species from these groups (e.g., Crommentuijn et al., 1997; AQUIRE database, www.epa.gov/ecotox/). This makes it probable that in microcosm and mesocosm experiments, observed reductions in densities of crustaceans, insects and fish at low concentrations can generally be considered as direct toxic effects. One should, however, be aware that insects, crustaceans and fish may also include relatively insensitive taxa (e.g., Dortland, 1980; Brock et al., 1992b; Lahr and Diallo, 1993; Giddings et al., 1996).

The categories 'Microcrustaceans', 'Macrocrustaceans', 'Insects' and 'Fish' include the sensitive organisms. The categories 'Rotifers', 'Other macroinvertebrates' and 'Algae & macrophytes' often include organisms that are indirectly affected but where the occurrence of direct effects cannot be excluded a priori.

Table 4. Reported negative effects on various taxonomic groups as a result of single applications of acetylcholinesterase-inhibiting insecticides in aquatic microcosms and mesocosms. The effects are arranged according to toxic units (TUmso) and expressed as a percentage of the cases ($n = x$) in which a reduction in numbers or biomass of one or more taxa within a taxonomic group was reported.

	TU_{mso}			
	0.01-0.1	0.1-1	1-10	10-100
Amphipoda	0% (*n*=4)	43% (*n*=7)	100% (*n*=7)	100% (*n*=7)
Cladocera	0% (*n*=5)	83% (*n*=12)	100% (*n*=17)	100% (*n*=11)
Copepoda	20% (*n*=5)	30% (*n*=10)	38% (*n*=13)	63% (*n*=8)
Isopoda	-	-	100% (*n*=1)	100% (*n*=2)
Ostracoda	0% (*n*=3)	14% (*n*=7)	38% (*n*=8)	67% (*n*=6)
Anostraca	-	-	0% (*n*=1)	-
Trichoptera	?** (*n*=1)	100% (*n*=1)	100% (*n*=1)	100% (*n*=1)
Ephemeroptera	0% (*n*=2)	75% (*n*=4)	100% (*n*=3)	100% (*n*=3)
Diptera	0% (*n*=3)	71% (*n*=7)	100% (*n*=7)	100% (*n*=8)
Hemiptera	-	-	100% (*n*=1)	100% (*n*=5)
Odonata	0% (*n*=1)	0% (*n*=2)	75% (*n*=4)	100% (*n*=6)
Coleoptera	-	-	100% (*n*=1)	67% (*n*=3)
Hydracarina	0% (*n*=1)	0% (*n*=2)	50% (*n*=4)	33% (*n*=3)
Pisces	0% (*n*=3)	67%* (*n*=3)	83% *(*n*=6)	100%* (*n*=3)
Rotifera	0% (*n*=3)	0% (*n*=6)	0% (*n*=7)	0% (*n*=4)
Mollusca	0% (*n*=2)	0% (*n*=5)	0% (*n*=6)	13% ***(*n*=8)
Annelida	0% (*n*=2)	0% (*n*=3)	0% (*n*=6)	13%*** (*n*=8)
Turbellaria	-	0% (*n*=1)	50% (*n*=2)	33%*** (*n*=3)
Plants	0% (*n*=2)	0% (*n*=5)	0% (*n*=9)	50%*** (*n*=6)

* direct as well as indirect effects reported
** data do not allow clear conclusions as to whether or not effects occurred
*** reported as indirect effects

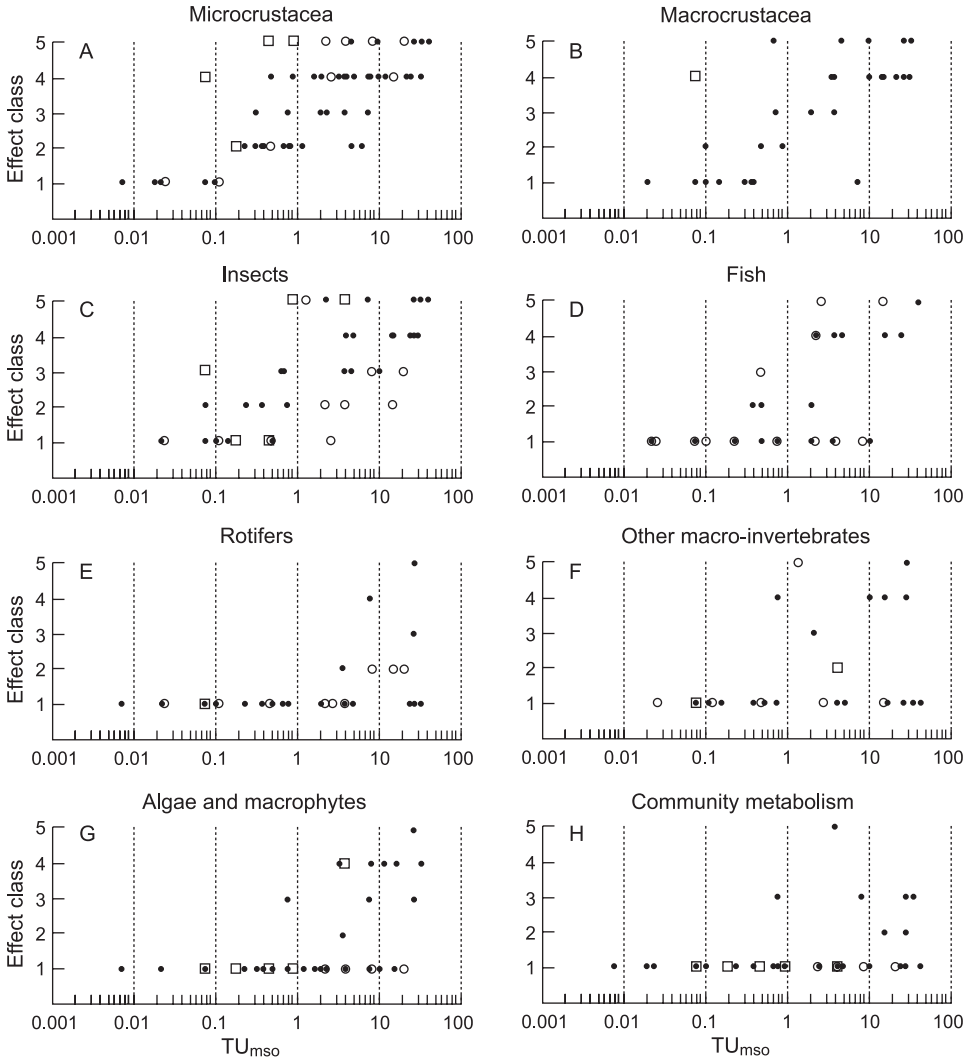

Fig. 1. Effects of insecticides with an acetylcholinesterase-inhibiting mode of action in microcosm and mesocosm studies. The figure includes observations of studies in stagnant water (single and multiple applications), and of chronic applications in stagnant as well as running water test systems. Effects are classified into several categories, structural endpoints (A to G) and a functional category (community metabolism; H). The effects are also classified (Effect class) according to magnitude and duration. 1 = no significant effect, 2 = slight effect, 3 = clear short-term effect (< 8 weeks), 4 = clear effect in short-term study (recovery moment unknown), 5 = clear long-term effect (> 8 weeks). Closed circles (●) indicate experiments with a single application. Open circles (○) and squares (□) indicate experiments with multiple applications or chronic exposure, respectively.

