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Impact of single and repeated applications of the insecticide chlorpyrifos on tropical freshwater plankton communities

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Abstract This paper describes the effects of a single and a repeated application of the organophosphorus insecticide chlorpyrifos on zooplankton and phytoplankton communities in outdoor microcosms in Thailand. Treatment levels of 1 μg L^{-1} were applied once or twice with a 2-week interval. Both treatments led to a significant decrease in cladocerans followed by an increase in rotifers, although the extent by which species were affected was different. *Ceriodaphnia cornuta* was the most responding cladoceran after the first treatment, while *Moina micrura* responded most to the second. This is explained by differences in the growth phase of *M. micrura* at the time of application and an increase in *Microcystis* abundance over the course of the experiment. Several phytoplankton taxa either increased or decreased as a result of the chlorpyrifos-induced changes in zooplankton communities. Even though chlorpyrifos disappeared fast from the water column, effects on plankton communities persisted till the end of the experiment (42 days) when the insecticide concentrations had dropped below the detection limit. This was presumably due to the increasing population trend of *Microcystis*, favouring rotifers over cladocerans.

Keywords Chlorpyrifos · Single application · Repeated application · Tropical · Plankton community

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Introduction

Before the late 1960s, the traditional agricultural practices in Thailand were in close interrelationship with the local environment. Occasional floods of rivers during the rainy season assured a continual fertility of the land and pesticides were hardly used (Heckman 1979; Tonmanee and Kanchanakool 1999). The Green Revolution led to an intensification of agricultural practises and the use of pesticides and fertilizers increased considerably throughout the years (Jungbluth 2000). As a consequence, pesticide contamination of soil, water and agricultural products have been reported throughout the country (Thapinta and Hudak 2000).

However, few studies have been carried out so far into the environmental side-effects of agrochemicals in tropical countries like Thailand (Lacher and Goldstein 1997; Gopal 2005). The Thai ecotoxicological literature consists almost entirely of determinations of LC50 values for various freshwater species, invariably conducted using static tests, and basic freshwater community interactions are still largely unknown (Campbell and Parnrong 2001).

In the present study, the fate and effects of a single and repeated application of the organophosphorous insecticide chlorpyrifos was evaluated in outdoor plankton-dominated microcosms in Thailand. Microcosms and mesocosms have frequently been used for the environmental risk assessment

of several chemicals, like insecticides (see Maltby et al. 2005 and Van Wijngaarden et al. 2005 for reviews). These test systems provide more ecological realism as compared to laboratory bioassays since they include ecological processes like interactions between plankton populations while still allowing an experimental set-up.

In a previous microcosm experiment using larger test systems, the fate and effects of a single chlorpyrifos application was studied (Daam et al. accepted pending revisions). However, farmers in Thailand administer pesticides with a high frequency on their land, whereby a biweekly interval is not uncommon (Van den Brink et al. 2003; Satapornvanit et al. 2004). In addition, the extent by which pesticide pollution may spread over watersheds has been reported to be high in the tropics due to heavy rain, intensive drainage practices and extensive systems of drainage canals where surplus water may flow into local streams and rivers (Henriques et al. 1997; Castillo et al. 2006). This implies that after pesticide application in an agricultural field, surrounding waterways may be subject to part of the pesticide load.

We, therefore, evaluated the effects of a single and repeated application of 1 μg chlorpyrifos/L on zooplankton and phytoplankton communities and their interactions in outdoor microcosms in Thailand. The interval between the two applications was set at 2 weeks to mimic local agricultural practices.

Materials and methods

Experimental set-up

Ten circular experimental microcosms, each with a diameter of 0.76 m and a water depth of 0.56 m (water volume approximately 250 L), were used for the experiment. The concrete tanks were coated with watertight non-toxic epoxy paint and set up outdoors at the hatchery of the Asian Institute of Technology (AIT), located approximately 42 km north of Bangkok (Thailand). The test systems were filled with water from the canal surrounding AIT after filtering though a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems. No sediment was added to keep the experimental set-up as simple as possible and, consequently, to facilitate interpretation of the (in)direct treatment effects. In the preparatory phase of the experiment (1 week prior to application), zooplankton was collected from the AIT canal and introduced into the microcosms. To this end, canal water was filtered over a net (mesh size 0.1 mm) to avoid fish and prawns entering the systems. Subsequently, the water was concentrated using a zooplankton net (mesh size 60 μm), collected in a bucket containing filtered canal water and divided over the microcosms in several subsamples after gently stirring the sample. In this period, the water was circulated two times by exchanging 100 L between the microcosms using a Perspex tube to achieve similarity between the communities in the systems. A nutrient addition of N (1.4 mg/L as urea) and P (0.35 mg/L as triple superphosphate) was applied twice a week during the entire experimental period. These nutrient concentrations were based on those used to induce plankton growth in ponds at the hatchery of AIT. Endpoints were monitored up to 4 weeks after the second application to ensure demonstration of direct and indirect effects.

Application and fate of the test substance

Chlorpyrifos was applied as an aqueous solution of Dursban (active ingredient 40%) to six microcosms at a concentration of 1 μg a.i./L. This concentration was chosen because it matches the LC50 of the temperate standard test species *Daphnia magna* and corresponds well with LC50 values of two other temperate cladocerans and the tropical cladoceran *Moina micrura*, which was deployed in a previously performed microcosm study in Thailand (Table 1; Daam et al., accepted pending revisions). In this study, a chlorpyrifos concentration of 1 μg/L was one of the treatment doses. Hence, the treatment level in the present study was set at 1 μg/L since treatment effects could be expected without completely eliminating the zooplankton community and to enable a comparison of fate and effects with this previous microcosm experiment. Four other systems were untreated to serve as controls. Two weeks after the first application, three of the six applied tanks received a second application of 1 μg/L chlorpyrifos. After applications, the water in the microcosms was gently stirred in order to mix the insecticide over the water column. Subsamples of the treatment solutions were taken and subsequently analysed as described below for calculations of nominal concentrations.

Depth-integrated water samples of approximately 10-L were collected in a glass container using a Perspex tube 1 h after application (initial concentration) and at various moments during the experiment (Table 2). Of these samples, 750 mL were transferred to glass bottles and shaken with 50 mL *n*-hexane (HPLC grade) for 1 h. A part of the upper liquid was collected and transferred to GC-vials for analysis by splitless injection of 3 μL on a HP 5890 Gas Chromatograph. GLC operating parameters: capillary column coated with HP-5, length 30 m, internal diameter 0.32 mm, film thickness 0.25 μm; initial oven temperature 70°C, increasing with 20°C/min until a final temperature of 280°C which was kept constant for 10 min (total run time 20.5 min); detector: Electron Capture Detector; nitrogen flow 1.5 mL/min. The detection limit and recovery of

Table 1 48 h-LC50 values for cladocerans and chlorpyrifos

Cladoceran	LC50 (in µg/L)	Reference
Daphnia magna	1.0	Kersting and van Wijngaarden (1992)
Daphnia longispina	0.8	Van Wijngaarden et al. (1993)
Simocephalus vetulus	0.8	Van Wijngaarden et al. (1993)
Moina micrura	0.6	Daam et al. (accepted pending revisions)

Table 2 Sampling frequency and units of the endpoints measured

Endpoint	Unit	Sampling frequency
Chlorpyrifos concentrations	µg/L	Days: 0*; 1, 4, 7, 14**, 15, 17, 21, 28
Physicochemical parameters		
DO; EC; pH; T	mg/L; µS/cm; - ; °C	Weeks: −0.5, 0, 1, 1.5, 2, 4, 5, 6
Zooplankton		
Species composition	#/L	Weeks: −0.5, 0,…5, 6
Phytoplankton		
Species composition	#/L	Weeks: 0.5, 0, 1,…5
Chlorophyll-a	µg/L	Weeks: 0.5, 0, 1,…6

* 1 h after the first application

** 1 h before as well as 1 h after the second application

chlorpyrifos were respectively 0.07 µg/L and 93.5 ± 6.7% (mean ± SD, $n = 6$). Correction for the recovery was made when calculating chlorpyrifos residues in water.

