**Pesticide-mediated trophic cascade and an ecological trap for mosquitoes**

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**Abstract**

Broad-spectrum pesticides can have immediate toxic effects on both target pest species and on non-target species. They may also have positive residual effects on mosquitoes after pesticide degradation, by altering the community structure – *i.e.* by reducing abundances of mosquito competitors and predators, and *via* a trophic cascade, which may increase food resources for mosquito larvae. Alternatively, if a pesticide-mediated trophic cascade results in toxic or inedible algae, the pesticide can act as an ecological trap for some taxa by attracting oviposition in sites where algae are abundant but unsuitable.

The present study assessed mosquito oviposition habitat selection, mosquito larval performance and community structure alterations after applications of various pesticides. The experiment was conducted in outdoor mesocosms assigned to one of four treatments: (1) control – no pesticides; (2) *Bacillus thuringiensis* var. *israelensis* (*Bti*), a narrow-spectrum bacterium well-known for its larvicidal activity on mosquitoes and other dipterans; (3) temephos, an organophosphate mosquito larvicide with community-wide spectrum effects; (4) pyriproxyfen, a pyridine-based Insect Growth Regulator (IGR) class with wide-spectrum effects. Soon after pesticide application, *Culex pipiens* oviposition was highest in the control pools. Invertebrate species richness and abundance were strongly reduced in the broad-spectrum pesticides treatments (temephos and pyriproxyfen) when compared to control. One month after pesticide application, *Cx. pipiens* oviposition was highest in the pyriproxyfen-treated pools, although larval survival remained lowest in pyriproxyfen-treated pools. Our results suggest that pyriproxyfen causes a chemically mediated trophic cascade and provides an ecological trap – i.e., attracting mosquito oviposition due to an altered community structure, but causing high mosquito larval mortality.

Keywords: mosquito oviposition habitat selection, larval performance, community interactions, temephos, pyriproxyfen, *Bacillus thuringiensis* var. *israelensis*

**Introduction**

Chemical control remains the most widely used approach for arthropod vector control. Although the need to develop effective systems for pesticide management has been emphasized to ensure judicious use of insecticides to manage insecticide resistance, and to reduce risks to human health and the environment (van den Berg et al. 2012, Matthews et al. 2015), the use of chemicals in the 10-year period between 2000 and 2009 has been extensive (WHO 2011). Many pesticides used for agricultural pest control find their way in exceptionally high concentrations into small water bodies such as temporary pools, which often serve as mosquito breeding sites, with considerably higher pesticide concentrations than in larger water bodies (Lorenz et al. 2017). Insecticides may alter competitive interactions in insect communities in favor of competitively inferior species (Rohr et al. 2006). When more than two interacting species are involved, it is possible to observe indirect effects in which one species affects the abundance of another species via a third, intermediate species (Relyea and Hoverman 2006). Such indirect effects can include top-down trophic cascades or bottom-up effects (Abrams et al. 1996), as insecticides can modify the effects of interspecific competition by increasing food resources (Rohr and Crumrine 2005). As an example, low concentrations of broad-spectrum insecticides can cause declines of herbivores, leading to phytoplankton blooms and decreased light transmission down through the water column. Consequently, periphyton declines can result in lower growth and survival of periphyton grazers (Bendis and Relyea 2016). Finally, while some insecticides can repel oviposition (Bentley and Day 1989), others can induce excitability after application, increase activity and change behavior of insects (Hardin et al. 1995), or provide ecological traps (Vonesh and Kraus 2009) - *i.e.* a low-quality habitat for reproduction or survival that animals prefer over other available higher quality habitats (Battin 2004).

Negative effects of several taxa on larval abundance of mosquitoes have been demonstrated in laboratory and field studies (Knight et al. 2004, Banerjee et al. 2010). Some mosquitoes are known to avoid antagonists – i.e., competitors (Blaustein and Kotler 1993, Stav et al. 2005, Duquesne et al. 2011) and predators or predator-released kairomones (Spencer et al. 2002, Blaustein et al. 2004, Eitam et al. 2004, Stav et al. 2005, Van Dam and Walton 2008, Silberbush et al. 2010) - when selecting breeding sites.

Insecticide applications in mosquito breeding habitats can greatly alter community structure and temporarily decrease biodiversity. Such disturbances can favor pioneer organisms such as mosquitoes by reducing the abundance of their antagonists (Duquesne and Liess 2010). The mechanism for emergence and re-emergence of mosquitoes resulting from anthropogenic factors is therefore very likely to be community alteration of mosquito breeding habitats – in particular, community simplification including reduced species richness (Staats et al. 2016). Indeed, mosquito populations can recover from very low abundance after treatment as population growth and development occur relatively quickly (Lichtenberg and Getz 1985). Mosquitoes are generally pioneer insects - *i.e*., rapid colonizers - and fast recolonization might lead to higher abundances of mosquitoes than in undisturbed sites, due to loss of predators and competitors in disturbed breeding sites (Duquesne and Liess 2010; Staats et al. 2016). Sensitivity to insecticides might be increased by biotic stressors (competitors, predators, pathogens) but also by abiotic stressors, such as salinity (Duchet et al. 2010a; Silberbush et al. 2014), hydroperiod length and drought (McLachlan 1985, Jeffries 1994), making insecticide effects difficult to predict due to interrelated processes (Duquesne and Liess 2010).

Several insecticides are used worldwide in mosquito control. Among these compounds, the bacterial larvicide, *Bacillus thuringiensis* var. *israelensis* (*Bti*), is generally considered as highly selective to Nematocera dipterans (Boisvert and Lacoursière 2004), although Poulin et al. (2010) observed changes in reproductive success of house martin populations (*Delichon urbicum*) after reductions in prey such as adult midges in a *Bti*-treated area. Temephos is a broad-spectrum organophosphate insecticide that blocks acetylcholinesterase, causing nervous and respiratory damage, leading to insect death (Fulton and Key 2001). Pyriproxyfen mimics the action of the juvenile hormones on a number of physiological processes, and is a potent inhibitor of embryogenesis, metamorphosis and insect adult formation (Ishaaya and Horowitz 1992).

We hypothesized that the negative impact of insecticides on the community will positively affect mosquito oviposition habitat selection (OHS) and possibly negatively affect mosquito larval performance, during the post-disturbance period of community recovery. We assessed this hypothesis in the present study in outdoor pools by evaluating OHS and mosquito larval performance during and after exposure to three different insecticides: *Bti*, temephos and pyriproxyfen.