Table 5. Reported negative effects on various taxonomic groups as a result of repeated application of pyrethroids in aquatic microcosms and mesocosms. The effects are arranged according to toxic units (TU_{mso}) and expressed as a percentage of the cases ($n = x$) in which a reduction in numbers or biomass of one or more taxa within a taxonomic group was reported.

	TU_{mso}			
	0.001-0.01	**0.01-0.1**	**0.1-1**	**1-10**
Amphipoda	-	100% (*n*=1)	100% (*n*=11)	100% (*n*=7)
Isopoda	-	-	80% (*n*=5)	100% (*n*=2)
Copepoda	0% (*n*=1)	60% (*n*=5)	56% (*n*=16)	73% (*n*=11)
Cladocera	0% (*n*=1)	0% (*n*=2)	50% (*n*=10)	86% (*n*=7)
Ostracoda	0% (*n*=1)	0% (*n*=1)	50% (*n*=2)	-
Trichoptera	0% (*n*=1)	67% (*n*=3)	86% (*n*=7)	83% (*n*=6)
Ephemeroptera	0% (*n*=1)	50% (*n*=6)	82% (*n*=17)	85% (*n*=13)
Diptera	0% (*n*=1)	33% (*n*=6)	82% (*n*=17)	100% (*n*=13)
Hemiptera	0% (*n*=1)	50% (*n*=2)	67% (*n*=6)	100% (*n*=2)
Odonata	0% (*n*=1)	33% (*n*=3)	36% (*n*=11)	50% (*n*=10)
Coleoptera	0% (*n*=1)	0% (*n*=2)	64% (*n*=11)	60% (*n*=10)
Hydracarina	0% (*n*=1)	100% (*n*=1)	100% (*n*=1)	-
Pisces	0% (*n*=1)	0% (*n*=5)	33% (*n*=6)	83% (*n*=6)
Rotifera	0% (*n*=1)	0% (*n*=3)	0% (*n*=13)	0% (*n*=11)
Mollusca	0% (*n*=1)	0% (*n*=3)	0% (*n*=12)	0% (*n*=10)
Annelida	0% (*n*=1)	0% (*n*=2)	0% (*n*=11)	0% (*n*=6)
Turbellaria	0% (*n*=1)	0% (*n*=1)	0% (*n*=7)	0% (*n*=3)
Plants	0% (*n*=1)	0% (*n*=5)	0% (*n*=13)	8% (*n*=12)

Effects of acetylcholinesterase inhibitors

In stagnant test systems, clear effects (Classes 3, 4 and 5) are observed in the endpoint categories 'Microcrustaceans', 'Macrocrustaceans, 'Insects' and 'Fish' from about 0.1 TU_{mso} (Fig. 1 A-D). Effects are hardly ever observed at insecticide concentrations below 0.1 TU_{mso}. One exception forms a study on a chronic exposure to chlorpyrifos (Van den Brink et al., 1995). For the previously mentioned four categories, more or less clear concentration-effect relationships are present (Fig. 1 A-D). The data also show that single applications were studied most often (Fig. 1). Effects are more severe in studies with repeated or chronic applications (Fig. 1 A, C).

Clear effects on 'Rotifers', 'Other macroinvertebrates', and 'Algae & macrophytes' generally occur from concentrations of 1 TU_{mso} and higher (Fig. 1 E-G). Usually, effects in community metabolism endpoints were observed at concentrations around 10 TU_{mso} and higher (Fig. 1 H). This indicates that the structure of the aquatic community is more sensitive to acetylcholinesterase inhibitors than functional characteristics of the ecosystem.

Few studies with acetylcholinesterase inhibitors have been done in running waters. Results are not incorporated in Figure 1 because of the deviating exposure regimes. A pulse of six hours with a concentration of 0.08 TU_{mso} chlorpyrifos had no effect on the abundance of fauna in experimental streams (Pusey et al., 1994). A clear effect on insect populations was observed in the same study for an equally long application of 3.85 TU_{mso}, after which recovery of the reduced populations occurred within eight weeks. Courtemanch and Gibbs (1980) found a clear decrease in the abundance of Plecoptera and Ephemeroptera for carbaryl in streams at a nominal pulse concentration of 5.7 TU_{mso}. Morrison and Wells (1981) studied pulse applications of fenitrothion in streams. At 0.1 TU_{mso} they found no effect at all, and at 1.7 TU_{mso} only a slight effect, especially in the form of drift of insects. Thus, the results of the lotic systems do not seem to differ very much from that of lentic systems with regard to the direct impact of acetylcholinesterase-inhibitor concentrations.

Effects of synthetic pyrethroids

The microcosm and mesocosm studies with pyrethroids in particular, concern effects of repeated applications in stagnant water. Effects are observed in the categories 'Microcrustaceans' and 'Insects' from about 0.01 TU_{mso} and higher (Fig. 2 A, C). In the range 0.01-0.1 TU_{mso} they relate especially to slight effects (Class 2). At higher exposure concentrations, in the range 0.1-1 TU_{mso}, clear effects (Classes 3, 4 and 5) are regularly reported for 'Microcrustaceans', 'Macrocrustaceans' and 'Insects', while for 'Fish' slight effects are reported in a limited number of studies (Fig. 2 A-D). In some studies, clear effects at concentrations lower than 1 TU_{mso} are also reported for the category 'Rotifers (Fig. 2 E). At concentrations higher than 1 TU_{mso}, effects can be observed in all categories of structural endpoints (Fig. 2 A-G).

After repeated exposure to pyrethroids and at final peak concentrations higher than 0.1 TU_{mso}, long-term (> 8 weeks after last application) effects on – in particular – crustaceans and insects cannot be excluded (Fig.2). The pyrethroid studies also indicated that the structure of the aquatic community is more sensitive to insecticides than functional characteristics of the ecosystem (Fig. 2 A-G vs 2 H).

Responses of the most sensitive endpoints

In a few cases, results clearly deviated from the general concentration-effect relationships for the sensitive endpoint categories 'Microcrustaceans', 'Macrocrustaceans', 'Insects', and 'Fish' (Figs 1 and 2). For example, in the study of Lahr and Diallo (1993) with fenitrothion, macrocrustaceans responded by a factor of 10 – 100 times less sensitive than in the other studies (No effects (Class 1) at 7.3 TU_{mso} in Fig. 1 B).