Endpoints

The sampling and measurement techniques of the measured endpoints are described below. Their sampling frequency and units are provided in Table 2.

Dissolved oxygen was measured in the morning with an YSI model 58 oxygen meter connected to an YSI 5739 probe approximately 10 cm under the water surface. Together with the oxygen, conductivity and temperature were measured with a WTW conductivity meter and pH with a CONSORT pH meter.

At several moments during the course of the experiment, a bulk water sample of 10-L was collected in a bucket by taking several depth-integrated water samples using a Perspex tube. From this bulk sample, a subsample of 1-L was taken to study the phytoplankton community and another 1-L for determination of the phytoplanktonic chlorophyll-a concentration. Then, the bucket was emptied until a remainder of 5 L was obtained which was transferred through a zooplankton net (mesh size 60 µm) to examine treatment effects on the zooplankton community.

The concentrated zooplankton sample was fixed with formol in a final concentration of 4%. The 1-L phytoplankton sample was stained with lugol and concentrated after sedimentation of 6 days. Additional lugol was added when needed to assure conservation of the samples. Subsamples of the zooplankton and phytoplankton samples were counted with an inverted microscope (magnification 100–400) and numbers were recalculated to numbers per litre microcosm water. Colony forming algae except *Microcystis aeruginosa* and *Microcystis incerta* were quantified by counting the number of colonies. *M. eruginosa* and *M. incerta* form large 3-dimensional colonies that, especially when occurring in high abundances, are difficult to quantify with high precision. Therefore, these two species were quantified as single cells in subsamples of the phytoplankton samples after disintegration of the colonies by ultrasonication as described by Kurmayer et al. (2003).

Phytoplanktonic chlorophyll-a measurements were made using the 1-L water sample taken as described above. A known volume was concentrated over a Whatman GF/C glass fibre filter (mesh size 1.2 µm) until the filter was saturated. Filters were then air dried and extracted the same day using the method of Moed and Hallegraeff (1987).

Data analysis

Abundance data of zooplankton and phytoplankton were $\mathrm{Ln}(Ax + 1)$ transformed prior to analysis, where x stands for the abundance value and Ax makes 2 by taking the lowest abundance value higher than zero for x. This was done to down-weight high abundance values and approximate a normal distribution of the data (for rationale, see Van den Brink et al. 2000).

Until the second chlorpyrifos application (14 days post first application), statistical significance of differences between the treatment and the control were calculated for all parameters using ANOVA. Analyses were performed with Community Analysis, version 4.3.05 (Hommen et al. 1994). Statistical significance was accepted at $p < 0.05$. After the second application, statistical significance between the

treatments and the control were calculated using the Dunnett's test and expressed as *p*-values.

The zooplankton and phytoplankton data sets were analysed by PRC (Principal Response Curves) using the CANOCO software package version 4.5 (Ter Braak and Smilauer 2002). PRC is based on the Redundancy Analysis ordination technique (RDA), the constrained form of Principal Component Analysis. The analysis results in a diagram showing the sampling day on the *x*-axis and the first Principal Component of the treatment effects on the community on the *y*-axis (Fig. 2). This yield a diagram showing the deviations *in time* of the treatments compared to the control. In this way PRC shows the most dominant community response to the treatment present in the data set. The species weights are shown in a separate diagram, and indicate the affinity the species have with this dominant response. The species with a high positive weight are indicated to show a response similar to the response indicated by PRC, those with a negative weight, one that is opposite to the response indicated by PRC. Species with a near zero weight are indicated to show a response very dissimilar to the response indicated by PRC or no response at all. The significance of the PRC diagram was tested by Monte Carlo permutation of the microcosms, i.e. by permuting entire time series in the partial redundancy analysis from which PRC is derived (Van den Brink and Ter Braak 1999). After the first PRC component, more can be extracted from the remaining variation analogous to as described by Van den Brink and Ter Braak (1998). The second PRC shows the most important deviations from the first PRC, present in the data set.

Monte Carlo permutation tests were performed to assess the significance of the differences in community composition between the treatments and the controls. This was done by testing every treatment against the controls per sampling date. For more information on Monte Carlo permutation tests and how they are used in the analysis of microcosm experiments we refer to Van Wijngaarden et al. (1995).

Results

Fate of chlorpyrifos

The standard deviations within treatments for initial and nominal concentrations as well as the concentrations during the course of the experiment were mostly lower than 5% and always lower than 10% of the respective concentrations. The concentrations of chlorpyrifos decreased rapidly after both applications (Fig. 1). Four and seven days after application, mean chlorpyrifos concentrations were

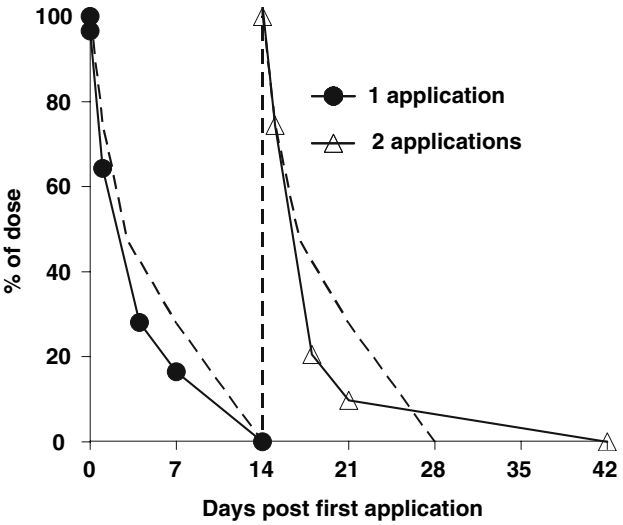

Fig. 1 Dynamics of the chlorpyrifos concentrations in the water as a percentage of the dose applied. The dashed line indicates the chlorpyrifos concentrations following application of 1 µg/L as measured in a microcosm study using larger test systems (Daam et al. accepted pending revisions; for explanation: see text)

respectively 28–21% and 17–10% (first-second application) of nominal concentrations.

Zooplankton

Before application, the dominant species in the zooplankton samples belonged to the groups of Rotifera and Copepoda, while Cladocera and Ostracoda occurred in low numbers. During the course of the experiment, Cladocera and Ostracoda increased in numbers in the control systems while Copepoda showed the opposite trend. By the end of the experiment, Cladocera and Ostracoda, and to a lesser extent the Rotifera, dominated the control zooplankton community.

Analysis using the PRC method indicated that forty-two percent of all variance could be attributed to the treatments. Of this variance, 40% is displayed on the vertical axis of a first PRC (Fig. 2a, $p < 0.01$) and another 18% on the vertical axis of a second PRC (Fig. 2b, $p < 0.01$). The PRC diagrams of the zooplankton dataset show that both chlorpyrifos treatments led to deviations from the controls, which are confirmed by the results of the Monte Carlo permutation tests (Table 3).