**Materials and Methods**

*Experimental Design*

To examine the effect of pesticides on mosquito oviposition, we conducted an experiment in the Hai Bar Nature Reserve, Mt Carmel, Israel (340 m asl; N32º45’18”; E35º00’54”). This area experiences a rainy season from about October through April, and then experiences a dry period for the remainder of the year. Twenty-four outdoor pools (40 L plastic tubs; length \* width \* height: 50 \* 40 \* 20 cm) were set up ~80 m from a large, temporary artificial pond created by the Israel Nature & Parks Authority that served as a source of insect and amphibian colonists of the pools. The tubs were dug into the ground, and filled with 20 L of aged tap water on 13 April 2013. We collected pond water from the nearby pond, removed predatory insects, and added 10 L of a homogenized mixture of this water to all the pools to serve as initial source of zooplankton, periphyton and phytoplankton and other microorganisms. Three days later (16 April 2013), we added two predatory taxa to all pools, to more closely simulate the invertebrate community commonly found in the nearby temporary rocky pools: two larval dragonflies (*Sympetrum* *fonscolumbii*) and seven backswimmer larvae (*Anisops sardea*). Predators colonized naturally the mesocosms only at the very end of the experiment, and the introduced predators were completely gone by 3 June 2013. The experiment ran from 22 April to 7 August 2013. The experimental pools were left uncovered to allow aerial colonisation and oviposition by insects. To avoid high water temperatures, the pools were shaded using a fine black meshed cloth placed to the South of each pool, and tilted at 45°. During the experiment, water level was maintained in the pools by adding aged tap water. The pools were arranged in a rectangular configuration with inter-mesocosm distance being 0.5 m.

*Pesticide applications*

Four treatments (controls plus three pesticide treatments) were randomly assigned to the experimental pools, with six replicate pools per treatment. On 23 April, we made larvicide applications: Lossquito® (*Bti*, 1200 International Toxic Units – ITU per mg; Biodalia, Yokneam, Israel) with a 30 mL/L solution (final concentration: 1.8 µL/L in the pools), Temeguard® 25% (temephos, Temeguard, Israel) as a suspension formulation containing 25 g active ingredient per liter, at 0.0625% (final concentration active ingredient (a.i.) 625 µL/L in the pools), and lastly, Rimi® 90 (0.5% pyriproxyfen; Rimi, Petach Tikva, Israel) at 20 mg/L (final concentration a.i.: 100 µg/Lin the pools). The initial insecticide concentrations applied were chosen according to the recommended rates for mosquito control treatment in Israel (source: Israeli Ministry of Health). Six pools remained as the untreated controls. During the initial pesticide application, while *Anisops* were present, there was no mosquito oviposition; the mosquitoes during this time of the season avoid ovipositing in the presence of *Anisops sardea* (Eitam et al. 2002) and *Anisops debilis* (Silberbush et al. 2014).

The maximum recommended field concentration of pyriproxyfen in horticulture is 75 mg a.i./L (Fogel et al. 2016), nearly 100-fold higher than the recommended concentration for mosquito control. When no buffer zones are observed nearby aquatic habitats (USDA 1987), pesticides may be deposited by drift (Bendis and Relyea 2016), mobilized from upland areas by runoff (Beyers et al. 1995), or tile drainage (i.e. drainage system used to remove excess water from soil) due to heavy rainfall (Leu et al. 2004; Taghavi et al. 2010; Bereswill et al. 2012) into water bodies nearby agriculture areas. Due to their lower dilution capacity, small water bodies are likely to receive higher pesticide concentrations than larger ones (Lorenz et al. 2017). Finally, the biological activity of pyriproxyfen may persist 2 months after a 0.1 lb/acre application (equivalent to 113 g/ha) due to adsorbtion onto organic matter (Schaefer et al. 1988).

Thus, based on this information, we decided to consider a worst-case scenario and a simulation of a high concentration of contamination that would affect the aquatic community in small temporary ponds. A second treatment was applied to the mesocosms on 5 June 2013, with insecticide concentrations applied at 10-fold larger concentration than the original application.

*Pesticide Concentration Analyses*

To determine insecticide concentrations in treated pools, water samples were collected at 1 hour, 2 days and 7 days after each treatment in 3 randomly chosen temephos and pyriproxyfen-treated microcosms. Samples were taken at mid-depth, using 250 mL glass amber bottles. They were stored at -20 °C until further analysis. Fifty mL of temephos and pyriproxyfen treated water samples were extracted with ethyl acetate (3 X 50 mL). The combined organic extracts obtained were concentrated *in vacuo*. The dried crude extract was mixed with methanol to obtain 1 mg/mL stock solution. A Waters spherisorb S10 ODS2 column (4.6 X 200 mm) was used for High Performance Liquid Chromatography (HPLC) (Thermo) analysis with a solvent gradient of 95:5 (A:B) to 0:100 (A:B), over 23 min at 1 mL/min(A=H2O, 0.1% HCOOH; B=90% CH3CN, 10% H2O) followed by an isocratic profile over 5 min with 100% B and then, 2.5 min with 5% of A. The pesticides were quantified using calibration curves, based on UV detection at 272 and 265 nm (pyriproxyfen and temephos respectively). Aliquots of pyriproxyfen standards, ranging from 0.00006 to 1 mg/mL, and temephos, ranging from 0.000015 to 1 mg/mL, were prepared and injected after the filtration (0.22 µm PTEE) in the column through a manual injector to record their peak area at 272 and 265 nm for pyriproxyfen and temephos, respectively. Injection was performed in triplicates to obtain mean peak area value to establish the calibration curve. The response was linear in the range of concentrations tested (data not shown). Under these conditions, the lowest concentration used for standard curve corresponds to the respective limit of detection. Similarly, 100 µL of treated extracts were injected in triplicates to record mean peak area value.

*Sampling*

Sampling began one day before the initial pesticide application (22 April 2013, Day 0). Further samples were taken on 1 May, 3 June, 12 June, 10 July, and 7 August 2013.

Abiotic parameters (temperature, pH, and conductivity) were monitored between 10:00 am and 12:00 pm using a pH/EC/TDS Combo testing meter (Hanna Instruments, Kehl am Rhein, Germany). On each sampling date, a 100 mL sample of water was taken from each pool for Chlorophyll *a* concentration measurements. Water samples were filtered through Whatman GF/C fiberglass filters. Pigments were extracted overnight using 1.5 mL of methanol. Chlorophyll *a* was quantified spectrophotometrically (Spectrophotometer Nanodrop 2000C, Thermo Fisher Scientific Inc., Waltham, MA USA) according to Ritchie (2006) to determine phytoplankton biomass. An additional 100 mL sample of water was taken from each pool for phytoplankton identification, and glass microscope slides (75 x 25 mm) suspended in each pool were scraped and preserved for periphyton identification. Algal samples were preserved in 4% formalin. Three samples randomly selected out of the six replicates per treatment, were identified for two sampling dates, 12 June and 7 August, by the Mediterranean Institute of Biodiversity in Marseille (France). Phytoplankton abundance measurements were performed under an inverted microscope (Olympus IX 70) using the Utermöhl method (Utermöhl 1958, AFNOR 2006). Whenever possible, taxa were identified at the species level according to monographs of the series Süßwasser von Mitteleuropa (Krammer and Lange-Bertalot 1986, Lange-Bertalot and Krammer 1988, Krammer and Lange-Bertalot 1991b, a, Komárek and Anagnostidis 1999, 2005, Komárek 2013).