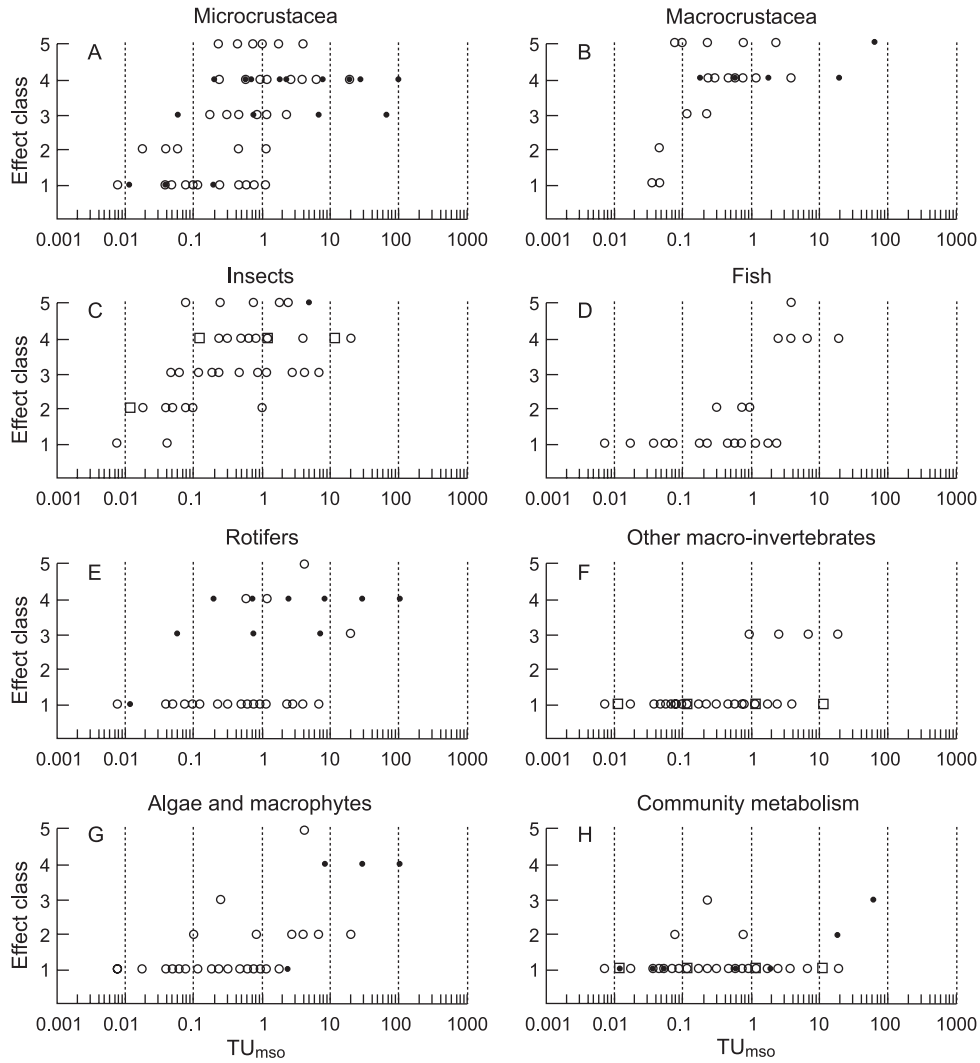

Fig. 2. Effects of insecticides with synthetic pyrethroids in microcosm and mesocosm studies. The figure includes observations of studies in stagnant water (single and multiple applications), and of chronic applications in stagnant as well as running water test systems. Effects are classified into several categories, structural endpoints (A – G) and a functional category (community metabolism; H). The effects are also classified (Effect class) according to magnitude and duration. 1 = no significant effect, 2 = slight effect, 3 = clear short-term effect (< 8 weeks), 4 = clear effect in short-term study (recovery moment unknown), 5 = clear long-term effect (> 8 weeks). Closed circles (•) indicate experiments with a single application. Open circles (○) and squares (□) indicate experiments with multiple applications or chronic exposure, respectively.

In this study, macrocrustaceans were only represented by the anostracan taxon *Streptocephalus* spp. which is relatively insensitive to fenitrothion. Overall, however, the study did not necessarily give deviating information on the ecological effects in the field because sensitive groups, in the form of insects and microcrustaceans, were still present. Lahr et al. (1995) studied the effects of a single application of deltamethrin at one relatively high concentration only (67.5 TU_{mso}). Here, Anostraca was shown to be the most sensitive group (Class 5, Fig. 2 B) while the short-cyclic cladocerans (Class 3, Fig. 2 A) and inflying hemiptera (Class 3, Fig. 2 C) rapidly recolonized the treated natural ponds.

To reduce the emphasis on slight effects, and to focus on the realistic worst-case scenario of the effects observed in the microcosm and mesocosm studies, we selected the most sensitive endpoints of each study and plotted observed effects against studied concentrations (Fig. 3). In the case of single applications, effects on the most sensitive endpoints are not usually observed at concentrations of $\leq$ 0.1 TU_{mso} (Fig.3A). At higher doses, slight to clear effects may be expected. In the case of microcosm and mesocosm studies, which typically simulate isolated water systems, there is a good chance that recovery of sensitive endpoints takes longer than eight weeks (Class 5 effects) at single doses resulting in exposure concentrations of 1 TU_{mso} and higher (Fig. 3A).

For repeated and chronic exposures, concentrations below 0.01 TU_{mso} have rarely been the subject of studies (Fig. 3B and C). Nevertheless, the results show that below 0.01 TU_{mso}, it is unlikely for any clear effects to be expected. Within the concentration range 0.01-0.1 TU_{mso} mainly slight (Class 2) to short-term clear effects (Class 3) are reported for the most sensitive endpoints. Above 0.1 TU_{mso}, clear and prolonged effects (Class 5) are to be expected in test systems that are repeatedly or chronically stressed with insecticides.

Regression analysis indicates that when comparing Class 1 effects with Classes 3, 4 and 5 effects, single applications at concentration levels of 0.13 TU_{mso} can be expected to induce clear effects (Classes 3 to 5) in the field in 50% of cases (Table 6). There is a small probability (FEC-5%) that effects occur at concentrations below 0.05 TU_{mso} (FEC-5%: Field Effect Concentrations which will affect the most sensitive endpoints with a probability of 5%). There is a high probability (FEC-95%) that clear effects will occur in microcosm and mesocosm situations at concentrations of 0.34 TU_{mso} and higher.

For the situation where we include slight effects (Class 2) in the 'no-effect class', FEC-50% for single applications increases to 0.26 TU_{mso} (Table 6). FEC-5%, however, stays more or less at the same concentration level, i.e. 0.04 TU_{mso} against 0.05 TU_{mso} in the previous scenario.

Regarding multiple or chronic applications, effects can be expected to occur at lower concentrations (Table 6). FEC-50% levels were 16 to 33% of those for single applications. Differences between multiple and chronic exposures were less significant

(Table 6). Probability calculations for chronic FECs, however, were less accurate since much less data were available (Table 6: no calculation possible; high range confidence limits). Nevertheless, it means that for an adequate risk analysis it is at least desirable to distinguish between exposure regimes resulting from single applications on the one hand, and that of multiple/chronic applications on the other.