After the first treatment, the first PRC diagram does not show a large deviation from control in the 2 weeks following application (Fig. 2a), whereas the curve of the second PRC clearly drops immediately after application (Fig. 2b). From 2 to 3 weeks onwards, both PRCs show deviations in zooplankton community from control for microcosms treated once with chlorpyrifos. The curve of the first PRC rises considerably after the second treatment,

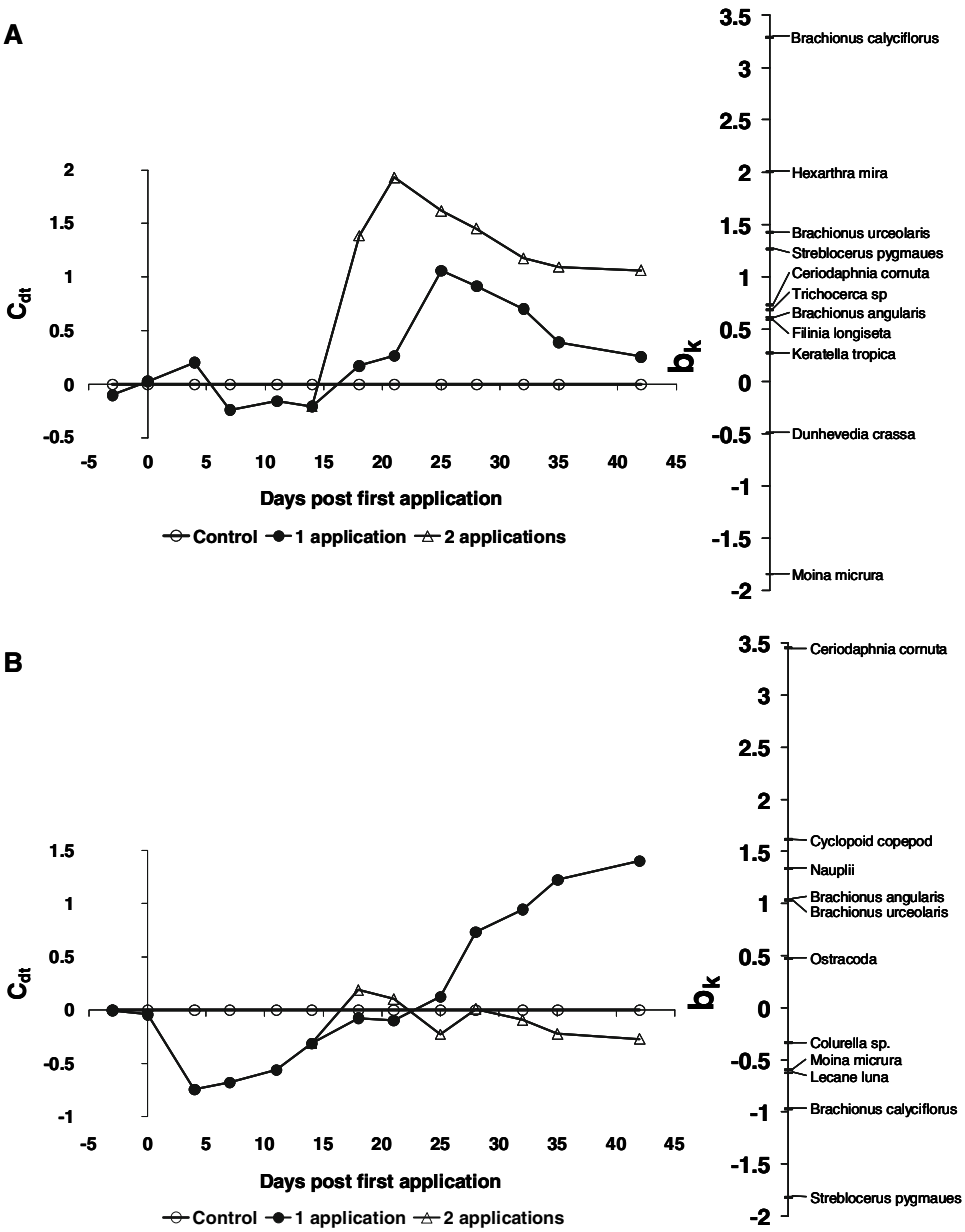

Fig. 2 First (**a**) and second (**b**) Principal response curves resulting from the analysis of the zooplankton data set, indicating the effects of one or two applications of 1 μg/L of the insecticide chlorpyrifos on the zooplankton community. Of all variance, 20% could be attributed to sampling date; this is displayed on the horizontal axis. Forty-two percent of all variance could be attributed to treatment level. Of this variance, 40% is displayed on the vertical axis of the first PRC (**a**) and 18% on the vertical axis of the second PRC (**b**). The lines represent the course of the treatment levels in time. The species weight (bk) can be interpreted as the affinity of the taxon with the Principal Response Curves. A Monte Carlo permutation test indicated that the treatment regime had a significant influence on the community structure ($p = 0.011$) and that a significant part of the variance explained by treatment level is displayed in the first ($p = 0.024$) and second ($p = 0.001$) PRC

whereas the curve in the second PRC stays close to zero. The broad pattern that emerges from this is that the dominant short-term effects of the first chlorpyrifos application are best described by the second PRC and longer-term effects by a combination of the two PRCs, whereas effects of the second application are mainly shown by the first PRC.

The indicated response pattern for individual species is obtained by multiplying the respective species weights with the treatment (c_{dt}) scores in the corresponding PRCs and then summing the two products (Van den Brink and Ter Braak 1998). This is thus especially relevant for the longer term effects of the first application. To facilitate the subtraction of the indicated response on species level, a plot of the weights of the different species on the first and second PRC is given in Fig. 3. Furthermore, calculated response curves with a ray of +45° and −45° from the horizontal axis are included in this diagram. Species coordinates that lay near the origin indicate that the corresponding species did not show a large response to the treatments. The species that are positioned along one of the axis have a response curve as indicated by the corresponding PRC. For instance, *Hexarthra mira* is located on the right side of the horizontal axis and thus has a response curve similar to the first PRC. *Streblocerus pygmaues* has a positive weight in the first PRC and a negative weight in

Table 3 Results of Monte Carlo permutation (in *p*-values) performed per sampling date for the zooplankton data set

Day	1 application	2 applications
−3	0.308	NP
0	0.083	NP
4	0.016	NP
7	0.014	NP
11	0.041	NP
14	0.261	NP
18	0.309	0.030
21	0.030	0.030
25	0.030	0.030
28	0.030	0.030
32	0.075	0.058
35	0.051	0.105
42	0.051	0.124

NP means calculation is not possible

the second PRC, so its response curve is a combination of the first PRC and the inversed curve of the second PRC (Fig. 3).

The dynamics of the taxa with a weight higher than 1.5 or lower than −1.5 with either one of the two PRCs are given in Fig. 4a though 4f, whilst all taxa for which a statistical significance of difference was calculated are presented in Table 4. The most susceptible taxa belonged to the Cladocera, although they were affected differently by the two chlorpyrifos applications. The first application led to a complete elimination of *Ceriodaphnia coruta*, and only a slight (though significant) decrease in numbers of *Moina micrura* (Fig. 4a and b). However, *M. micrura* was completely eliminated by the second application, while numbers of *Streblocerus pymaues* increased in abundances compared to controls (Fig. 4c). Except an isolated case of decreased abundance of *Brachionus urceolaris* 7 days after the first application, rotifer species increased in abundances after the first and, more pronounced, after the second application (Table 4; Fig. 4d and e). Calanoid and cyclopoid (Fig. 4f) copepods decreased in numbers 4 days after the first application and ostracod abundances were higher in all treated microcosms compared to controls at the end of the experiment (Table 4).