The invertebrate community was sampled after mixing the water, with a 9.5 \* 7 cm plankton net (250 µm mesh size) swept through the water (volume sampled: 1.8 L). Invertebrates were then preserved in 80% ethanol in the laboratory. Fixed specimens were counted under a stereomicroscope (Leica M125 stereomicroscope, Leica Microsystems, Wetzlar, Germany) and identified to species level when possible, using identification keys (Johannsen and Thomsen 1937, Pennak 1978, Amoros 1984).

Every 3 days, egg rafts and mosquito larvae were counted. Egg rafts were detected visually; identification relied on hatching the eggs and identifying the larvae. After several days, only a few larvae from each egg raft were kept in the laboratory and grown for eventual identification. The remaining larvae from each egg raft were returned to the mesocosm. The larvae present in the pools were sampled with the plankton net swept through the water (volume sampled: 1.8 L), transferred to a container, counted, and identified to species level (Rioux 1958, Harbach 1985 ) before returning to the pool.

*Data analyses*

The identified taxa were categorized as active (Culicidae, Chironomidae, Ceratopogonidae, and Ephemeroptera) or passive dispersers (zooplankton: calanoids, cladocerans, and ostracods). Biomass was estimated for each taxon according to literature (see Table S1 in Supporting Information). The taxa were categorized as grazers/scrapers (ostracods, Chironomidae, Ceratopogonidae, and Ephemeroptera), which would compete largely with *Culiseta longiareolata* larvae (Blaustein & Margalit 1994), or filter feeders (calanoids and cladocerans), which would compete also with *C. longiareolata* but compete most strongly with filter-feeding *Culex* larvae (Stav, Blaustein & Margalit 2005).

A Principal Component Analysis (PCA) was run to examine differences in algal species composition among treatments and time. PCA was performed using ade4 package for R software version 2.15.2.

Before analysis, the sum of mosquito egg raft abundances, and the sum of mosquito larvae abundances were calculated for each week and for each experimental pool. Survivorship of mosquito larvae was estimated by the number of late instar larvae counted on week *i* divided by the number of egg rafts counted on week *i*-1 (similar to that of Eitam et al. 2004).

To analyse the effect of larvicide treatment on the different dependent variables, we performed a repeated measures ANOVA (RM-ANOVA). Data were log-transformed (y = log(x + 1)) prior to analysis in order to satisfy assumptions of parametric analysis. A Greenhouse–Geisser correction for sphericity was used when necessary. When RM-ANOVA indicated a significant difference among treatments, Duncan’s post-hoc test was used to identify the differences between treatments and control. In the case of mosquito egg rafts, larvae and survivorship analysis, data were weekly sums. ANOVA tests were performed using Statistica Version 2.9.0 (Statsoft). Statistical significance was accepted at p < 0.05 for all tests.

In order to investigate the possible mechanisms for pesticide effects on community structure, we carried out a path analysis. We coded the three pesticide treatments as exogenous binary variables, and analysed their effects on the endogenous variables filter feeders biomass, chlorophyll *a*, oviposition (the weekly sum of egg raft abundances) and *Culex* larval biomass. In order to keep all variances on similar scales, we centered and scaled all the endogenous variables. We included the following direct effects:

1. Pesticide treatments on filter feeders, oviposition and larval biomass, because pesticides, particularly if broad-spectrum, are likely to affect population growth for these organisms.
2. Filter feeders on chlorophyll *a* (because grazers consume algae), oviposition (because adults may be able to detect filter feeders) and larval biomass (because of potential interference competition).
3. Chlorophyll *a* on oviposition and larval biomass, because adult mosquitoes may be able to detect algae, and algae provide food for larvae.
4. Oviposition on larval biomass, because eggs hatch into larvae.

We did not include any latent variables, because the number of parameters for the hypotheses above (19, including intercepts) would make it difficult to fit anything more complicated, given a sample size of 24 pools. We did not include any nondirected arcs representing correlations due to unmeasured common causes, for the same reason. In order to distinguish between short- and long-term mechanisms, we fitted this model separately to the data from 12 June (a week after treatment) and 10 July (approximately a month after treatment). We did not analyze the data from 7 August, because by this time changes in the weather meant that there was very little oviposition. We fitted the model by maximum likelihood using the sem() function in the R package lavaan version 0.5-23.1097 (Rosseel 2012). We report path diagrams with standardized coefficients in the main text, and full tables of coefficients as supplemental material (Tables S2 and S3).

After the first pesticide treatment, mosquito egg raft abundance was very low and did not differ significantly among treatments (data not shown). We attribute this low oviposition rate to the presence of *Anisops sardea,* which initially were present in all pools, and strongly inhibits oviposition by *Culiseta and Culex* mosquitoes (Eitam et al. 2002). There were no *A. sardea* remaining by the time of the second pesticide application. Therefore, only the results of the second treatment are presented.

**Results**

*Pesticide exposure concentrations*

Concentrations of pesticides degraded rapidly within the first 48 h in the pools, both for temephos and pyriproxyfen (Fig. 1). High concentrations of pyriproxyfen and temephos were 60% and 80% degraded in the water, respectively, 2 days after the application, and were not detected in the water samples 7 days after the application.

*Abiotic parameters*

Values of the abiotic parameters are given Table 1. Time x treatment interactions were statistically significant for pH (*P* < 0.001; Table 2), and conductivity (*P* <0.001; Table 2), but not for water temperature (*P* = 0.094; Table 2). pH was significantly lower (*P* < 0.001) in the pyriproxyfen treatment (8.9) compared to the control (9.4) one week after the treatment (12 June). Conductivity was significantly higher (*P* < 0.001) in the pyriproxyfen treatment (5.72 mS/cm) compared to the control (2.56 mS/cm) on 12 June.

*Algal community*

Chlorophyll *a* concentrations remained under 20 µg/L in the control pools throughout the experiment (Fig. 2A). There was a significant time x treatment interaction (*P* = 0.029; Table 3). Chlorophyll *a* concentrations did not differ significantly from control in *Bti*- and temephos-treated pools, whereas chlorophyll *a* concentrations were significantly higher in pyriproxyfen-treated pools (30 µg/L) in comparison to control (3 µg/L) one month after the treatment, on 10 July (*P* = 0.019; Fig. 2A).

