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Chapter 3

Predicting the Effects of Insecticide Mixtures on Non-Target Aquatic Communities

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/53356

1. Introduction

In this study two questions will be posed: firstly, how can single-species, single-compound toxicity test data on non-target aquatic insects predict patterns in stream communities exposed to the same compounds individually and jointly? Secondly, can mixtures of two or three insecticides be treated additively using a concentration addition, Toxic Unit (TU) approach in an aquatic community context? To evaluate these questions, the following studies examined the responses of field-collected benthic (bottom-dwelling) invertebrates exposed to mixtures of organophosphorus insecticides (chlorpyrifos and dimethoate) in detail as well as a preliminary investigation of the effects of adding a third insecticide to the mixture, the neo-nicotinoid (imidacloprid).

Non- target aquatic organisms are routinely exposed to pesticides because these compounds are widely used and are regularly detected during stream biomonitoring [1]. Mixtures of insecticides are particularly worrisome because these compounds can directly alter the abundance and diversity of aquatic insects; consequently, these effects can reshape aquatic food webs. Organophosphorus insecticides are particularly relevant for consideration because they are extensively used in agriculture worldwide and, for example, constitute ~ 40% of the insecticides applied in the United States [2]. In this study, two organophosphorus insecticides were selected, chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridinyl) phosphorothioate) and dimethoate (O,O-dimethyl S-[2-(methylamino)-2-oxoethyl] phosphorodithioate) to examine in detail because both are among the most commonly used in North America. Both are also routinely applied jointly or sequentially for the protection of more than 40 crops globally [2,3].

Chlorpyrifos and dimethoate are also highly toxic to non-target, aquatic species. According to van Wijngaarden *et al.* [4], the 48-h LC_{50} (median lethal concentration to affect 50% of the

population) for chlorpyrifos on the non-target mayfly, *Cloeon dipterum* is approximately 1 μg/L and similarly, Baekken and Aanes [5], report that the 96-hr LC_{50} for the mayfly, *Baetis rhodani*, is in the range of 7 μg/L for dimethoate. The third insecticide, imidacloprid (1-((6-Chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine), is also highly toxic to non-target aquatic species (e.g., the mayfly, *Epeorus longimanus* 24-h LC50 = 2.1 ± 0.5 μg/L, see [6]). Unlike chlorpyrifos and dimethoate however, the primary mode of action of imidacloprid is semi-permanent binding to the acetylcholine receptor rather than the ACh enzyme [7]. This difference may increase toxicity of the ternary mixture because all three insecticides bind the same enzyme and receptor system.

Organophosphorus insecticides are thought to primarily target the acetylcholinesterase (AChE) enzyme, preventing the removal of acetylcholine (ACh) by the enzyme from the post-synaptic gap [8]. Therefore, excessive acetylcholine is bound and continuous nerve signals are sent to cholinergic receptors, which can result in trembling, respiratory duress and ultimately death [8]. Notably, in order for most organophosphorus compounds to become toxic they must first be transformed into their active form, an oxon [9,10]. However, insecticides such as chlorpyrifos and dimethoate are chemically diverse and are able to interact with multiple metabolic pathways and targets. Therefore, indirect biochemical or ecological effects of these compounds may be responsible for observed differences in their toxicity [8,9,10].

In this study, two organophosphorous insecticides (chlorpyrifos and dimethoate) with the same primary mode of action were tested individually and jointly on a natural, macroinvertebrate assemblage using a toxic unit approach. The primary question asked was whether the joint-action of these two insecticides can be reasonably evaluated at a community level using additive assumptions of toxicity. This question was evaluated by determining the appropriate concentrations in toxic units of chlorpyrifos and dimethoate by compiling single-species toxicity test data for orders of insects commonly thought to be sensitive indicators in aquatic biomonitoring of streams and rivers namely, Ephemeroptera, Plecoptera and Trichoptera, or E.P.T. taxa. A 20 day artificial stream experiment was conducted where field-collected benthic (bottom-dwelling) macroinvertebrate assemblages were exposed to four toxic unit (TU) doses of either chlorpyrifos or dimethoate individually (control, 0.2, 0.4 and 0.8 TU) and two, 1:1 mixture doses (0.2 + 0.2 TU and 0.4 + 0.4 TU) of both insecticides applied jointly. Subsequently, responses in the benthos in a community were examined using Principle Components Analysis (PCA). Macroinvertebrate abundance, richness and guild structure was assessed using a factorial ANOVA and a chi-square (χ^2) approach to compare observed responses to control values as well as to predicted responses to treatment across a toxic unit gradient.