Phytoplankton

The PRC diagram resulting from the analysis of the phytoplankton data set is given in Fig. 5, whilst the results of the Monte Carlo permutation tests are given in Table 5. Most species have a positive weight in the diagram, indicating that most species decreased in abundances after the first application and increased slightly after the second application. Only *Microcystis aeruginosa/incerta* has a relatively high negative weight and is thus expected to show the opposite trend. These findings are confirmed by the univariate analysis, which calculated four negative treatment-related responses after the first application and four positive-related treatment effects after the second application (Table 4). In addition, a negative response on abundances of *Microcystis aeruginosa/incerta* was found after the second treatment (Fig. 6a). Interestingly, *Scenedesmus quadricauda*, *Coelastrum astroideum* and *Oocystis borgei* were found to decrease after the first application and to increase the second application. Their dynamics are presented in Fig. 6b–d.

Chlorophyll-a

Chlorophyll-a concentrations in control and microcosms that received only one application of 1 µg/L chlorpyrifos were high and rather constant during the experimental period (mean ± SD: 112 ± 38 and 105 ± 48 µg/L, respectively; Fig. 7a). The second insecticide application resulted in a decrease in chlorophyll-a content to a concentration as low as 11 µg/L. Although levels remained lower than controls and once applied microcosms until the end of the experimental period, significant differences in chlorophyll-a levels between the different treatments were noted only up to 3 weeks after the second treatment (Table 4).

Physicochemical conditions

The overall trend in dissolved oxygen (DO) concentration during the experiment is visualized in Fig. 7b. By the end of the experiment, oxygen levels were 65–70% lower compared to the initial phase of the experiment in controls and singly applied tanks. Microcosms that received two chlorpyrifos treatments, remained high levels of DO leading to a significant increase over controls until 3 weeks after the second treatment. No other significant treatment effects were found on physicochemical parameters.

Discussion

Fate of chlorpyrifos in the water

Chlorpyrifos disappeared fast from the water layer with dissipation rates slightly higher than those reported in a microcosm study carried out in Thailand evaluating single chlorpyrifos applications (Daam et al., accepted pending revisions, Fig. 1). This may be explained by the fact that in the latter study, deeper test systems (length 1 m, width 1 m, water depth 1 m) were used, meaning that the surface

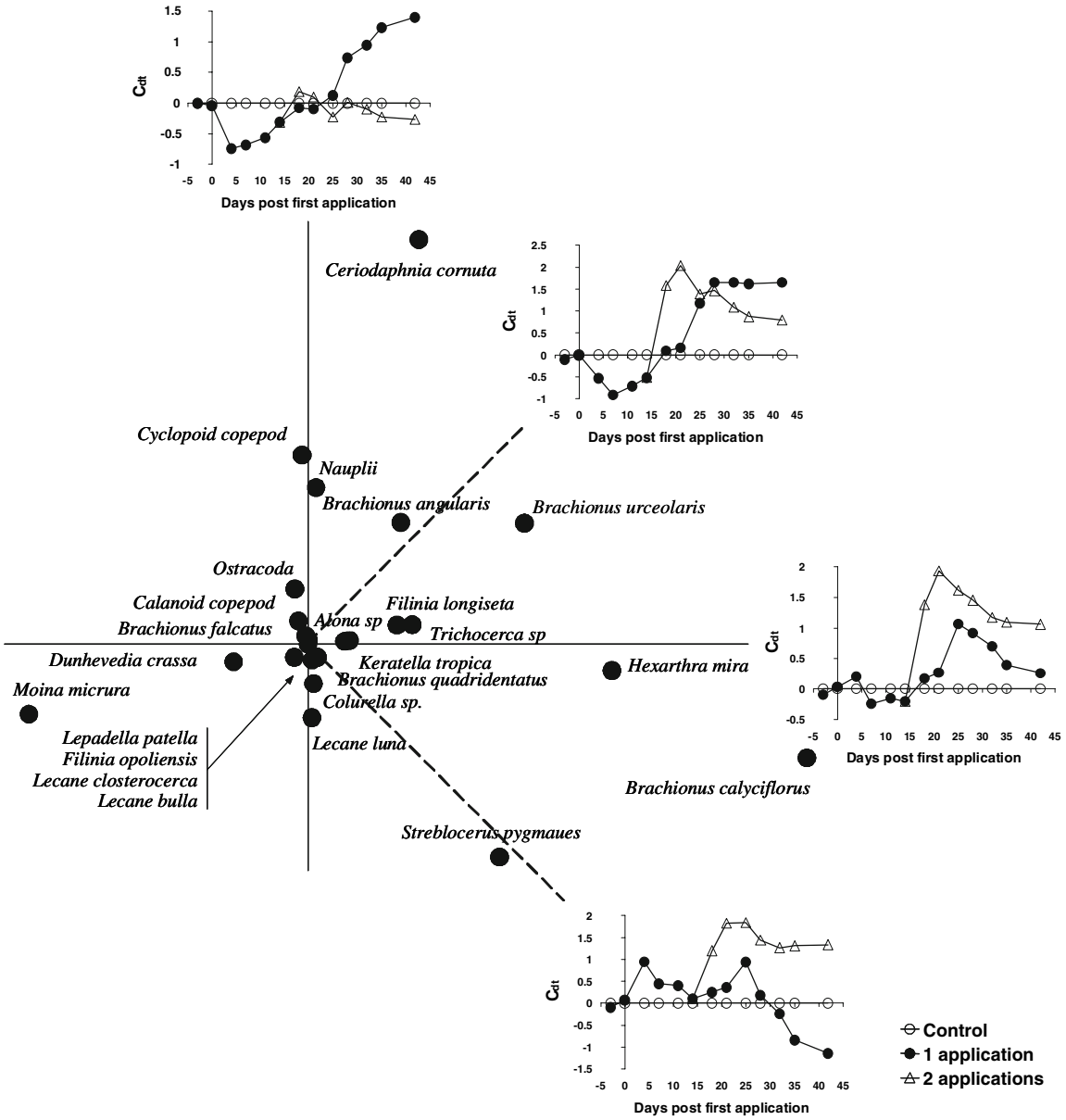

Fig. 3 Two dimensional plot of the weights of the zooplankton taxa on the first (horizontal axis) and second (vertical axis) PRC, as given in Fig. 2. The diagram in the corner applies to the taxa that have equal weights on the two PRC's

to volume ratio of the water in the present study was higher (2.3 times). This implies a relatively higher surface area for evaporation of chlorpyrifos from the water to the air, which has been demonstrated to play a significant role in the loss of chlorpyrifos from aquatic systems (Racke 1993).

Representativeness of the zooplankton and phytoplankton communities

Rotifera was the most biodiverse group, among others represented by 5 *Brachionus* taxa and 3 *Lecana* taxa. Rotifera have indeed been reported to generally dominate tropical zooplankton communities (Segers 2001). Furthermore, the warm water adapted Rotifera species *Filinia opoliensis*, *Hexarthra mira* and *Keratella tropica* (Kutikova 2002) were found regularly in the zooplankton samples. Cladocera had a composition characteristic for tropical Asian freshwaters, i.e. *Daphnia* was absent and the smaller limnetic species *Moina micrura* and *Ceriodaphnia cornuta* dominated the cladoceran community (Dumont 1994). Fernando (2002) concluded that tropical freshwater fishes have adapted to smaller sized zooplankters since they all feed on zooplankton in a broad sense at some stage of their life, e.g. young zooplanktivorous stages. All zooplankton species identified in the present study were previously recorded in Thailand (Sanoamuang 2001). Based on an evaluation of zooplankton