Twenty seven taxa of algae were identified in the pools, distributed among Rhodophyta, Dinophyta, diatoms, Chlorophyta, Cyanobacteria and Xanthophyta. Diatoms and Chlorophyta were the most diverse groups in the pools, with 10 and 8 identified species respectively, and Chlorophyta and Cyanobacteria were the most abundant phyla in term of counted cells.

The main algal groups in the different treatments are shown in Fig 2B. The first ordination axis of the PCA ordination plot clearly differentiates between Chlorophyta and the other groups and explains 47% of the variability in the algal community. In the ordination plot, Chlorophyta was the major component in all four treatments in June. In contrast, in August, the four treatments were different: Xanthophyta and Dinophyta were most abundant in the *Bti*-treated pools; diatoms, Rhodophyta and Cyanobacteria were more abundant in the temephos-treated pools and control, and Chlorophyta was the main group in the pyriproxyfen-treated pools (Fig. 2B).

*Invertebrate community*

Twenty invertebrate taxa were identified from 3 June to 7 August. The aquatic invertebrate community mainly comprised crustaceans (7 species including calanoids, cladocerans and ostracods with *Arctodiaptomus similis*, *Moina* sp., *Alona* sp., *Heterocypris* sp., and *Potamocypris* sp. being the most abundant), and insects, which included Ephemeroptera, and 6 families: Ephydridae, Dysticidae, Libellulidae, Culicidae, Chironomidae, and Ceratopogonidae. Larvae of the three last families the most abundant were: *Culex pipiens*, *Chironomus* sp. and *Dasyhelea* sp.

There were significant time x treatment interactions for taxa richness of both active dispersers and passive dispersers (*P* = 0.009 and *P* = 0.027, respectively; Table 3). Taxa richness of active dispersers decreased drastically in both pyriproxyfen and temephos-treated pools after the treatment and was significantly lower than control on 12 June (*P* < 0.001 for both pesticides; Fig. 3A) and 10 July (*P* = 0.035 and *P* = 0.003, respectively; Fig. 3A). Taxa richness of passive dispersers was significantly lower in pyriproxyfen and temephos-treated pools than in control pools, on 12 June (*P* = 0.004 and *P* = 0.002, respectively; Fig. 3B). However, there were no significant differences at the end of the experiment between the treatments and the control. The taxa richness of active dispersers and passive dispersers did not differ significantly from the control in *Bti*-treated pools (Fig. 3A-B).

There were significant time x treatment interactions for biomass of both filter feeders (calanoids, cladocerans and *Culex* larvae) and grazers-scrapers (ostracods, Chironomidae, Ceratopogonidae, Ephemeropteraand *Culiseta* larvae; *P* = 0.020 and *P* = 0.010, respectively; Table 3). Temephos and pyriproxyfen treatments had a negative effect on biomass of filter feeders on 12 June (*P* < 0.001 for both temephos and pyriproxyfen treatments; Fig 3C), and on 10 July for the temephos treatment (*P* = 0.037; Fig. 3C). Biomass of grazers-scrapers was significantly lower in temephos and pyriproxyfen treatments in comparison to the control on 12 June (*P* = 0.006 for both pesticides; Fig. 3D), and was significantly lower than the control in temephos-treated pools on 10 July (*P* = 0.049; Fig. 3D). However, no differences were observed between control and treated pools at the end of the experiment (7 August; Fig. 3D).

The biomass of filter feeders and grazers/scrapers did not differ from the control in *Bti*-treated pools, except on 7 August, when grazers/scrapers’ biomass was higher in *Bti*-treated pools than in control pools (*P* = 0.049; Fig. 3D).

*Mosquito oviposition habitat selection and larval performance*

*Culex* *pipiens* was the main mosquito species present in the experiment pools after the second pesticide treatment. Therefore, the results presented here focus on this species.

There were statistically significant time x treatment interactions for egg raft abundance (*P* < 0.001; Table 4; Fig. 4A). Two weeks after the treatment, *Cx. pipiens* egg raft abundance was lower in all the treated pools in comparison to control pools (*P* < 0.05; Fig. 4A), and remained significantly lower in the temephos-treated pools until Week 8 (*i.e.* 8th week after the treatment; Fig. 4A). In the pyriproxyfen-treated pools, *Cx. pipiens* oviposition was significantly higher than in control pools on Weeks 4, 6, 7, and 8 (*P* < 0.05; Fig. 4A). From Week 3 to Week 9, egg raft abundance was not significantly different between control and *Bti*- treated pools, except on Week 8 where egg raft abundance was lower in *Bti*-treated pools than in control pools (*P* = 0.024; Fig. 4A).

There were statistically significant time x treatment interactions for *Cx. pipiens* larval abundance (*P* < 0.001; Table 4). The temephos and pyriproxyfen applications significantly decreased larval abundance for 3 weeks after treatment (*P* < 0.001; Fig.4B), and it remained statistically lower in the temephos-treated pools than in the control pools, until the end of the experiment (*P* < 0.01; Fig. 4B). Larval abundance in the *Bti*-treated pools did not differ significantly from control pools, however, it was lower in *Bti*-treated pools than control pools on Week 4 and Week 5 (*P* = 0.029 and *P* = 0.002, respectively; Fig. 4B).

There was also a statistically significant time x treatment interaction for the survivorship ratio (*P* = 0.032; Table 4). Survivorship in the *Bti*-treated pools was not significantly different from control pools during the entire experiment, except on Week 2, where the survivorship was significantly higher in *Bti*-treated pools than in control (*P* = 0.028; Fig. 5). Survivorship was significantly lower in temephos and pyriproxyfen-treated pools than in control pools on Weeks 7, 8 and 9 (*P* < 0.05; Fig. 5).

One week after treatment, there were strong direct negative effects of all three pesticides on *Culex* larval biomass, and a strong direct positive effect of filter feeder biomass (calanoids and cladocerans, without *Cx. pipiens* larvae for this analysis) on oviposition (Fig. 6a, Table S2). Approximately one month after treatment, the direct negative effects of all three pesticides remained, and there was a strong direct positive effect of chlorophyll *a* on oviposition (Fig. 6b, Table S3). There was little evidence for indirect effects of pesticides on larval biomass via filter feeders, chlorophyll *a* and oviposition on either date. However, since filter feeder biomass was close to zero in all but seven pools one week after treatment, and in all but two pools approximately one month after treatment, it is perhaps unlikely that such effects could have been detected. Overall, the models fitted poorly (12 June chi-square statistic 7.77, df = 3, *P* = 0.051, 10 July chi-square statistic 32.52, df = 3, *P* < 0.0005). In both cases, most of the variation in larval biomass was explained (12 June *R*2 = 0.84, 10 July *R*2 = 0.72), but the models were less successful in explaining variation in oviposition (12 June *R*2 = 0.27, 10 July *R*2 = 0.59) and performed very poorly for chlorophyll *a* (12 June *R*2 = 0.02, 10 July *R*2 <0.005) and filter feeder biomass (12 June *R*2 = 0.01, 10 July *R*2 = 0.11). Again, the fact that filter feeder biomass was close to zero in most pools is the most likely cause of this poor performance.