2. Methods

This 20-d study was conducted from 12 July to 2 August, 2007 at the Environment Canada mesocosm facility 10-km southeast of Fredericton (New Brunswick, Canada). Aquatic inver-

http://dx.doi.org/10.5772/53356

tebrates were collected in the Nashwaak River (sampling location: 46º14294´N, 66º36722´W). The Nashwaak River is a relatively pristine tributary of the larger Saint John River and runs more than 100 km through forested and rural communities of less than 500 inhabitants in central New Brunswick.

Subsampled invertebrate assemblages were inoculated into 88 outdoor, artificial streams (Figure 1, see also [11,12]). Each partial flow-through stream was circular and had a planar area of 0.065 m^2 and a 10-L volume. Three treatments of organophosphorus insecticides ($n_{replicates\ per\ treatment}$ = 8) were examined in detail: chlorpyrifos (control, 0.2, 0.4 and 0.8 TU), dimethoate (control, 0.2, 0.4 and 0.8 TU) and a 1:1 mixture of both insecticides (0.1 + 0.1, 0.2 + 0.2 and 0.4 + 0.4 TU). An additional ternary 1:1:1 mixture of all three insecticides was also examined as a pilot study and included imidacloprid as well as chlorpyrifos and dimethoate (0.1 + 0.1 + 0.1 TU). Treatment solutions were housed in polyethylene reservoirs and manifolds were used to distribute the treatment solutions at uniform flow rates to each replicate stream. Groundwater from the extensive Saint John River aquifer was used to provide water to the artificial streams. Wastewater from each stream was passed through carbon filters (Culligan Inc.; activated carbon filter cylinder, Moncton, NB, CAN) to remove all contaminants before any water was discharged to the environment.

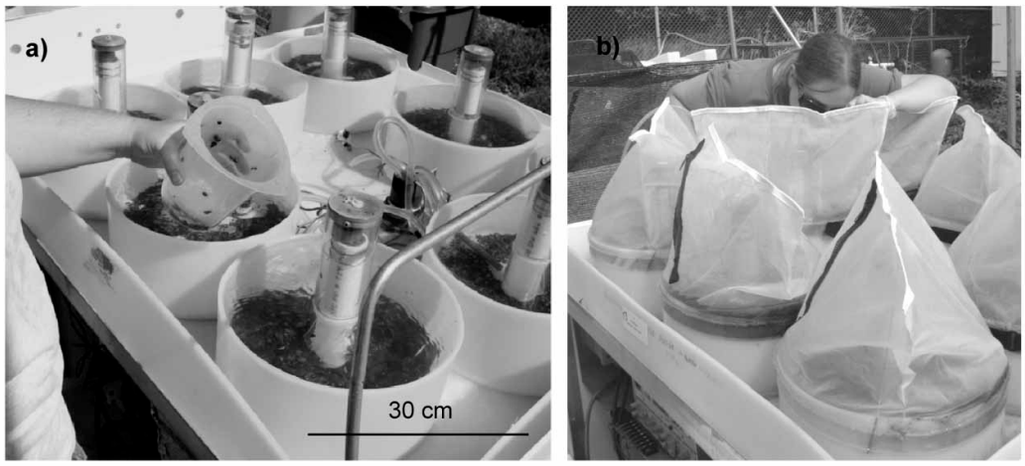

Figure 1. Cylindrical artificial streams. We inoculated 88 outdoor, artificial streams with a field-collected benthic invertebrate assemblage. Each flow-through stream was circular with a planar area of 0.065 m2 and a 10-L volume. In Fig. 1a, 8 streams were inoculated with gravel (coarse and fine) as well as 5 cobbles per stream. Protruding from the centre of each replicate stream is a motorized, rotating paddle that regulated the velocity of water in each stream. In Fig. 1b, streams post inoculation where each stream is covered with mesh to facilitate the collection of adult emergent insects.