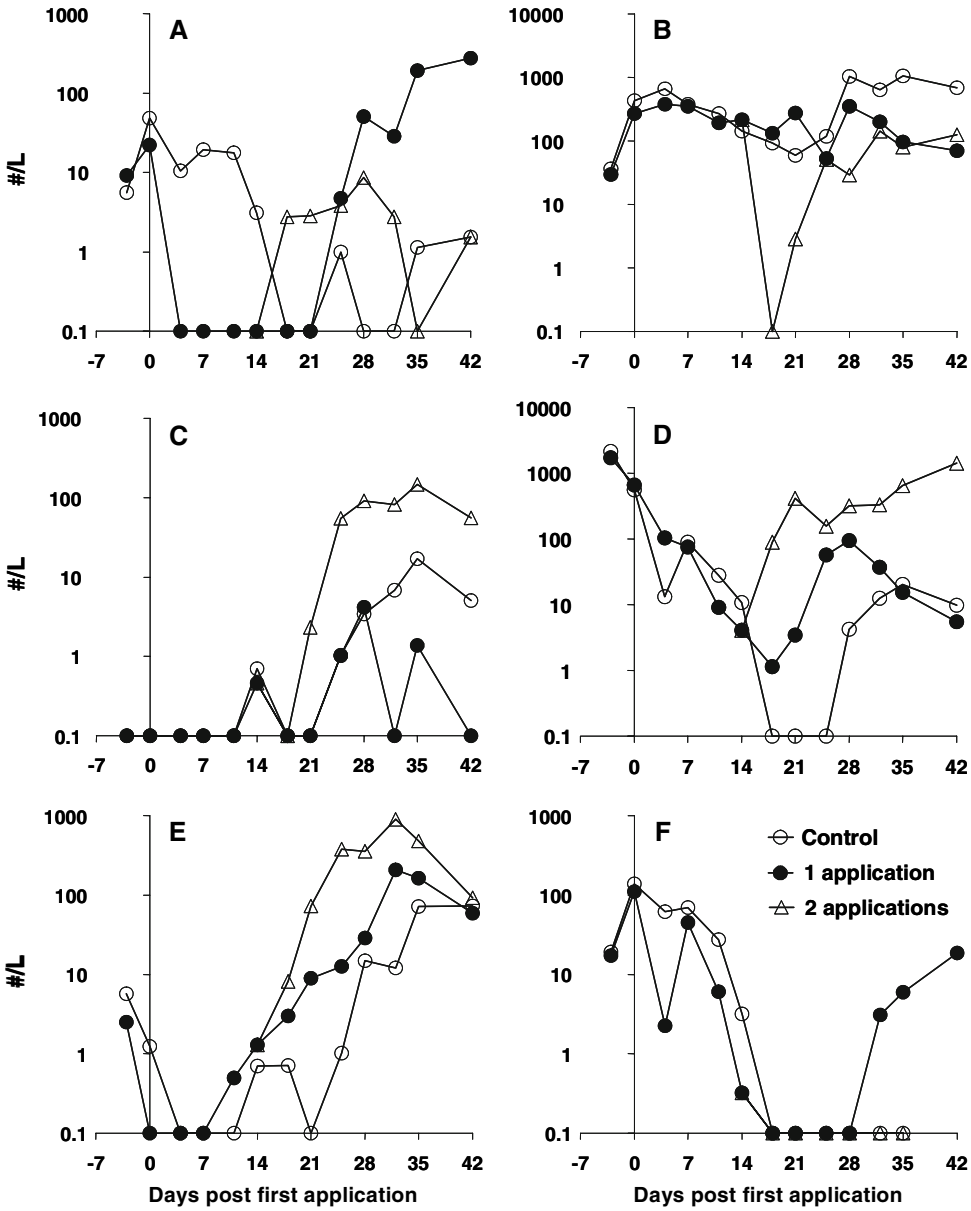

Fig. 4 Dynamics in numbers of the zooplankton taxa with a species weight higher than 1.5 or lower than −1.5 in the two PRCs of the zooplankton dataset. **a**–**f** show the geometric means of the abundances of the cladocerans *Ceriodaphnia cornuta* (**a**), *Moina micrura* (**b**), and *Streblocerus pygmaues* (**c**); the rotifers *Brachionus calyciflorus* (**d**) and *Hexarthra mira* (**e**); and cyclopoid copepods (**f**). In the figures, a value of 0.1 denotes the absence of the taxon

communities in several waterbodies, Boonsom (1984) associated zooplankton species with different habitats in Thailand. By comparing the zooplankton species in the present study with those listed for the different habitats, it can be concluded that the zooplankton communities in the microcosms were characteristic for Thai irrigation tanks and to a lesser extent for fish fields, rather than rivers and reservoirs.

In the phytoplankton samples, 27 of a total number of 41 taxa belonged to the phylum Chlorophyta. In line with this, chlorophyte biomass in the tropics has been reported to be high (Kalff and Watson 1986) and Chlorophyta was the most diverse phytoplankton phylum in field studies carried out in different parts of Thailand (Pongswat et al. 2004; Ariyadej et al. 2004; Peerapornpisal 1996). The cyanophyte *Microcystis aeruginosa* became the dominant species along the course of the experiment (Fig. 6a). This dominance may be explained by the fact that lentic systems were used, since water bodies with a high degree of water column stability favour *Microcystis*. This is because *Microcystis* colonies can regulate their buoyancy, implying that during periods of water stability they have an advantage over other phytoplankton for nutrients and especially light (Dokulil and Teubner 2000; Bonnet and Poulin 2002). The experiment was carried out at the end of the rainy season, when direct sunlight is often blocked by cloud cover (Heckman 1979), indicating that light may indeed be a limiting factor during this time of the year. In line with this, Vijanakorn et al. (2004) found *Microcystis* blooms in a reservoir in Thailand in the rainy season of 2002.

Table 4 NOECs (No Observed Effect Concentration) for zooplankton and phytoplankton populations, chlorophyll-a and water quality parameters that showed a significant response ($p \leq 0.05$)

	Days post start first application													
	−3	0	4	7	11	14	18	21	25	28	32	35	42	Figure
Zooplankton														
Cladocera	>	>	0(−)	>	>	>	1(−)	>	>	>	>	>	>	
Ceriodaphnia cornuta	>	>	0(−)	0(−)	0(−)	>	>	>	>	>	>	>	>	4a
Moina micrura	>	>	0(−)	>	>	>	1(−)	1(−)	>	1(−)	>	>	>	4b
Streblocerus pygmaues	>	>	>	>	>	>	>	>	1(+)	1(+)	>	>	>	4c
Rotifers	0(−)	>	0(+)	>	0(+)	>	1(+)	0(+)	0(+)	0(+)	1(+)	>	>	
Brachionus calyciflorus	>	>	>	>	>	>	1(+)	1(+)	0(+)	0(+)	>	>	1(+)	4d
Brachionus urceolaris	>	>	>	0(−)	>	>	>	1(+)	>	>	>	>	>	
Colurella sp.	>	>	>	>	0(+)	>	>	>	>	>	>	>	>	
Hexarthra mira	>	>	>	>	>	>	>	1(+)	1(+)	>	0(+)	>	>	4e
Keratella tropica	>	>	>	>	>	>	>	1(+)	>	>	>	>	>	
Trichocerca sp.	>	>	>	>	>	>	>	1(+)	>	>	>	>	>	
Copepoda	>	>	>	>	>	>	>	>	>	>	>	>	>	
Calanoid copepod	>	>	0(−)	>	>	>	>	>	>	>	>	>	>	
Cyclopoid copepod	>	>	0(−)	>	>	>	>	>	>	>	>	>	>	4f
Ostracoda	>	>	>	>	>	>	>	>	>	>	0(+)	0(+)	>	
Phytoplankton														
Scenedesmus quadricauda (4)	>	>	nm	0(−)	nm	>	nm	1(+)	nm	>	nm	>	nm	6b
Coelastrum astroideum	>	>	nm	0(−)	nm	>	nm	0(+)	nm	>	nm	>	nm	6c
Coelastrum microporum	>	>	nm	0(−)	nm	0(−)	nm	>	nm	>	nm	>	nm	
Oocystis borgei	>	>	nm	0(−)	nm	>	nm	>	nm	1(+)	nm	>	nm	6d
Microcystis aeruginosa/incerta	>	>	nm		nm	>	nm	1(−)	nm	>	nm	>	nm	6a
Nitzschia palea	>	>	nm		nm	>	nm	1(+)	nm	>	nm	>	nm	
Chlorophyll-a	>	0(−)	nm	>	nm	>	nm	1(−)	nm	1(−)	nm	>	>	7a
Water quality														
Dissolved oxygen	>	>	nm	>	>	>	nm	>	nm	1(+)	nm	>	>	7b