**Discussion**

Our results showed that pyriproxyfen negatively affected *Culex* population even after the insecticide was below detectable levels 7 days after the application, through ecological interactions. Indeed, the path analysis showed a positive effect of chlorophyll *a* concentrations on *Cx. pipiens* oviposition. However, pyriproxyfen at a high dosage caused a chemically mediated trophic cascade and also caused an ecological trap, attracting mosquito oviposition due to altered community structure but causing high mosquito larval mortality.

An ecological trap is a low-quality habitat for reproduction or survival that animals prefer over other available higher quality habitats, often modified by human activities (Battin 2004). In environments that have been altered by humans, reliable cues observed by animals to make behavioral and life history decisions might no longer be associated with adaptive outcomes. In such cases, organisms can become ‘trapped’ by an error in habitat assessment resulting from some mismatch between the environmental cues animals use to select habitat, and actual habitat quality (Donovan and Thompson III 2001, Kokko and Sutherland 2001, Schlaepfer et al. 2002). Ecological traps impact a broad taxonomic range of animals, from mammals (Zugmeyer and Koprowski 2009) to insects (Ries and Fagan 2003, Horváth 2010). Ecological traps have been observed with the *Culex* mosquito after application of carbaryl (Vonesh and Kraus 2009). Vonesh and Kraus (2009) observed that *Culex* showed a strong preference for ovipositing in carbaryl-contaminated pools, although mosquito larvae abundance was lower than in control pools. They concluded that the pesticide carbaryl they used for their experiment could act as an ecological trap for some taxa, attracting colonizer insects to habitats that are ultimately lethal for them.

Path analysis did not show evidence for an indirect effect of the insecticides on the chlorophyll *a* *via* a negative effect on the zooplankton. However, chlorophyll *a* concentrations increased in pyriproxyfen-treated pools. By drastically reducing the numbers of herbivores (zooplankton and *Culex* larvae), the insecticide likely had an indirect impact on the algal community. After application of pyriproxyfen, the algal community was mainly comprised of Chlorophyta (*Chlorella* sp, *Kirchneriella* sp., and *Scenedesmus planctonicus*), commonly consumed by zooplankton. Some of these algal species can be toxic or indigestible for *Culex* larvae. Indeed, Marten (2007) showed that some green algae in the order Chlorococcales are indigestible, and *Coelastrum*, *Kirchneriella*, and *Scenedesmus* species always killed *Cx. quinquefasciatus* larvae. Further experiments should be conducted to explore the link between insecticide applications and algae community, and evaluate the effects of green algae on mosquito larvae.

High mosquito larval densities in the pyriproxyfen treatment can also explain the poor survival of the mosquito larvae. Indeed, high oviposition in pyriproxyfen might have led in return, to intraspecific competition. In fact, in the larval habitat, food availability and space regulate competition among conspecifics (Jannat and Roitberg 2013), as larval developmental time and mortality increase when food is scarce (Renshaw et al. 1993, Mahmood et al. 1997). Suleman (1982) demonstrated the negative effect of densities on mosquito larval mortality as larval mortality increased as a function of decreasing amount of food per larvae, whereas greater larval food supply led to enhance larval and production and larger mosquitoes with longer longevity and higher biting frequency (Araújo and Gil 2012).

In contrast, despite the negative effect of temephos on the invertebrate community, we did not observe any top-down effects, *i.e.* increase of algal populations. Indeed, except on 7 August, chlorophyll *a* concentrations remained very low as temephos can induce inhibition of photosynthesis (Birmingham and Colman 1977). Significant decreases in growth rates of the green alga *Chlorella pyrenoidosa* and the diatom *Navicula minima* were observed after treatment with 10 µg/L temephos (Birmingham and Colman 1977). The low quantity of food resources might explain the low oviposition (even after breakdown of the pesticide). Transformation products are commonly more toxic than the parent compound (Fielding 1992). Through oxidation, the major photolytic route of pesticide degradation, temephos is converted to its oxon and sulfoxide. Oxidation products of the pesticides (oxygen analogues, sulfoxides, sulfones) are commonly more toxic than the parent compound, and can persist for a longer period than the parent compound (Lacorte et al. 1996, Kamel et al. 2009).

Temephos and pyriproxyfen applications negatively affected taxa richness, and biomass of dipterans (chironomids and ceratopogonids) and of crustaceans (*Moina, Ceriodaphnia* and ostracods). The organophosphate temephos and the juvenile hormone mimic pyriproxyfen are broad-spectrum insecticides. Application of temephos at the recommended rate for mosquito control affects crustaceans (Brown et al. 2000, Milam et al. 2000), mosquitofish *Gambusia affinis* (Milam et al. 2000), and insects (Pinkney et al. 2000, Marina et al. 2014). Acute exposures to pyriproxyfen negatively affects survival and reproduction of crustaceans (Tuberty and McKenney 2005, Linton et al. 2009), Odonata (Schaefer and Miura 1990), and Chironomidae (Schaefer and Miura 1990).

However, in our case, the community tended to recover by the end of the experiment. Two days after the insecticide application, 20% of temephos and 40% of pyriproxyfen were detected in the samples, and concentrations of both insecticides were below detection after one week. The rapid degradation within one week of both insecticides, in water pond, is consistent with the literature (Lacorte et al. 1995; Schaefer et al. 1988). The maintenance of diversity through disturbance is thought to be due to a fundamental tradeoff between competitive ability and resistance to disturbance (Rohr et al. 2006). Disturbances tend only to increase diversity at low to intermediate intensities (Rohr et al. , Grime 1973, Connell 1978, Rohr et al. 2006). In the case of strong stress periods, diversity is reduced as only a few species can tolerate it. Insecticides can induce direct or indirect effects on insects. In the case of direct effects, insecticides increase mortality, but sublethal exposure can also affect behavior and induce excitability, and increase of the activity due to attractiveness from some pesticide products (Elzen 1989). Colonization and oviposition behavior of invertebrate species play an important role in the assembly of individual aquatic communities (Blaustein 1999, Kraus and Vonesh 2010, Resetarits and Binckley 2014). By affecting colonizing taxa differently and increasing richness, the contaminant may alter the ecological context through indirect effects of contaminant exposure (Vonesh and Kraus 2009), and processes such as oviposition and habitat selection can be significantly affected by pesticides as well as recovery and colonization (Duquesne and Liess 2010).