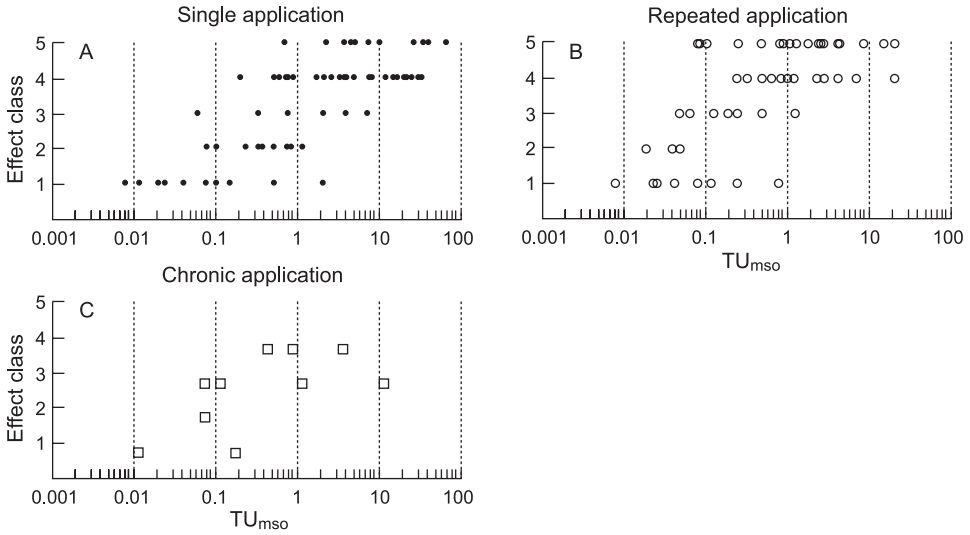

Fig. 3. Responses of the most sensitive endpoint in microcosm and mesocosm studies performed with acetylcholinesterase inhibiting or pyrethroid insecticides, based on the data presented in Figures 1 and 2. The effects on the most sensitive endpoints are presented for a single application (A), multiple applications (B), and chronic exposure (C). The effects are also classified (Effect class) according to magnitude and duration. 1 = no significant effect, 2 = slight effect, 3 = clear short-term effect (< 8 weeks), 4 = clear effect in short-term study (recovery moment unknown), 5 = clear long-term effect (> 8 weeks).

Table 6. Field Effect Concentrations (FEC) as calculated by means of logistic regression. FECs, with 95%-confidence limits, are expressed in TU_{mso}. FECs were expressed as 5, 50 and 95 percentages of probability of effects occurring on the most sensitive endpoints for achetylcholinesterase inhibiting and pyrethroid insecticides (Fig. 3). FECs were calculated for two scenarios; one where no effects are placed against clear effects (Effect Class 1 versus Effect Classes 3, 4 and 5) and one where no and slight effects are placed against clear effects (Classes 1 and 2 versus Classes 3, 4 and 5). Results were based on responses found in studies using single, multiple and chronic insecticide applications. x = no calculation possible due to a lack of data.

		Estimate	(95%-Confidence limits)
No Effects vs Clear Effects			
Single	FEC5%	0.049	(0.016 - 0.154)
	FEC50%	0.130	(0.068 - 0.249)
	FEC95%	0.341	(0.093 - 1.257)
Multiple	FEC5%	0.016	(0.003 - 0.095)
	FEC50%	0.043	(0.020 - 0.094)
	FEC95%	0.118	(0.043 - 0.320)
Chronic	FEC5%	x	(x - x)
	FEC50%	x	(x - x)
	FEC95%	x	(x - x)
No & Slight Effects vs Clear Effects			
Single	FEC5%	0.036	(0.007 - 0.198)
	FEC50%	0.261	(0.126 - 0.541)
	FEC95%	1.862	(0.502 - 6.914)
Multiple	FEC5%	0.023	(0.007 - 0.070)
	FEC50%	0.052	(0.032 - 0.085)
	FEC95%	0.119	(0.050 - 0.284)
Chronic	FEC5%	0.003	(0.000 - 4.868)
	FEC50%	0.043	(0.003 - 0.665)
	FEC95%	0.544	(0.010 - 29.01)

Table 7. $NOEC_{eco}$ and $LOEC_{eco}$ values (µg/L) for microcosm and mesocosm studies with single or multiple applications of acetylcholinesterase-inhibiting insecticides. $LOEC_{eco}$ values are divided into slight effects (Class 2) and more severe effects (Classes 3 to 5). $NOEC_{eco}$ represents the 'no effect class'(Class 1).

Active ingredient	Dose	$NOEC_{eco}$ (Class 1)	$LOEC_{eco}$ (Class 2)	$LOEC_{eco}$ (Class 3,4,5)	Reference
Stagnant water systems					
Azinphos-methyl	single	0.2	0.72	--	Stay & Jarvinen, 1995
	single	0.2	--	1.0	Knuth et al., 1992
	single	--	--	1.0	Tanner & Knuth, 1995
	multiple	0.22	--	0.95	Giddings et al., 1994
Chlorpyrifos	single	0.1	0.3	1.0	Biever et al., 1994
	single	0.1	--	0.9	Van den Brink et al., 1996
	single	0.1	--	1.0	Van Wijngaarden et al., 2005b
	single	--	--	0.5	Brazner *et al.*, 1989; Siefert et al., 1989; Brazner & Kline, 1990
	single	--	0.5	5	Stay et al., 1989
	single	--	--	5	Brock et al., 1992a, b, 1993
	single	--	--	10	Hughes et al., 1980
	single	--	--	35	Van Donk et al., 1995; Brock et al 1995b; Cuppen et al., 1995
	continuous	--	--	0.1	Van den Brink et al., 1995
Diazinon	multiple	--	--	2.4	Giddings et al., 1996
Fenitrothion	single	--	--	80	Lahr & Diallo, 1993
	multiple	--	--	14.3	Fairchild & Eidt, 1993
Parathion-ethyl	continuous	0.2	--	0.5	Dortland, 1980
Parathion-methyl	single	--	--	10	Crossland, 1988
	single	--	--	100	Crossland, 1984
Phorate	single	--	--	23	Dieter et al., 1996
Bendiocarb	single	--	--	24	Lahr et al., 1995
Carbaryl	single	--	2	20	Havens, 1994, 1995
Carbofuran	single	5	--	25	Wayland, 1991
Running water systems					
Chlorpyrifos	single	0.1	--	5	Pusey et al., 1994
	continuous	--	--	0.1	Ward et al., 1995
Fenitrothion	single	1.1	--	18.7	Morrison & Wells, 1981
	single	--	--	30.8	Poirier & Surgeoner, 1988
	single	--	--	460	Yasuno et al., 1981
Carbaryl	single	--	--	34	Courtemanch & Gibbs, 1980

Comparing $NOEC_{eco}$ with regulatory criteria

For the acetylcholinesterase inhibitors, most LOECs from the reviewed studies were in Classes 3 to 5 (Table 7). $NOEC_{eco}$s could be derived for five acetylcholinesterase inhibitors, and Class 2- $LOEC_{eco}$s for three compounds (Table 7). These usually concerned exposure regimes resulting from single applications. Comparing $NOEC_{eco}$s with first tier Uniform Principles (UP) criteria (EU, 1997) shows that these NOEC values were about a factor of 10 or more, higher than set acceptable concentrations (Table 8).

Most of the pyrethroid studies also yielded Classes 3 to 5-$LOEC_{eco}$ values only (Table 9). A $NOEC_{eco}$ could be derived for three pyrethroids. These NOECs did not deviate much from the first tier UP criteria (Table 10). NOECs were equal to, or less than, a factor of five higher than set safety criteria. Hence, the margin between UP criteria and $NOEC_{eco}$s observed in the field was less for synthetic pyrethroids than for acethylcholinesterase inhibitors. This can be explained by the fact that some non-target organisms in the field are relatively more sensitive to pyrethroids than to acetylcholinesterase inhibitors, at least when compared with the standard test species of *Daphnia* and fish (Schroer et al., 2004).

Overall, the established $NOEC_{eco}$s indicate that set safety factors and criteria for protecting aquatic organisms as described in the EU Uniform Principles seem to be adequate for both groups of insecticides, and possibly over-protective for single applications acetylcholinesterase inhibitors.