0: NOEC = control; 1: NOEC = 1 application; >: NOEC ≥ 1 application (days −3 till 14) or 2 applications (days 18 till 42); (+): significant increase compared to controls; (−): significant decrease compared to controls; nm: not measured

Direct treatment effects of chlorpyrifos on zooplankton

Both chlorpyrifos applications had pronounced but different effects on the zooplankton community. After the first application, the cladoceran *Ceriodaphnia cornuta* was eliminated and only a relatively small effect on *Moina micrura* was found (Fig. 4a and b, Table 4). After the second application, however, *M. micrura* was the most responding zooplankton species and *C. cornuta* started to re-emerge even though this species was absent in control and singly applied microcosms at that time. This may be explained by differences in growth phase between these species at the time of application. Abundances of *C. cornuta* were relatively low at the time of the first application and showed a decreasing trend in controls, while *M. micrura* was relatively abundant and showed an increasing trend. In the period before the second application, *C. cornuta* was still absent and abundances of *M. micrura* were decreasing. In line with this, Hanazato and Yasumo (1990) demonstrated that zooplankton populations were less susceptible for the insecticide carbaryl when applied in their growth phase than in their decreasing phase. A possible explanation for this is that increasing cladoceran populations contain more neonates, who have been demonstrated to be less sensitive to chlorpyrifos that older animals (Naddy et al. 2000). This may also elucidate why the first chlorpyrifos application significantly reduced the numbers of mature stages of copepods (cyclopoid, calanoid), while numbers of their immature stages (nauplii) were unaffected. No negative treatment effects were found after the second application on either mature or immature stages of copepods because they were absent in the controls.

Another reason for the larger impact on *M. micrura* after the second application compared to the first application may be the increased dominance over the experimental period by *Microcystis aeruginosa/incerta* (Fig. 6a). This is

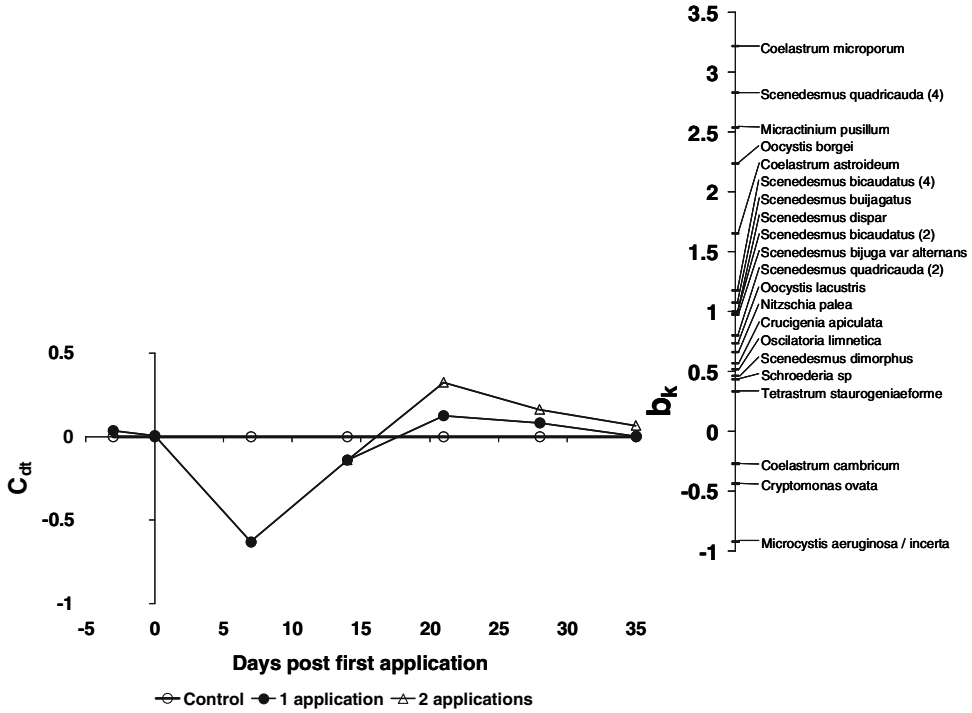

Fig. 5 First Principal response curves resulting from the analysis of the phytoplankton data set, indicating the effects of one or two applications of 1 μg/L of the insecticide chlorpyrifos on the phytoplankton community. Of all variance, 58% could be attributed to sampling date; this is displayed on the horizontal axis. Ten percent of all variance could be attributed to treatment level. Of this variance, 42% is displayed on the vertical axis of the first PRC. The lines represent the course of the treatment levels in time. The species weight (bk) can be interpreted as the affinity of the taxon with the Principal Response Curves. A Monte Carlo permutation test indicated that the treatment regime had a significant influence on the community structure ($p = 0.001$) and that a significant part of the variance explained by treatment level is displayed in the first ($p = 0.005$) PRC. (2): 2-cell colony; (4): 4-cell colony

Table 5 Results of Monte Carlo permutation (in *p*-values) performed per sampling date for the phytoplankton data set

Day	1 application	2 applications
−3	0.962	NP
0	0.803	NP
7	0.005	NP
14	0.409	NP
21	0.265	0.030
28	0.574	0.183
35	0.669	0.888

NP means calculation is not possible

because growth and reproduction of *M. micrura* has been reported to be severely reduced when reared with *Microcystis*, even when mixed with *Chlorella* (Hanazato and Yasuno 1987). Other studies also concluded that cladocerans are affected by *Microcystis*, while rotifers and copepods are less vulnerable (Lampert 1987; Ferrão-Filho 2002). These studies report toxic effects of microcystins and mechanical interference of small colonies and filaments with the filtering process as possible underlying mechanisms.

Indirect effects of the insecticide

The decrease in cladoceran abundances led to increased abundances of several rotifers, followed by ostracods as a result of decreased competition and mechanical interference. The cladoceran population was more affected after the second chlorpyrifos application and therefore led to more pronounced effects on rotifers compared to the first application as well as an increase of the tolerant cladoceran *Streblocerus pygmaues*.

Although cladoceran and copepod populations seemed recovered within 3 weeks after each application, rotifers and ostracods remained significantly increased in numbers up to 5 weeks post application (Table 4). This was presumably due to the increasing population trend of *M. aeruginosa/incerta* over the course of the experiment, which favoured rotifers over cladocerans as explained above. Ostracods have been reported to be indicative of stressed environments (Victor 2002), implying that the plankton community was affected for a prolonged period even after the pesticide had completely disappeared from the microcosms.