Our results did not show long-term negative effects of *Bti* on the community. Mosquito oviposition was lower in *Bti*-treated pools than in control pools, but the effect on larval abundance was of short duration and immediate after the treatment at high dosage. Kroeger et al. (2013) showed in natural ponds that high densities of cladocerans combined with *Bti* treatment at a recommended rate have a negative and persistent effect on *Culex* sp. populations and oviposition. However, other studies have shown that *Bti* can enhance the oviposition rate of *Aedes albopictus* in small containers (Stoops 2005, Carrieri et al. 2009). *Bti* is well-known for its high selectivity to Nematocera dipterans (Boisvert and Lacoursière 2004). Laboratory tests and field studies have shown that *Bti* may be safe to the environment due to its selectivity (Mulla et al. 1982, Barnes and Chapman 1998, Boisvert and Lacoursière 2004, Duchet et al. 2010b, Duchet et al. 2015). However, in a recent study, Lajmanovich et al. (2015) demonstrated negative effects of *Bti* on Glutathion-S-Transferase activities, and histological anomalies of intestines of tadpoles of the South American common frog (*Leptodactylus latrans*), but at a very high dosage (between 4- to 80-fold the recommended application rate). Most wetlands studies, including long-term monitoring (6 years or more), did not show any significant effects of *Bti* on aquatic invertebrate communities (Vijverberg 1980, Russell et al. 2009, Vinnersten et al. 2009, Lundström et al. 2010, Vinnersten et al. 2010, Caquet et al. 2011, Lagadic et al. 2014, Lagadic et al. 2016), although results of *in situ* studies on non-target organisms remain controversial (Hershey et al. 1995, Hershey et al. 1998, Liber et al. 1998, Niemi et al. 1999, Poulin et al. 2010).

The community context is important for evaluating toxicity effects, and plays an important role in shaping contaminant effects as synergistic interactions might occur between insecticide exposure and biotic stressors (Rohr et al. 2006). Therefore, it is important to understand how aquatic community assembly is altered by changes in response to contaminants, and how behavioral shifts in response to pesticide treatments could alter the ecological context. Green algae are likely to affect mosquito larvae survival, although so far this has only been demonstrated under laboratory (Marten 2007, Rey et al. 2009). Our study suggests that this effect may be important in natural systems, in which algal species composition has been indirectly affected by pesticides. Further research needs to be carried out in outdoor pools and in the field at the community level, to evaluate green algae toxic properties as an alternative to broad-spectrum insecticides in mosquito control management.

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**Table 1.** Overview of the abiotic parameters measured in control and treated pools at each sampling date. **Mean** +/- standard error are presented (n=6 for each treatment)

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Abiotic  Parameters | Treatment | 3 June | 12 June | 10 July | 7 Aug |
| pH | Control | **9.2**  ± 0.08 | **9.4**  ± 0.07 | **9.1**  ± 0.08 | **8.8**  ± 0.09 |
| *Bti* | **9.3**  ± 0.09 | **9.6**  ± 0.11 | **9.1**  ± 0.10 | **8.7**  ± 0.08 |
| Temephos | **9.3**  ± 0.04 | **9.6**  ± 0.08 | **9.2**  ± 0.10 | **9.0**  ± 0.10 |
| Pyriproxyfen | **9.3**  ±0.03 | **8.9**  ± 0.09 | **9.0**  ± 0.05 | **9.0**  ± 0.04 |
| Conductivity  (mS/cm) | Control | **2.38**  ± 0.04 | **2.56**  ± 0.05 | **3.44**  ± 0.09 | **3.92**  ± 0.12 |
| *Bti* | **2.29**  ± 0.07 | **2.37**  ± 0.06 | **3.15**  ± 0.16 | **3.47**  ± 0.25 |
| Temephos | **2.37**  ± 0.07 | **2.60**  ± 0.08 | **3.46**  ± 0.16 | **3.80**  ± 0.34 |
| Pyriproxyfen | **2.23**  ± 0.19 | **5.72**  ± 0.27 | **3.61**  ± 0.23 | **3.87**  ± 0.21 |
| Temperature  (°C) | Control | **24.3**  ± 0.52 | **19.0**  ± 0.54 | **23.4**  ± 0.19 | **23.7**  ± 0.14 |
| *Bti* | **23.7**  ± 0.41 | **19.1**  ± 0.25 | **23.0**  ± 0.26 | **24.1**  ± 0.15 |
| Temephos | **24.6**  ± 0.38 | **20.1**  ± 0.40 | **25.0**  ± 0.10 | **24.4**  ± 0.09 |
| Pyriproxyfen | **23.8**  ± 0.42 | **19.4**  ± 0.29 | **24.2**  ± 0.38 | **24.6**  ± 0.22 |

**Table 2.** Results of repeated-measures ANOVA for the abiotic variables during the second pesticide treatment part of the experiment. Bold p-values indicate statistically significant differences.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Source of variation | df | *F* | *P* |
| *pH* | |  |  |  |
|  | Treatment | 3, 20 | 5.20 | **0.008** |
|  | Time | 2, 40 | 40.90 | **< 0.001** |
|  | Time \* Treatment | 6, 40 | 7.60 | **< 0.001** |
| *Conductivity* | |  |  |  |
|  | Treatment | 3, 20 | 12.53 | **< 0.001** |
| Time | 2, 40 | 25.39 | **< 0.001** |
| Time \* Treatment | 6, 40 | 41.20 | **< 0.001** |
| *Water temperature* | |  |  |  |
|  | Treatment | 3, 20 | 5.60 | **0.006** |
| Time | 2, 40 | 511.20 | **< 0.001** |
| Time \* Treatment | 6, 40 | 2.00 | 0.094 |

*Values were log(y + 1) transformed prior to analysis*

**Table 3.** Results of repeated-measures ANOVA for the biotic variables during the second pesticide treatment of the experiment. Bold p-values indicate statistically significant differences.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Source of variation | df | *F* | *p* |
| *Chlorophyll* a *concentrations* | |  |  |  |
|  | Treatment | 3, 20 | 1.39 | 0.275 |
|  | Time | 1.49, 29.83§ | 4.23 | **0.034** |
|  | Time \* Treatment | 4.47, 29.83§ | 3.03 | **0.029** |
| *Active dispersers abundance* | |  |  |  |
|  | Treatment | 3, 20 | 21.26 | **< 0.001** |
| Time | 2, 40 | 17.03 | **< 0.001** |
| Time \* Treatment | 6, 40 | 3.38 | **0.009** |
| *Passive dispersers abundance* | |  |  |  |
|  | Treatment | 3, 20 | 5.19 | **0.008** |
| Time | 1.43, 28.60§ | 6.37 | **0.010** |
| Time \* Treatment | 4.29, 28.60§ | 3.13 | **0.027** |
| *Filter feeders biomass* | |  |  |  |
|  | Treatment | 3, 20 | 9.63 | **< 0.001** |
| Time | 1.27, 25.31§ | 6.66 | **0.011** |
| Time \* Treatment | 3.80, 25.31§ | 3.59 | **0.020** |
| *Grazers-scrapers biomass* | |  |  |  |
|  | Treatment | 3, 20 | 7.32 | **0.002** |
| Time | 1.99, 39.84§ | 9.93 | **< 0.001** |
| Time \* Treatment | 5.98, 39.84§ | 3.29 | **0.010** |