2.1. Establishment of the aquatic community

2.1.1. Mimicking in-stream habitats

Prior to initiating the experiment, benthic substrates were introduced into each replicate stream. A realistic benthic substrate was created by inoculating each stream with a mix-

ture of 25% fine gravel (2 - 4 mm) and 75% gravel (4 - 30 mm) that was obtained from gravel beds adjacent to the invertebrate sampling site on the Nashwaak River (Figure 1a). Cobblestones (7-10 cm) were also collected from this site with five stones randomly assigned to each replicate stream. Cobble and gravel were gently washed to remove any attached invertebrates while maintaining the periphyton community. This procedure established a lotic substrate consisting of a 2-3 cm layer of gravel-cobble plus surface stones that were covered with periphyton and was similar to the original habitat of the benthic community examined (Figure 1a).

2.1.2. Field collection

Benthic invertebrates were collected in a single riffle upstream of the gravel collection site on the Nashwaak River with U-nets (area = 0.06 m^2). The subsampling procedure consisted of the collection of twenty-five (25) U-nets collected 8 times by 5 samplers working systematically upstream within the riffle. Twenty-five U-nets were selected to slightly increase (~10%) the ambient density of aquatic invertebrates in the artificial streams, thus offsetting any mortality due to transport from the river to the test site. Each set of 25 U-nets were divided into 16 community subsamples with 5 reference subsamples from each set retained to determine the initial composition of the aquatic community. Streams were systematically inoculated with a subsample from each of the 8 sets of the 25 U-nets collected. Such that each of the 11 treatments levels (low, medium, high or chlorpyrifos, dimethoate, binary mixture, as well as a single comparison of a low ternary mixture and the control) received a portion of the same stream assemblages collected in the field (Figure 2).

2.2. Establishment of treatments

The 96-h LC50s (as 95% C.I.) were estimated for chlorpyrifos (4.68 – 5.69 μg/L) and dimethoate (23.96 – 26.57 μg/L) by curve-fitting single-species, single-compound toxicity test data compiled from public databases (U.S. Environmental Protection Agency Ecotox database [13], Figure 3). Appropriateness of doses was also assessed using tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River [14]. For imidacloprid (96-h LC50 0.8 – 3.1 μg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous artificial stream studies in our region [15]. Only genera of the orders Ephemeroptera, Plecoptera and Trichoptera (E.P.T. taxa) were included in the estimated riverine community 96-h LC50 (the median lethal concentration that will affect 50% of E.P.T. taxa) because the abundance of these insects is generally thought to be indicative of healthy streams and is widely used in stream biomonitoring [16].

Insecticide solutions were mixed in agricultural grade stock tanks, a 2000-L stock tank for chlorpyrifos, a 520-L stock tank for dimethoate and a 200–L stock tank of each component of the ternary mixture. All solutions were mixed using groundwater from the extensive Saint John River aquifer. Stock solutions of chlorpyrifos (70 μg/L) were made by serial dilution of Lorsban -4E© (NAF-163, Dow AgroSciences, Indianapolis, IN, USA). Stock solutions of dimethoate (200 μg/L) were made by serial dilution of Lagon 480E © (9382, United Agri Prod-

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ucts Canada Inc., Dorchester, ON, Canada) and finally, imidacloprid (240 μg/L) by dilution of Admire 240® (Bayer CropScience, Calgary, AB, CAN). The insecticide-treated groundwater was delivered to one of eleven treatment reservoirs by positive displacement pumps (Viking Pumps, Pulsefeeder 25-H duplex pump, Cedar Falls, IA, USA). Secondary pumps then delivered the treatment solutions from each reservoir through a manifold to generate uniform flow rates into the base of each partial flow-through, replicate stream.