Abundances of *Scenedesmus quadricauda* (4-cell colonies), *Coelastrum astroideum*, *C. microporum* and *Oocystis*

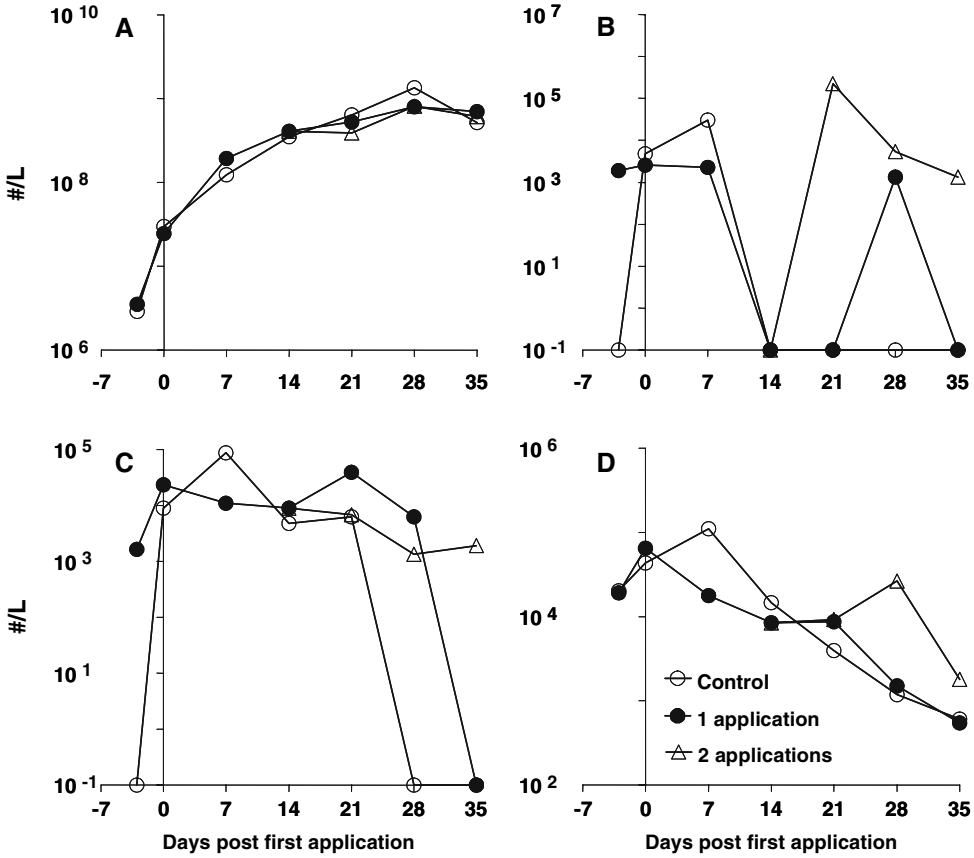

Fig. 6 Dynamics in numbers of *Microcystis aeruginosa/incerta* (**a**), which dominated the phytoplankton community, as well as the dynamics in numbers of the three phytoplankton species that showed a decrease after the first chlorpyrifos application and an increase after the second treatment: *Scenedesmus quadricauda* 4-cell colonies (**b**), *Coelastrum astroideum* (**c**) and *Oocystis borgei* (**d**). A value of 10^{-1} denotes absence of the taxon

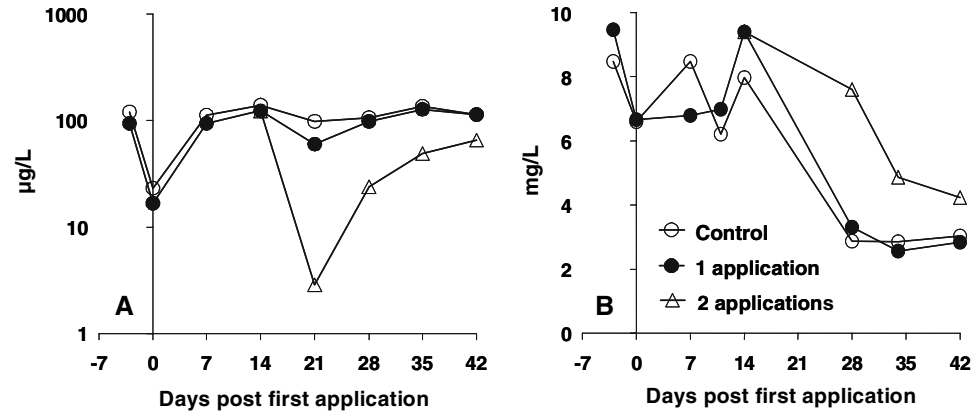

Fig. 7 Dynamics of chlorophyll-a values (**a**) and morning dissolved oxygen levels (**b**)

borgei decreased in applied tanks 1 week after the first chlorpyrifos treatment. It is unlikely that this was the result of direct toxicity of chlorpyrifos since reported EC50 values of chlorpyrifos for algae are more than a thousand-fold higher than the concentration tested (Van Donk et al. 1992). The decrease of these phytoplankton taxa was probably the result of an increased grazing pressure by *Moina micrura* to maintain its population size. In line with this, three of these species (*S. quadricauda*, *C. astroideum* and *O. borgei*; Fig. 6b–d), increased in abundances after *M. micrura* was completely eliminated by the second chlorpyrifos application. This further stimulated the growth of the tolerant cladoceran *Streblocerus pygmaues*. Growth of the rotifers, however, is not likely to have increased further due to the increase in these phytoplankton taxa, which may be explained by differences in edible phytoplankton particles between rotifers and cladocerans. *M. micrura* and other small cladocerans have been considered to feed on particles smaller than 40 µm (Hanazato and Yasuno 1987), while rotifers can handle particles up to 25 µm (Bergquist et al. 1985). *O. borgei*

occurred in the phytoplankton samples as broad ellipsoidal colonies of mostly 4 cells with a length between 30 μm and 40 μm, indicating that this species could indeed be grazed by cladocerans, but not by rotifers. *S. quadricauda* 4-cell colonies had a maximum length of approximately 20 μm, which implies that these colonies could be grazed upon by cladocerans as well as rotifers. However, this species has two spines of 10–15 μm on each terminal cell, presumably hampering the grazing by rotifers. This is supported by Bergquist et al. (1985), who recorded an increase in *S. quadricauda* in the presence of small zooplankton and also ascribed this to the presence of its spines. *C. astroideum* and *C. microporum* formed compact colonies of mostly 16 and 32 cells and occasionally colonies with 8 cells and, only for *C. microporum*, 64 cells occurred. Although size varied considerably, colony size was around 30 μm for 16-cell colonies and 50 μm for 32-cell colonies, indicating that rotifers and cladocerans could filter colonies up to 16 and 8 cells, respectively. Interestingly, colony size of *C. microporum* increased from 17 ± 1 in controls to 30 ± 7 (means ± SD; data not shown) in applied microcosms 1 week after the first application, which is in agreement with the hypothesis that *M. micrura* grazing increased on this species to recover its population size. The increase in *C. astroideum* after the second application, however, was calculated for all treated microcosms (NOEC = control) and no differences in colony size between treatments were found. This indicates that a factor other than *M. micrura* grazing was involved since abundances of the latter species in once applied tanks were comparable to controls. As a result of its elongated shape, the increase in numbers of *N. palea* (Length ± 30–60 μm; Width ± 3–5 μm) is also not likely to be the result of decreased *M. micrura* grazing alone.

The increased abundances of rotifers led to increased grazing on *Microcystis aeruginosa/incerta*, which subsequently decreased in abundance. Although the PRC indicates a decrease for both chlorpyrifos applications on day 21 and 28 (Fig. 5), a statistical significant decrease could only be demonstrated for two applications on day 21 (Dunnett's test, $p < 0.05$). This corresponded to a decrease in abundance of approximately 40% compared to controls. Though not significant in the Dunnett's test, abundances of *Microcystis aeruginosa/incerta* in once applied microcosms were as much as 20% and 40% lower than control values on day 21 and 28, respectively.