*Values were log(y + 1) transformed prior to analysis*

§ *with the Greenhouse-Geisser correction*

**Table 4.** Results of repeated-measures ANOVA for *Culex* sp. Abundance during the second pesticide application period of the experiment.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Source of variation | Degrees of Freedom | *F* | *p* |
| *Egg raft abundance over time* | |  |  |  |
|  | Treatment | 3, 20 | 16.84 | **< 0.001** |
|  | Time | 8, 160 | 14.49 | **< 0.001** |
|  | Time\*Treatment | 24, 160 | 5.43 | **< 0.001** |
| *Mosquito larval abundance over time* | | |  |  |
|  | Treatment | 3, 20 | 14.06 | **< 0.001** |
|  | Time | 4.24, 84.82§ | 15.53 | **< 0.001** |
|  | Time\*Treatment | 12.72, 84.82§ | 5.72 | **< 0.001** |
| *Survivorship ratio* | |  |  |  |
|  | Treatment | 3, 12 | 12.80 | **< 0.001** |
|  | Time | 3.31, 39.83§ | 9.70 | **< 0.001** |
|  | Time\*Treatment | 9.96, 39.83§ | 2.28 | **0.032** |

*Values were log(y + 1) transformed prior to analysis*

§*with the Greenhouse-Geisser correction*

**Figure legends**

**Figure 1.** Changes in pyriproxyfen (square symbols) and temephos (triangle symbols) mean concentrations (±SE; n = 3) measured in water. Day 0 corresponds to sampling 2 hours after pesticide applications.

**Figure 2.** Algae community. (A) Chlorophyll a concentrations in treated (*Bti*, temephos and pyriproxyfen) and control pools. Mean values (± SE, n = 6) are given. The black arrow indicates the larvicide application on 5 June. \*: Significant differences compared to control (Duncan’s post hoc test following RM ANOVA, p < 0.05). (B) Principal Component Analysis of the algae community in treated and control pools, showing relationships between the main groups of algae identified and passively projected pesticide applications in comparison to control. Squares represent the treatments in June (J) and in August (A) (TEME = temephos, PYRI = pyriproxyfen, Bti = *Bti* and CONT = Control), circles represent the groups of Algae. The first and second ordination axes explain 75% of the data variability.

**Figure 3.** Descriptors of invertebrate communities in control, *Bti*-, temephos-, and pyriproxyfen-treated pools. Mean values (± SE, n = 6) are presented. The black arrow indicates the larvicide application on 5 June. (A) Active dispersers taxa richness; (B) Passive dispersers taxa richness; (C) Biomass of filter feeders; (D) Biomass of grazers/scrapers. \*: Significant differences compared to control (Duncan’s post hoc test following RM ANOVA, p < 0.05).

**Figure 4.** Egg raft abundances of *Culex* (A) and density of *Culex* larvae (B) in control, *Bti*-, temephos-, and pyriproxyfen-treated pools. Mean values (± SE, n = 6) are shown. The black arrow indicates the larvicide application on 5 June. \*: Significant differences compared to control (Duncan’s post hoc test following RM ANOVA, p < 0.05).

**Figure 5.** Survival of *Culex* larvae in control, *Bti*-, temephos-, and pyriproxyfen-treated pools. Mean values (± SE, n = 6) are shown. The black arrow indicates the larvicide application on 5 June. \*: Significant differences compared to control (Duncan’s post hoc test following RM ANOVA, p < 0.05).

**Figure 6.** Path diagrams with standardized coefficients for (a) June, one week after treatment and (b) 10 July, approximately one month after treatment. Solid arrows represent positive coefficients, and dashed arrows negative coefficients. Thick arrows are those whose coefficients were significantly different from zero at the 0.05-level, based on *z*-tests.