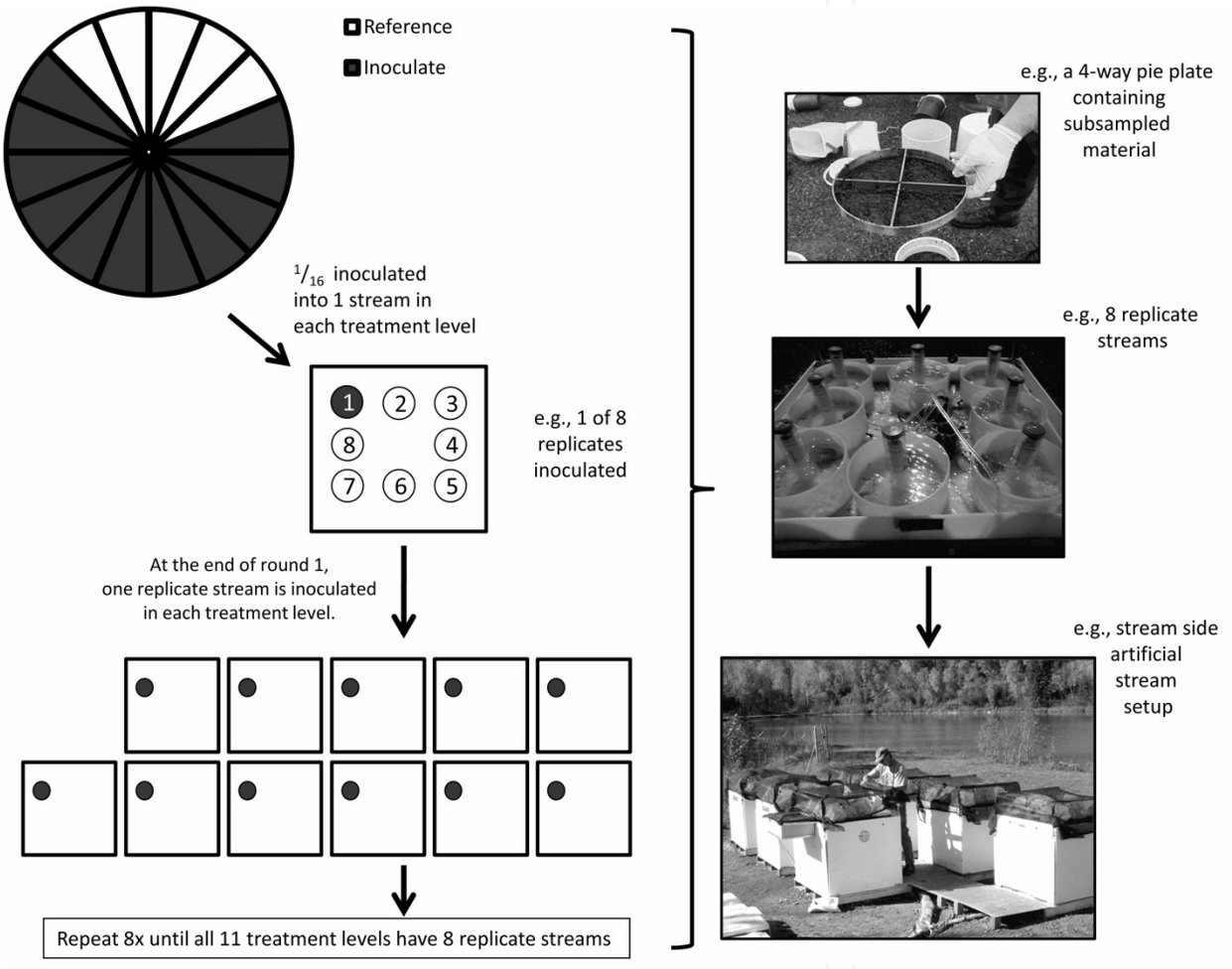

Figure 2. Benthic community subsampling and inoculation procedure for 88 replicate streams (11 treatments each containing 8 replicates). Sets of 25 U-nets (5 samplers collecting 5 U-nets each) were subsampled into 16 equal parts using a pie-plate made from 44 μm mesh. One sixteenth (1/16) of every 20 U-nets collected was inoculated into one replicate stream in every treatment level. This procedure was repeated eight times with each additional set of 25 U-nets systematically inoculated into adjacent replicate streams (one per treatment level). Thus, if the initial stream community had been significantly different in composition differences would have been allocated between treatments. Differences in community composition were not detected between subsamples (*Wilks-L* > 0.86; *P* > 0.99, in both cases).