Thus, as a result of decreased competition with *Microcystis aeruginosa/incerta*, numbers of *N. palea* and *C. astroideum*, as well as *S. quadricauda* and *O. borgei*, increased in numbers. In addition, as a consequence of the decreased *M. aeruginosa/incerta* biomass, chlorophyll-a levels decreased. The decreased phytoplankton biomass as indicated by the chlorophyll-a levels led to increased DO levels as measured in the morning due to reduced respiration in the night (Fig. 7b).

Implications for risk assessment and recommendations for future research

The assessment of the risk of pesticides to the aquatic environment is currently based on dose-effect response studies using either single or continuous exposure regimes (e.g. EU 1997). In normal agricultural practises, however, pesticides are generally applied repeatedly to ensure a sufficient protection of their crops. Hence, aquatic ecosystems surrounding agricultural fields are subject to repeated pesticide loads, which may influence toxic effect cascades on aquatic life. Indeed, Hanazato and Yasuno (1990) reported an increase in the magnitude of effects on the zooplankton community in experimental ponds after repeated applications compared to a single application of the insecticide carbaryl. After a single application of carbaryl, cladocerans were reduced but recovered soon and consequently suppressed rotifers through competition. Repeated applications suppressed cladocerans for a prolonged period, which induced the occurrence of abundant rotifers (Hanazato and Yasuno 1990).

Also in the present study, different effect patterns were observed after the first and second chlorpyrifos application. It appeared, however, that the larger impact of chlorpyrifos on the cladoceran *Moina micrura* after the second treatment was a result of its population dynamics at the time of application and the increase in *Microcystis* dominance, rather than an accumulation of toxicity. Due to the relatively larger reduction in total numbers of cladocerans, indirect effects on rotifers and the phytoplankton community composition were indeed more pronounced after the second treatment.

The apparent absence of increased toxicity on the cladoceran populations after the second application may be explained by a combination of the rapid degradation rate of chlorpyrifos and the time interval between the applications. In the experiment by Hanazato and Yasuno (1990), the repeated carbaryl application regime consisted of 10 applications every other day. The interval of 2 weeks used in the present study to mimic realistic Thai agricultural practises appears to be sufficient to allow recovery of the cladoceran populations although effects on zooplankton community level lasted longer. In line with this, Naddy et al. (2000) demonstrated that daphnids could survive two 6-h 0.5 μg/L chlorpyrifos pulses if a minimum interval of 3 days was used between the treatments. These authors further stipulated that relationships among variables of pulsed exposures, including concentration, duration, interval, and frequency, need to be better evaluated and understood. This will not only allow investigating the

response of organisms under more environmentally pragmatic exposure conditions, but may also provide additional information, such as the potential for recovery, resistance, or latent effects. This may be especially relevant for agricultural common practises in tropical countries like Thailand, where application frequency is high (Van den Brink et al. 2003; Satapornvanit et al. 2004). Thus, additional experimental research is required evaluating repeated applications of pesticides with different degradation rates and application intervals relevant for local agricultural practices to come to a better understanding of pesticide freshwater ecotoxicology in tropical countries like Thailand. In addition, since several pesticides are often applied together as a mix to specific crops (Jungbluth 2000; Van den Brink et al. 2003; Satapornvanit et al. 2004), crop-based experiments mimicking specific pesticide treatment packages are needed to evaluate the actual ecological risk of pesticides for freshwater life.

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Appendix

Appendix A Geometric means of the abundances of zooplankton and phytoplankton species (in #/L) per treatment prior to the applications

Species/treatment	Prior to first application		Prior to second application		
	Control	1 application	Control	1 application	2 applications
Zooplankton					
Brachionus calyciflorus	562	660	11	3	5
Brachionus angularis	0	5	0	0	0
Brachionus falcatus	80	85	0	0	0
Brachionus urceolaris	31	25	1	0	0
Brachionus quadridentatus	0	0	0	0	0
Lecane bulla	74	33	0	0	0
Lecane luna	1	0	0	0	2
Lecane closterocerca	0	0	0	0	0
Keratella tropica	3	1	0	0	0
Trichocerca sp.	0	0	17	26	25
Hexarthra mira	1	0	1	2	1
Lepadella patella	0	0	0	0	0
Filinia longiseta	0	4	0	0	0
Filinia opoliensis	0	1	0	0	0
Colurella sp.	0	0	9	13	4
Nauplii	93	57	2	0	0
Cyclopoid copepod	139	110	3	1	0
Calanoid copepod	189	138	1	0	0
Moina micrura	427	270	142	258	181
Ceriodaphnia cornuta	49	22	3	0	0
Alona sp.	0	0	0	0	0
Streblocerus pygmaues	0	0	1	0	1
Dunhevedia crassa	0	0	0	0	0
Ostracoda	0	2	15	22	23
Phytoplankton					
Scenedesmus bicaudatus (4-cell colony)	3826	739	54905	39397	62766
Scenedesmus bicaudatus (2-cell colony)	1263	739	43562	13155	72057

Appendix A continued

Species/treatment	Prior to first application		Prior to second application		
	Control	1 application	Control	1 application	2 applications
Scenedesmus dispar	0	1925	1263	0	0
Scenedesmus quadricauda (4-cell colony)	4738	2533	0	0	0
Scenedesmus quadricauda (2-cell colony)	0	0	1263	0	0
Scenedesmus dimorphus	51900	52690	0	0	0
Scenedesmus denticulatus var. linearis	0	0	0	2705	1925
Scenedesmus buijagatus	0	0	0	0	0
Scenedesmus bijuga var alternans	0	0	0	0	0
Coelastrum astroideum	9032	23402	4738	8879	8814
Coelastrum microporum	3826	1925	71910	10302	8879
Coelastrum sphaericum	0	0	1263	1925	15778
Coelastrum cambricum	0	0	0	1925	0
Pediastrum tetras	0	0	0	0	0
Oocystis borgei	43504	64943	14715	6604	10643
Oocystis pusilla	3826	1323	0	0	0
Oocystis lacustris	0	0	1263	0	0
Golenkinia sp.	0	0	0	0	0
Ankistrodesmus falcatus	0	2533	0	0	0
Ankistrodesmus nanoselene	9032	739	0	0	0
Crucigenia apiculata	0	3475	0	0	0
Crucigenia tetrapedia	0	3826	0	0	0
Crucigenia rectangularis	6544	2972	0	0	0
Monoraphidium sp.	0	0	0	0	0
Schroederia sp.	1263	0	1711	0	0
Micractinium pusillum	2017	739	7113	4076	0
Tetrastrum staurogeniaeforme	0	739	0	0	0
Tetraedron trigonium	0	739	0	0	0
Trebouxia	0	0	0	0	0
Oscilatoria limnetica	0	0	0	0	0
Oscilatoria tenius	3826	0	0	5856	1925
Pseudoanabaena mucicola	0	0	0	0	0
Merismopedia tenuissima	0	739	0	0	0
Microcystis aeruginosa	29840754	24476414	353148566	515064859	324301073
Microcystis incerta	0	0	0	0	0
Nitzschia palea	51011	33426	0	0	1925
Nitzschia amphibia	0	739	0	0	0
Cyclotella sp.	1263	739	0	0	0
Mallomonas sp.	0	0	0	0	0
Trachelomonas sp.	0	739	1263	0	0
Chilomonas sp.	0	739	0	0	0
Cryptomonas ovata	66360	50167	0	0	0
Cryptomonas pyrenoidifera	1711	4417	0	0	0

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