**Figure 1.**

**Figure 2.**

**A**

**\***

**B**

**Figure 3.**

**A**

**\***

**\***

**\***

**B**

**\***

**\***

**C**

**\***

**\***

**D**

**\***

**\***

**\***

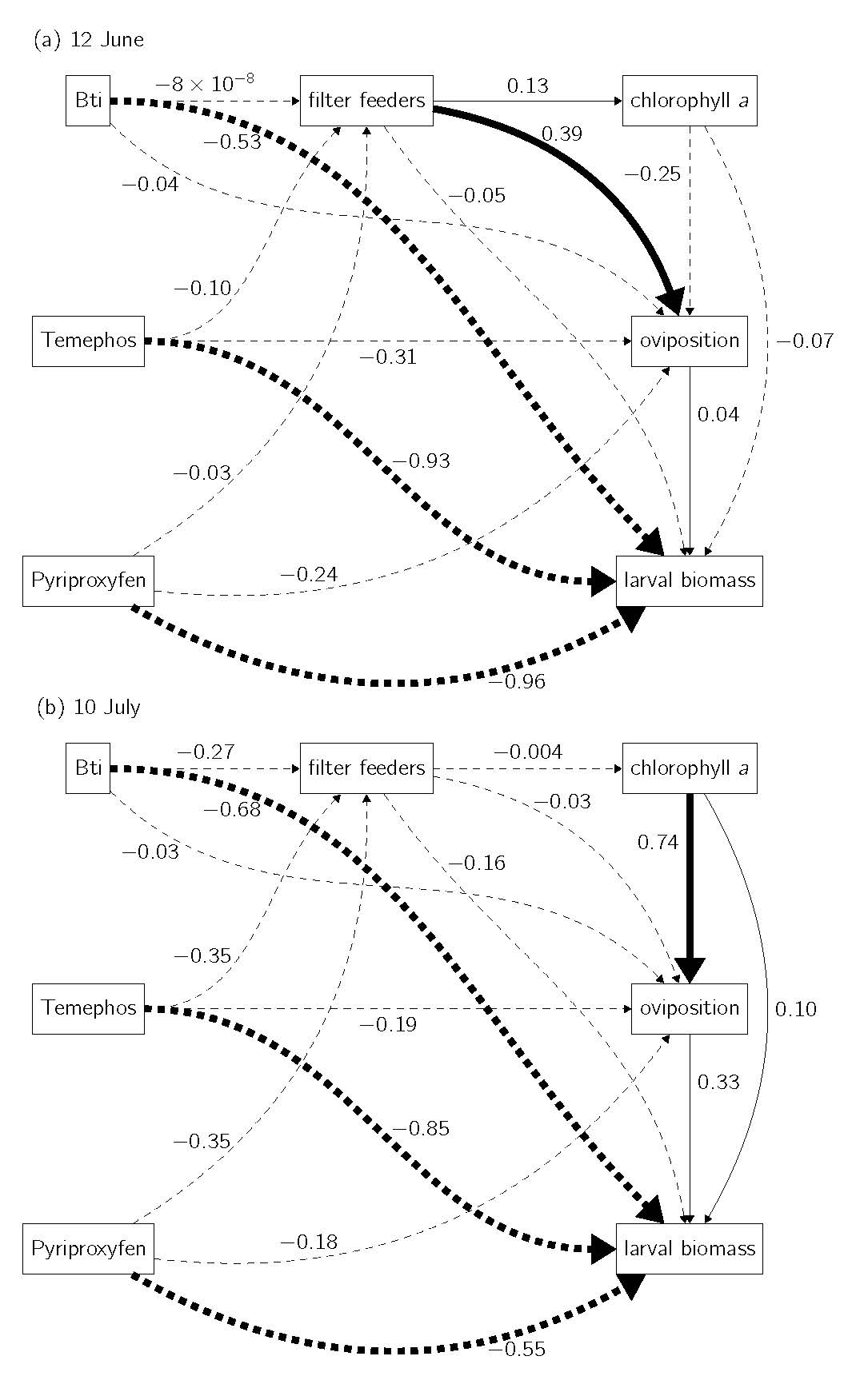
**Figure 4.**

**A**

**B**

**Figure 5.**

Figure 6.



**Appendix S1**

**Supplemental Materials**

**Table S1.** Estimated dry weights for the taxa identified into the experimental pools

|  |  |  |
| --- | --- | --- |
| Taxa | dry weight / individual (µg) | references |
| *Arctodiaptomus* sp. | 15 | Dumont, Van de Velde & Dumont 1975 |
| *Moina* sp. | 8.5 | Dumont, Van de Velde & Dumont 1975 |
| *Alona* sp. | 1.6 | Dumont, Van de Velde & Dumont 1975 |
| *Ceriodaphnia* sp. | 6.3 | Dumont, Van de Velde & Dumont 1975 |
| *Heterocypris* sp. | 70 | Widbom 1984 |
| *Potamocypris* sp. | 15 | Widbom 1984 |
| *Dasyhelea* sp. | 100 | Sota, Mogi & Kato 1998 |
| *Chironomus* sp. | 130 | Dawson, Jenson & Norberg-King 2000 |
| *Culex* sp. | 1130 | Duquesne *et al.* 2011 |
| Ephemeroptera | 1530 | Gupta, Michael & Gupta 1993 |
| Anisops | 9800 | Smock 1980 |
| Odonates | 5000 | Smock 1980 |
| Dysticidae | 20000 | Smock 1980 |

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|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Table S2.** Path coefficients for 12 June, estimated by maximum likelihood. Root mean square error of approximation 0.257. Response variable | Estimate | Coefficient | Standard error | *z*-score | *P*-value | Standardized coefficient |
| Larval biomass | Oviposition | 0.032 | 0.087 | 0.368 | 0.713 | 0.036 |
|  | Chlorophyll *a* | -0.056 | 0.074 | -0.755 | 0.450 | -0.066 |
|  | Grazer biomass | -0.018 | 0.030 | -0.586 | 0.558 | -0.054 |
|  | Bti | -0.506 | 0.097 | -5.208 | <0.0005 | -0.530 |
|  | Pyriproxyfen | -0.916 | 0.100 | -9.189 | <0.0005 | -0.959 |
|  | Temephos | -0.891 | 0.102 | -8.764 | <0.0005 | -0.932 |
| Oviposition | Chlorophyll *a* | -0.238 | 0.168 | -1.415 | 0.157 | -0.250 |
|  | Grazer biomass | 0.141 | 0.065 | 2.174 | 0.030 | 0.386 |
|  | Bti | -0.037 | 0.229 | -0.161 | 0.872 | -0.035 |
|  | Pyriproxyfen | -0.256 | 0.229 | -1.115 | 0.265 | -0.239 |
|  | Temephos | -0.334 | 0.230 | -1.451 | 0.147 | -0.312 |
| Chlorophyll *a* | Grazer biomass | 0.050 | 0.077 | 0.645 | 0.519 | 0.131 |
| Filter feeders biomass | Bti | -2×10-7 | 0.728 | -2×10-7 | 1.000 | -8×10-8 |
|  | Pyriproxyfen | -0.096 | 0.728 | -0.132 | 0.895 | -0.033 |
|  | Temephos | 0.289 | 0.728 | 0.397 | 0.692 | 0.099 |

**Table S3.** Path coefficients for 10 July, estimated by maximum likelihood. Root mean square error of approximation 0.640.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Response variable | Estimate | Coefficient | Standard error | *z*-score | *P*-value | Standardized coefficient |
| Larval biomass | Oviposition | 0.276 | 0.143 | 1.937 | 0.053 | 0.328 |
|  | Chlorophyll *a* | 0.088 | 0.149 | 0.589 | 0.556 | 0.098 |
|  | Grazer biomass | -0.378 | 0.276 | -1.371 | 0.170 | -0.159 |
|  | Bti | -1.756 | 0.355 | -4.948 | <0.0005 | -0.678 |
|  | Pyriproxyfen | -1.432 | 0.370 | -3.867 | <0.0005 | -0.553 |
|  | Temephos | -2.211 | 0.371 | -5.965 | <0.0005 | -0.853 |
| Oviposition | Chlorophyll *a* | 0.787 | 0.140 | 5.638 | <0.0005 | 0.741 |
|  | Grazer biomass | -0.077 | 0.394 | -0.195 | 0.845 | -0.027 |
|  | Bti | -0.076 | 0.507 | -0.151 | 0.880 | -0.025 |
|  | Pyriproxyfen | -0.563 | 0.517 | -1.088 | 0.277 | -0.183 |
|  | Temephos | -0.569 | 0.517 | -1.101 | 0.271 | -0.185 |
| Chlorophyll *a* | Grazer biomass | -0.012 | 0.543 | -0.021 | 0.983 | -0.004 |
| Filter feeders biomass | Bti | -0.289 | 0.256 | -1.127 | 0.260 | -0.266 |
|  | Pyriproxyfen | -0.385 | 0.256 | -1.503 | 0.133 | -0.354 |
|  | Temephos | -0.385 | 0.256 | -1.503 | 0.133 | -0.354 |