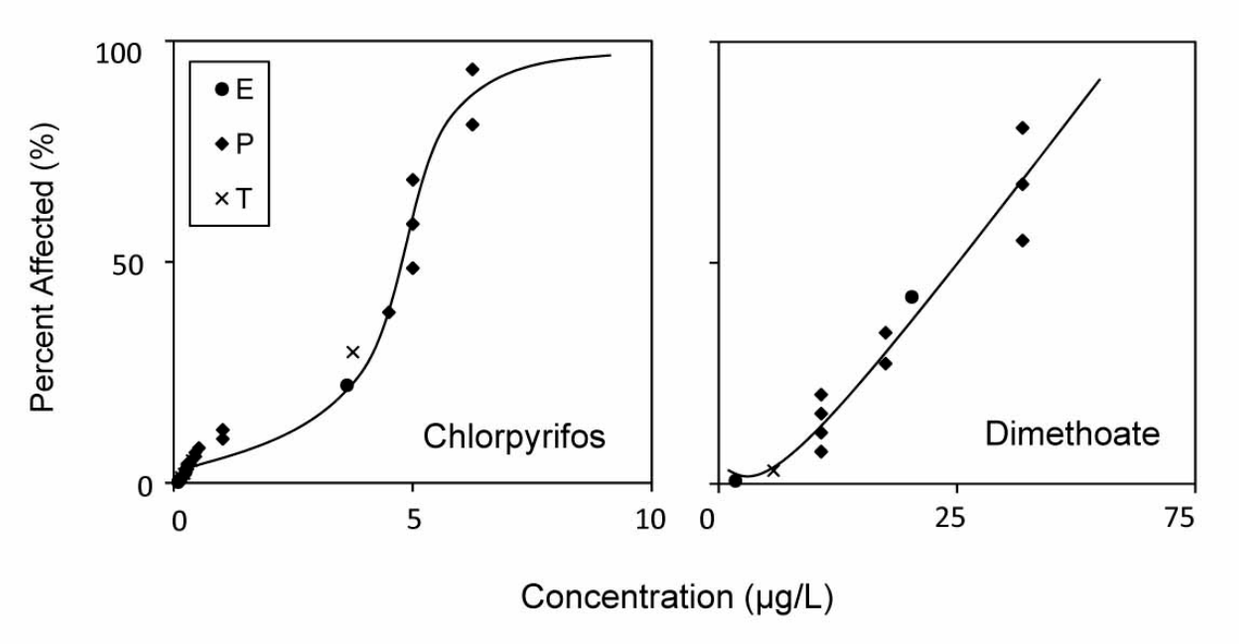

Figure 3. Percent Affected (96-h) of E.P.T. taxa as reported in the literature for the insecticides chlorpyrifos and dimethoate. For imidacloprid (96-h LC50 0.8 – 3.1 µg/L 95% C.I.), where less data was available, appropriate doses were determined in comparison to previous studies in our region [6,15]. Additional, tandem laboratory testing of chlorpyrifos and dimethoate on laboratory-reared *Chironomus tentans* and field-collected Heptageniidae mayflies from the Nashwaak River further corroborated dose selection [14]. Only genera of the E.P.T. Orders (Ephemeroptera, Plecoptera and Trichoptera) were used because the abundance of these insects is thought to be indicative of healthy stream conditions.

Chemical analysis determined the actual concentrations (Table 1) of the three insecticides individually and in mixture. Analyses were conducted at the National Water Research Institute (Environment Canada) in Saskatoon (SK, Canada) using a Waters 2695 Alliance HPLC System interfaced to a Micromass Quattro Ultima triple quadrupole mass spectrometer (LC-MS-MS) equipped with an electrospray ionization interface set to positive ion mode. For chlorpyrifos and dimethoate, chromatography was achieved using a Waters Xtera MS C_{18} (100 mm x 2.1 mm i.d., 3.5-µm particle size, Milford, MA, USA) analytical column and an aqueous acetonitrile mobile phase containing 0.1% formic acid (v/v). For imidacloprid, the mobile phase contained 40% aqueous acetonitrile and 0.2% formic acid (v/v). Water samples were collected in each treatment level on three occasions (July 13, 14, 17 in 2007) during the 96-h insecticide exposure period which began at noon on 13 July. Samples were collected in 500-mL amber vials (EPA vials, Fisher scientific, Fair Lawn, NJ, USA) and stored at 4ºC until shipment to Saskatoon for analysis. The samples were subjected to solid-phase (dimethoate) or liquid-phase (chlorpyrifos) extraction, the extracts taken to dryness, and the extract residue dissolved in deionized water (1.0 mL) prior to analysis by LC-MS-MS. All of the actual concentrations overlapped the target concentrations (Table 1) with an even distribution of under- and over- dosing for each target. Therefore, concentrations were comparable to those determined by laboratory bioassays in the published literature.