In this paper we specifically focussed on the regulatory implications of the outcome of model ecosystem studies for first tier risk assessment procedures as applied in the EU. Like the EU-member states, many other countries from all over the world use OECD guidelines for toxicity testing and apply safety factors in one way or another as a first step in aquatic risk assessment (e.g., US-EPA, 1998). In the case of acetylcholinesterase inhibitors and synthetic pyrethroids, the OECD standard test species *D. magna* and standard test fishes were good representatives of sensitive species in the field. When one accepts to rank toxicity of acetylcholinesterase inhibitors and synthetic pyrethroids to these standard species, then exposure – toxicity ratio methods like for example applied in the USA (hazard quotient method (Urban and Cook 1986)), also seem to be protective towards aquatic ecosystems.

General discussion and conclusions

The ecological risk of eighteen insecticides in freshwater ecosystems is discussed in this paper. They form 15% of the 123 pesticides with similar modes of action that are, or were, available on the market for agricultural pest management programmes (Tomlin, 2000). Nevertheless, given the range of responses reported among these pesticide studies, they appear to represent general ecological effects for acetylcholinesterase inhibitors and synthetic pyrethroids in aquatic ecosystems (Fig. 3).

Table 8. Summarised $NOEC_{eco}$ and $LOEC_{eco}$ values for acetylcholinesterase-inhibiting insecticides in microcosm and mesocosm studies. Concentrations in µg/L. First tier acceptable concentrations (UP) were derived from the EU-Uniform Principles (Table2). $LOEC_{eco}$ values are divided into slight effects (Class 2) and more severe effects (Classes 3 to 5). $NOEC_{eco}$ represents the 'no effect class'(Class 1). Cl = Class. TUR shows the $NOEC_{eco}$ or $LOEC_{eco}$ - first tier acceptable concentration ratio (Toxicity - UP Ratio).

Active ingredient	Exposure regime	UP	Actual nominal concentrations			TUR		
			$NOEC_{eco}$ (Cl 1)	$LOEC_{eco}$ (Cl 2)	$LOEC_{eco}$ (Cl 3-5)	$NOEC_{eco}$ (Cl 1)	$LOEC_{eco}$ (Cl 2)	$LOEC_{eco}$ (Cl 3-5)
Stagnant water systems								
Azinphos-methyl	single	0.02	0.2	0.72	1	10	36	50
	multiple	0.02	0.22	--	0.95	11	--	48
Chlorpyrifos	single	0.013	0.1	0.3	0.5	7.7	23.1	38.5
	continuous	0.013	--	--	0.1	--	--	7.7
Diazinon	multiple	0.01	--	--	2.4	--	--	240
Fenitrothion	single	0.11	--	--	80	--	--	727
	multiple	0.11	--	--	14.3	--	--	130
Parathion	continuous	0.011	0.2	--	0.5	18	--	45.5
Parathion-methyl	single	0.014	--	--	10	--	--	714
Phorate	single	0.015	--	--	23	--	--	1533
Bendiocarb	single	0.74	--	--	24	--	--	32.4
Carbaryl	single	0.056	--	2	20	--	35.7	357
Carbofuran	single	0.33	5	--	25	15	--	76
Running water systems								
Chlorpyrifos	single	0.013	0.1	--	5	7.7	--	385
	continuous	0.013	--	--	0.1	--	--	7.7
Fenitrothion	single	0.11	1.1	--	18.7	10	--	17
Carbaryl	single	0.056	--	--	34	--	--	607

Normalisation of reported field concentrations to TU_{mso} enables a comparison to be made between studies with insecticides that have working mechanisms in common. The use of TU_{mso} has been shown to be an adequate reference for estimating field responses due to direct toxic effects. It should be kept in mind, however, that for these compounds standard species are relatively good representatives of sensitive species. If standard species are not representative of the sensitive taxonomic groups, then the choice of TU_{mso} will be less successful.

The studies were done in various parts of the world and under various experimental conditions. However, $NOEC_{eco}$s and Class 2-$LOEC_{eco}$s were still shown to be very consistent regardless of study location, at least when similar exposure regimes are considered (Table 11). Leeuwangh (1994) compared the outcome of various microcosm and mesocosm studies done with chlorpyrifos. He concluded that direct effects on susceptible species are often concentration-related and not dependent on system scale or geographical location. Considering the consistency of the threshold values of several compounds (Table 11) this conclusion seems to be applicable to other pesticides as well.

Table 9. $NOEC_{eco}$ and $LOEC_{eco}$ values (µg/L) for microcosm and mesocosm studies with single or multiple applications of a pyrethroid insecticide. $LOEC_{eco}$ values are divided into slight effects (Class 2) and more severe effects (Classes 3 to 5). $NOEC_{eco}$ represents the 'no effect class'(Class 1).

Active ingredient	Dose	$NOEC_{eco}$	$LOEC_{eco}$ (Class 2)	$LOEC_{eco}$ (Class 3,4,5)	Reference
Stagnant water systems					
Cyfluthrin	multiple	--	--	0.036	Johnson et al., 1994; Morris et al., 1994
Cypermethrin	multiple	--	--	0.07	Farmer et al., 1995
	multiple	--	--	0.16	Hill, 1985
Deltamethrin	single	--	--	0.2	Morrill & Neal, 1990
	single	--	--	2.7	Lahr et al., 1995
Esfenvalerate	single	0.01	0.05	0.15	Stay & Jarvinen, 1995
	multiple	0.01	--	0.25	Webber et al., 1992
	multiple	--	0.01	0.08	Lozano et al., 1992
	multiple	--	--	0.25	Fairchild et al., 1992b
Fenvalerate	single	0.01	--	0.05	Day et al., 1987
Lambda-cyhalothrin	multiple	0.0016	--	0.016	Hill et al., 1994b
	multiple	--		0.017	Farmer et al., 1995
	multiple	--	0.01*	0.025	Roessink et al., 2005 Van Wijngaarden et al., 2005a
	multiple	--	0.01*	0.025	Roessink et al., 2005
	multiple	--	0.01*	0.025	Van Wijngaarden et al., 2005a
Permethrin	single	--	--	0.5	Kaushik et al., 1985
Tralomethrin	multiple	--	0.0027	0.0092	Mayasich et al., 1994
Running water systems					
Fenvalerate	continuous	--	0.01	0.1	Breneman & Pontasch,1994

* Longer-term effects on one pre-dominant species. For the community as a whole, NOECs calculated were 0.01 µg/L.

In the case of acetylcholinesterase inhibitors and pyrethroids, Arthropoda contain the species most sensitive to these compounds. In the different types of ecosystems, both natural and model, sensitive representatives of this group are usually available and generally form a predominant part of aquatic communities. This overall presence of one or a few sensitive taxa in microcosm and mesocosm studies carried out with these types of insecticides, explains why such studies have a certain robustness and a general predictive value for ecological risk assessment in the field.

Table 10. Summarised $NOEC_{eco}$ and $LOEC_{eco}$ values from studies with pyrethroids in microcosm and mesocosm experiments. Concentrations in µg/L. First tier acceptable concentrations (UP) were derived from the EU-Uniform Principles (Table2). $LOEC_{eco}$ values are divided into slight effects (Class 2) and more severe effects (Classes 3 to 5). $NOEC_{eco}$ represents the 'no effect class'(Class 1). Cl = Class. TUR shows the $NOEC_{eco}$ or $LOEC_{eco}$ - first tier acceptable concentration ratio (Toxicity - UP Ratio).

Active ingredient	Exposure regime	UP	Actual nominal concentrations			TUR		
			$NOEC_{eco}$ (Cl 1)	$LOEC_{eco}$ (Cl 2)	$LOEC_{eco}$ (Cl 3-5)	$NOEC_{eco}$ (Cl 1)	$LOEC_{eco}$ (Cl 2)	$LOEC_{eco}$ (Cl 3-5)
Stagnant water systems								
Cyfluthrin	multiple	0.0015	--	--	0.036	--	--	24
Cypermethrin	multiple	0.0068	--	--	0.07	--	--	10
Deltamethrin	single	0.0004	--	--	0.2	--	--	500
Esfenvalerate	single	0.0025	0.01	0.05	0.15	4	20	60
	multiple	0.0025	0.01	0.01	0. 08	4	4	32
Fenvalerate	single	0.008	0.01	--	0.05	1.25	--	6.25
Lamda-cyhalothrin	multiple	0.0021	0.0016	0.01	0.025	0.76	4.8	11.9
Permethrin	single	0.0065	--	--	0.5	--	--	77
Tralomethrin	multiple	0.0015	--	0.0027	0.0092	--	1.8	6.1
Running water systems								
Fenvalerate	continuous	0.008	--	0.01	0.1	--		12.5

Above threshold levels, studied endpoints show wide concentration ranges (in TU) per effect class between experiments. For example, concentrations inducing Class 3 effects ranged over approximately two orders of magnitude in TU for the frequently measured endpoints 'Microcrustaceans' and 'Insects' (Figs. 1 and 2). This high variability relates to ecological properties of the test systems, the experimental set-up and frequency of observations used, organisms studied and taxonomic level of identification, and ecotoxicological profile of the insecticides. Differences in environmental behaviour of the insecticides, resulting in differences in bioavailability, can be expected to be another source of observed variation in response concentrations.

Only a limited number of studies appeared to be suitable for validation of the first tier risk assessment criteria. $NOEC_{eco}$ values could be established for eight compounds only. Many of the studies were simply not designed to give this type of information. Obtained $NOEC_{eco}$s and Class 2-$LOEC_{eco}$ data, however, suggest that the safety factors as calculated in this paper generally offer aquatic organisms and ecosystem functions adequate protection against adverse effects related to usage of organophosphorous and pyrethroid insecticides. These studies also show that it seems to be significant to distinguish between exposure regimes; for a single application of non-persistent insecticides it seems possible to be a factor of ten more lenient than for repeated and chronic exposures to the same chemicals.

Table 11. Threshold concentrations ($NOEC_{eco}$/Class2-$LOEC_{eco}$) in relation to experimental set-ups and locations of model ecosystem studies with several insecticides.

Compound	Dose	Experiment	Location	NOECeco or Class 2-LOECeco (µg/L)	References
Azinphos-methyl	single	microcosms	lab	0.2	1
Azinphos-methyl	single	littoral enclosures	USA Minnesota	0.2	2
Chlorpyrifos	single	outdoor microcosms	USA Kansas	0.1	3
Chlorpyrifos	single	experimental ditches	NL	0.1	4
Chlorpyrifos	single	microcosms simulating Mediterranean conditions	lab	0.1	5
Esfenvalerate	multiple	outdoor mesocosms	USA Alabama	0.01	6
Esfenvalerate	multiple	littoral enclosures	USA Minnesota	0.01	7
Lambda-cyhalothrin	multiple	outdoor mesocosms	USA N-Carolina	0.002	8
Lambda-cyhalothrin	multiple	plankton-dominated enclosures	NL	0.01	9
Lambda-cyhalothrin	multiple	macrophyte-dominated enclosures	NL	0.01	9
Lambda-cyhalothrin	multiple	enclosures, spring vs late-summer	NL	0.01	10

1: Stay and Jarvinen (1995). 2: Tanner and Knuth (1995). 3: Biever et al. (1994). 4: Van den Brink et al. (1996). 5: Van Wijngaarden et al. (2005b). 6: Webber et al. (1992). 7: Lozano et al. (1992). 8: Hill et al. (1994b). 9: Roessink et al. (2005). 10: Van Wijngaarden et al. (2005a)

The most sensitive endpoints for direct effects of the insecticides studied were structural ecosystem characteristics and usually concerned population densities of crustaceans and insects. These direct effects can generally be well predicted on the basis of laboratory tests with similar species as studied in the microcosm and mesocosm experiments (e.g., Crossland and Wolff, 1985; Fairchild et al., 1992a; Van Wijngaarden et al., 1996; Maund et al., 1998; Sheratt et al., 1999; Schroer et al., 2004). Different studies conducted with the same insecticide (e.g., chlorpyrifos, esfenvalerate, lambda-cyhalothrin) also yield similar critical threshold values (Tables 7 and 9). This may imply that $NOEC_{eco}$s and Class 2-LOECs of adequate model ecosystem studies can be used to validate the cut-off values such as the HC_5 or HC_{10} values of Species Sensitivity Distribution curves (Solomon et al., 2001; Van den Brink et al., 2002a; Postuma et al., 2002) based on laboratory tests with standard and additional species. As it cannot be excluded that taxa that may be sensitive to a pesticide in a natural system are not screened in the laboratory because they are not easily cultured, held or tested.

Indirect effects of insecticides seem to be much more variable (e.g., Leeuwangh, 1994; Brock et al., 1992b, 2000b). Such types of effects are steered more by experimental conditions and stochastic processes than in the case of direct effects.

However, when indirect effects were summarised, general response patterns could be recognised (Table 12). The studies show that the frequency of reported indirect effects increased with increasing concentrations. Indirect effects on functional endpoints were less frequently reported, which on the one hand supports the idea that functional aspects of the ecosystems are less sensitive to toxic stress by compounds studied. On the other hand, however, it cannot be excluded that functional endpoints have been less frequently reported because they are not often measured in these types of studies. Indirect effects on structural endpoints are to be expected from exposure concentrations in the range of 0.1-1 TU_{mso} and higher (Table 12). Although it seems difficult to predict accurately which specific species will suffer indirect effects due to insecticide stress, aggregation of biological taxa into functional groups allows food-web modelling and the prediction of overall ecological responses that will follow direct toxic effects (Traas et al., 1998; Baird et al., 2001).

Table 12. Indirect effects summarised from studies in stagnant waters after a single application of an organophosphorous insecticide, a carbamate, or a pyrethroid. The nominal concentrations reported in the studies are expressed in TU_{mso}.

Range TU_{mso}	**Structural aspects**		**Functional aspects**	
	Shifts in animal populations	Shifts in algae and higher plants	Decrease in decomposition	Shifts in community metabolism
10-100	X[1,3,4,5,8,9,10]	X[4,5,8,10]	X[3,4,5]	X[3,4]
1-10	X[1,2,3,4,6,7,10,13,14]	X[1,10, 14]		X[14]
0.1-1	X[1,2,11,13,14]	X[1,14]		X[14]
0.01-0.1	X[12]			

Organophosphorous compounds

[1] Siefert et al. '89; Brazner and Kline '90
[2] Biever et al. '94
[3] Van den Brink et al. '96; Kersting and Van denBrink '97
[4] Brock et al. '92[a]; '92[b]; '93
[5] Van Donk et al. '95; Brock et al. '95[b]; Cuppen et al. '95
[6] Hughes et al. '80
[7] Fairchild & Eidt '93
[8] Crossland '84
[9] Crossland '88
[14] Van Wijngaarden et al., '05b

Carbamates

[10] Havens '95
[11] Wayland '91

Pyrethroids

[12] Day et al. '87
[13] Kaushik et al. '85

Many of the studies evaluated were stopped before recovery times of sensitive populations could be established (Class 4 observations in Figs. 1 and 2). Nevertheless, on the basis of the remaining studies, a general picture of the recovery of sensitive invertebrates can be given. In stagnant waters sensitive species having short life-cycles (microcrustaceans), usually recovered within eight weeks after a single exposure of less than 10 TU_{mso} (Fig. 1A). Fewer data are available on the recovery rate in systems that are repeatedly exposed. Fig. 2A, however, suggests that also after repeated applications, recovery generally occurs within eight weeks of the last application as long as this last application was less than 10 TU_{mso}.

Duration of effects and recovery of stressed ecosystems is an important issue in higher tier risk evaluation (Campbell et al., 1999). Testing pesticides in outdoor (model) ecosystems has the advantage that this type of research may provide information on the recovery of the systems after pesticide contamination has ceased. Actual recovery of sensitive populations depends on the instant that concentrations reach non-toxic levels again, in combination with an array of biological and ecological characteristics (e.g., Giesy et al., 1999; Brock and Budde, 1994; Lahr et al., 2000). The microcosm and mesocosm studies demonstrate that recovery after pesticide contamination is expected to be rapid in the real world when (a) the compound is not persistent, (b) the physicochemical environment is not altered, or is quickly restored, (c) the generation times of vulnerable populations are short, and/or (d) when there is immigration from residual populations in nearby unaffected areas.

Model ecosystems are generally of smaller dimensions than the aquatic ecosystems they aim to simulate. In addition, model ecosystem studies are restricted in duration (of the 51 evaluated studies, 26 lasted from two to six months, three made observations in the following growing season, and the rest lasted for less than two months after the (last) treatment). Because of these characteristics, it may be expected that organisms on the microscale (e.g., plankton) are better adapted to dimensions and time-scale of the experiments than organisms on the macroscale (e.g., macroinvertebrates, fish). Hence, predominantly small-sized species, especially when they also have short generation times (e.g., plankton, multi-voltine invertebrates), have an ecological advantage over other life-history traits in these types of studies. It should therefore be taken into account in the interpretation of effects and recovery of species from model ecosystem studies whether or not the experimental circumstances provide unrestricted conditions for studying the effects and recovery of species of interest.

This review of ecological effects studied under quasi-natural conditions shows that some estimations of direct effects in the field can be made by taking the acute EC_{50} of the most sensitive standard test species (TU_{mso}) as a reference concentration, and by classifying the effects. Modelling the observed responses of the most sensitive endpoints (Fig. 3) provides a way of extrapolating results of microcosm and mesocosm observations to probabilities of effect occurrences in the field at predicted or measured environmental concentrations (Table 6). Using the same regression

model, the outcome of low risk concentration values can be varied by choosing either a strict, or a more lenient, scenario (considering Effect Class 1 only or, considering Effect Classes 1 and 2 as the 'no-effect' classes). Other options are to choose higher or lower probability levels (e.g. FEC5% or FEC10%) as a criterion and/or take the lower 95% confidence limit into account to set safe concentrations.

Recently, this approach has been further explored by developing the empirical model PERPEST (Van den Brink et al., 2002b). PERPEST makes use of the database described in this paper, and that of microcosm and mesocosm data of insecticides that have other modes of action (Brock et al., 2000b), plus that of herbicides (Brock et al., 2000a), to predict ecological effects of pesticides on freshwater ecosystems. The PERPEST model searches for situations in the database that are analogous to a case in question.

Our effect classification system was shown to be helpful in evaluating treatment-related effects of different insecticides as observed in various ecosystem experiments that were made available in the open literature. The effect classification system can be equally well-applied in future higher tier risk evaluations. Recently, it was advocated that protection goals should be formulated more specifically and to specify more clearly what must be considered as 'unacceptable damage' to the ecosystem (Van Dijk et al., 2000; Giddings et al., 2002). When site-specific protection goals, and consequently target images become available, the effect classification system can be of help in the decision-making process. In this context, the classification system may be used to derive more than one 'regulatory acceptable concentration'. Eco-ethical principles may be used to derive acceptable concentrations in a landscape-ecological context, e.g., dependent on the functionality and vulnerability of the freshwater ecosystem concerned (see, e.g., Brock, 2001). Defining effect classes and differentiated protection goals, also has the advantage that the different stake-holders involved in the process of authorising pesticides, can discuss more transparently the decision-making of 'Ecologically Acceptable Concentrations (EACs)' from model ecosystem studies.

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8 Use of model ecosystems for risk evaluations

Role of model ecosystems in risk evaluation

With regard to regulatory risk assessment frameworks, higher-tier approaches are important steps in the risk assessment process (e.g. Campbell et al., 1999; European Commission, 2002). Data from higher-tier studies reduce the uncertainty associated with the conservative first tier risk characterisation since they bring more realistic information into the risk evaluation. This refined information can then be used to re-evaluate the conclusions of a first tier risk assessment. On the proviso that micro- and mesocosms comprise of complex natural assemblages and are appropriately designed, these studies are considered suitable to estimate community NOECs and no observed ecologically adverse effect concentrations (NOEAEC; sorry for this unpronounceable acronym) for these individual studies (Campbell et al., 1999). On the basis of these community NOECs or NOEAECs, and other information available, an uncertainty factor may be applied to yield the so-called Ecologically Acceptable Concentration (EAC) (European Commission, 2002). The EAC is the regulatory acceptable concentration for pesticides, i.e., the maximum concentration that is permissable in aquatic environments resulting from the agricultural use of plant-protection products. Where NOEAECs are used as the reference for applying the uncertainty factor, these are often set on Class 2 or on Class 3 effects (as defined in Chapter 7). The choice of which effect class to use may depend on the time-span of effects considered acceptable and on the protection level considered relevant by authorities (Campbell et al., 1999).

Safety factors in the context of experimental variation of individual compounds

Currently, uncertainty factors applied to model ecosystem-generated threshold levels for setting regulatory acceptable concentrations range from 1 (US EPA) to a variable factor, which is based on a case-by-case evaluation of studies (EU) (Solomon, 2005/in prep). The review of the model ecosystem studies (Chapter 7) summarises threshold levels and effects of a broad spectrum of micro- and mesocosm experiments. These studies represent spatio-temporal and experimental variation as all of these studies were performed under different conditions (different types of systems, different locations, different climates, different seasons) and therefore may be of use to derive an observation-based uncertainty factor. This factor may then be applied as an extrapolation factor to set regulatory acceptable concentrations.

It appears from the model ecosystem experiments performed with similar compounds that, regardless of type of test system, concentrations specifically in the range of 'no' to 'slight and transient' effects (Effect Class 1 - 2) are remarkably

consistent (Table 1 and 2). Remarkably, because it is known that even in standardized laboratory single species tests with similar compounds, variability in responses may be a factor of 3 or more (Sprague, 1985; Baird et al., 1989). On the one hand, this consistency in the findings indicates that the threshold level for 'no to slight effects' earns confidence as an indicator of safe concentrations in the field (at least, when studies contain representatives of sensitive taxonomic groups and when exposure regimes are more or less similar). On the other hand, it appears from the data presented in Tables 1 and 2 that the margin between the Effect Classes 2 and 3 is only a factor of 2.

When Class 3 effects are considered as NOEAECs, then an uncertainty factor is certainly needed. The chlopyrifos studies show that concentrations resulting in Class 3 effects may also lead to more severe effects in other experiments (Effect Class 4 – 5) (Table 1). The lambda-cyhalothrin studies gave similar information; concentrations causing Class 3 effects also caused Class 4 and Class 5 effects in other studies (Table 2). Consequently, the studies with single applications of chlorpyrifos in lentic test systems suggest that an extrapolation factor of 2 would suffice to make it likely that a Class 3 NOEAEC of a single study will not result in Class 4 – 5 effects in other, untested, systems. And, in case of multiple applications with lambda-cyhalothrin, an extrapolation factor of 4 probably would avoid Class 4 – 5 effects in other systems.

The studies discussed above indicate that a precautionary extrapolation factor of 2 to 4 may be sufficient when Class 3 NOEAECs are used as the reference for the determination of the regulatory acceptable concentration for short-term exposures to non-persistent insecticides. These factors, however, are not necessarily generally applicable. For example, Brock et al. (EXPECT/2005) determined uncertainty factors for a broader array of compounds, namely, the herbicide atrazin, the metal copper and the surfactants dodecyl trimethyl ammonium chloride (C_{12} TMAC) and linear alkylbenzene sulfonate (LAS). In these cases, the uncertainty factors were based on the spread (ratio of the upper and lower limit of the 95% confidence interval) as a measure of the variability of threshold concentrations after long-term exposures. The outcome was that uncertainty factors ranged from 1.4 to 5.4 (C_{12} TMAC: 1.4; Cu: 1.8; atrazine; 2.5; LAS: 5.4). Blanck et al. (2003) studied variability in zinc tolerance in periphyton communities sampled from 15 European river stretches. The regional uncertainty factor, based on the spread, ranged from 1.7 to 4.3 and when extrapolating from river to river, the uncertainty factor ranged from 2.4 to 8.6. Nevertheless, although these factors may vary depending on factors like the compound, the exposure regime, and the choice of Class 2 or Class 3 NOEAECs as the reference level, extrapolation for experimental variation in many cases seems to be covered with a factor of 3.

Besides accounting for experimental variation, additional considerations for setting an extrapolation factor for deriving regulatory acceptable concentrations might be (1), the incorporation of the protection goals that should be met, and (2), giving

weight to the information quantity and quality provided by model ecosystem study under concern.

Table 1. Effect concentrations (µg/L) in relation to experimental designs and locations of model ecosystem studies with chlorpyrifos. Effects resulted from short-term exposures. Effect Class 1: no treatment related effects demonstrated; Effect Class 2: slight transient effects; Effect Class 3: clear short-term effects on sensitive endpoints, recovery within 8 weeks after the application; Effect Class 4: clear effects, no full recovery at end of study; Effect Class 5: clear effects, recovery taking longer than 8 weeks after the application (see Chapter 7 for details of effect classification).

Application regime	Effect Class 1	Effect Class 2	Effect Class 3	Effect Class 4	Effect Class 5	Test system	Location Reference
6 h pulse	0.1	--	(5.0)*	--	--	experimental streams	Australia Pusey et al., 1994
single	0.1	0.3	1.0	--	3.0	outdoor microcosms	Kansas, USA Biever et al., 1994
single	0.1	--	-	--	0.9	experimental ditches	Netherlands Van den Brink et al., 1996
single	0.1	--	1.0	10	--	microcosms; eutrophic; *c.* 16 °C	Indoor Van Wijngaarden et al., 2005
single	0.1	--	1.0	--	--	microcosms; eutrophic; *c.* 26 °C	Indoor Van Wijngaarden et al., 2005
single	0.1	--	--	1.0	--	microcosms; hypertrophic; *c.* 26 °C	Indoor Van Wijngaarden et al., 2005
single	--	--	0.5	6.3	--	pond enclosures	Minnesota, USA Siefert et al., 1989
single	--	--	--	--	5.0	microcosms	Indoor Brock et al. 1992 a & b

* Recovery is relatively fast due to continuous input of propagules in experimental streams.

Table 2. Effect concentrations (ng/L) in relation to experimental designs and locations of model ecosystem studies with lambda-cyhalothrin. Effects resulted from repeated short-term exposures. Effect Class 1: no treatment related effects demonstrated; Effect Class 2: slight transient effects; Effect Class 3: pronounced short-term effects on sensitive endpoints, recovery within 8 weeks after the last application. Effect Class 4: clear effects, no full recovery at end of study; Effect Class 5: clear effects, recovery taking longer than 8 weeks after the last application (see Chapter 7 for details of effect classification).

Application regime	Effect Class 1	Effect Class 2	Effect Class 3	Effect Class 4	Effect Class 5	Test system	Location Reference
12 x (weekly) [1]	2.7	--	--	--	27.4	Pond mesocosms	USA, N. Carolina Hill et al. 1994
2 x (4 wk interval) [2]	4.0	--	16	--	85	Experimental ditches	Netherlands Arts et al. (accepted)
5 x (weekly) [2]	--	10	--	25	--	Lab microcosms	Indoor Van Wijngaarden et al. 2004
3 x (weekly)	--	10	--	25	--	Ditch enclosures Plankton/spring	Netherlands Roessink et al (2005)
3 x (weekly)	--	10	25	50	--	Ditch enclosures Macroph/ summer	Netherlands Van Wijngaarden et al. (in press)
3 x (weekly)	--	10	50	--	--	Ditch enclosures Macroph/spring	Netherlands Roessink et al. (2005)
4 x (biweekly)	--	--	--	--	17	Pond mesocosms	UK Farmer et al. 1995

[1] Experiment was characterized by both spray drift and run-off applications. As exposure concentrations, the median between nominal spray drift and run-off applications was used.

[2] More pesticides applied.

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Curriculum vitae

René van Wijngaarden werd geboren op 2 april 1957 te Willemstad, Curaçao N.A. In 1976 behaalde hij het HAVO-diploma te Zoetermeer. Vervolgens werd de Hogere Landbouwschoolopleiding met als specialisatie Milieukunde afgerond in 1981. Stage en afstudeeropdracht bij deze opleiding stonden in het kader van hydrobiologisch onderzoek. Respectievelijk, onderzoek naar de relatie tussen de zuurstofhuishouding en de samenstelling van de macrofauna (Rijksinstituut voor Natuurbeheer, afd. Hydrobiologie) en onderzoek naar macrofaunalevensgemeenschappen in Drentse bronnen (Planologische dienst, Drenthe). Als vervangende dienst (1981 – 1983) werd de auteur ingezet bij macrofaunadeterminaties voor de afdeling Landschapsecologie van het Rijksinstituut voor Natuurbeheer. In 1984 kwam hij in dienst bij het toenmalige Instituut voor Onderzoek van Bestrijdingsmiddelen (IOB), één van de onderzoeksinstituten die na een reeks van fuseringen uiteindelijk het huidige Alterra WUR vormen. Sindsdien is de auteur betrokken bij ecotoxicologisch onderzoek naar effecten van bestrijdingsmiddelen op zoetwaterecosystemen. Een belangrijk deel van zijn werkzaamheden hadden en hebben betrekking op experimenteel werk in modelecosystemen, zowel in het laboratorium als in het veld. Dit werk vormt de basis van het voorliggende proefschrift.

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