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Treatment in Toxic Units (TU)	0.2 TU	0.4 TU	0.8 TU	
Target [chlorpyrifos]	0.94 – 1.14	1.87 – 2.28	3.74 – 4.55	
Actual [chlorpyrifos]	0.47 – 1.31	1.64 – 2.70	2.41 – 6.89	
Target [dimethoate]	3.79 – 5.31	9.58 – 10.63	19.17 – 21.26	
Actual [dimethoate]	1.04 – 4.80	9.32 – 12.07	19.93 – 22.96	
Target [imidacloprid]	N/A	N/A	N/A	
Actual [imidacloprid]	N/A	N/A	N/A	
Mixtures in Toxic Units (TU x n)	**0.1 TU x 2**	**0.2 TU x 2**	**0.4 TU x 2**	**0.1 TU x 3**
Target [chlorpyrifos]	0.24 - 0.57	0.94 – 1.14	1.87 – 2.28	0.24 - 0.57
Actual [chlorpyrifos]	0.19 - 0.86	0.78 – 1.61	1.39 – 4.02	0.12 - 0.38
Target [dimethoate]	2.40 - 2.66	4.79 – 5.31	9.58 – 10.63	2.40 - 2.66
Actual [dimethoate]	2.13 – 3.54	2.36 – 5.88	8.18 – 16.43	2.18 - 2.80
Target [imidacloprid]				0.24 - 0.57
Actual [imidacloprid]				0.47 - 0.69

Table 1. Comparison of treatments in toxic units (TU) with respect to the 95% confidence interval (95% CI) of the estimated range of targeted doses and the actual concentrations for chlorpyrifos, dimethoate and the 1:1 binary (x2) mixtures of chlorpyrifos and dimethoate compared to 1:1:1 ternary (x3) insecticide mixtures of chlorpyrifos, dimethoate and imidacloprid. All concentrations are in µg/L. Target concentrations for each insecticide are presented as ranges to reflect the uncertainty in the LC50 estimate.

2.3. Final data collection

At the end of the 20-d experiment, the streams were dismantled and the contents collected. Water samples, periphyton samples and invertebrates were collected from each replicate stream. Benthic macroinvertebrates were collected from each stream and preserved (10% formalin, transferred to 70% ethanol after 1 week) for subsequent laboratory sorting and identification using dissecting microscopes (Leica© Microsystems Ltd., Cambridge, UK). Aquatic specimens were sorted and identified to genus at the end of the experiment according to Environment Canada protocols, with a minimum of 20% of the collected material checked by a certified taxonomist to achieve 95% confidence in the identifications [17]. Some taxa were only identified to Order given time constraints and available expertise (e.g., Oligochaeta, Nematoda, Gastropoda, Collembola and 1st instar Plecoptera). Guilds were inferred from the literature in order to infer the habits of organisms [16,18]. Adult insects were also collected over the course of the 20-d experiment in 2-d intervals and in some cases were used to corroborate the presence of cryptic genera.

2.4. Statistical approaches

Community responses were examined in the factorial portion of the experiment (chlorpyrifos x dimethoate) using Principal Components Analysis (PCA) because the data were continuous with respect to both of the treatment level factors of interest (e.g., actual

concentrations of insecticides) as well as the density of in-stream macroinvertebrates [19]. A correlation matrix was used to prevent the different variances in the variables to influence the analysis. Responses in different taxa and guilds were also examined using factorial ANOVA (for chlorpyrifos and dimethoate only) and chi-square (χ^2) approaches. In this study, factorial ANOVA approaches examined response variables with respect to explicit treatment categories: a gradient of toxic units (TU, throughout); different insecticide treatments (I) and the interaction between the dose and the insecticide treatments (TU x I). Post-hoc testing, where applicable, was conducted using 1-tailed Dunnett's tests [20] and compared specific treatments to control levels (ANOVA approach, marked 'a' in corresponding figures). Where necessary (e.g., total and scraper abundance), data were transformed to satisfy assumptions (ln transformation, [21]). Whether the treatments initiated predictable reductions in abundance (of taxa, groups or guilds) was examined by comparing observed differences to those expected (or predicted) using chi-square (χ^2) tests. Expected values were determined by calculating the predicted reduction compared to control values for each invertebrate metric, in abundance from the toxic unit treatment range. Predicted values with respect to control appear throughout and significant deviations from predicted values by the χ^2 approach are marked 'c' in the corresponding figures. Preliminary comparisons of differences between the low binary (0.1 TU x 2) and low ternary (0.1 TU x 3) mixtures (1-way ANOVA) are also made for the six response variables of interest with respect to control, predicted, binary and ternary mixture treatment levels. To simplify, although differences in density per cm^2 were tested for significance, the responses are shown as the percent reduction in response between the ternary and the binary mixtures at 0.1 TU.

3. Results

3.1. Responses to treatment with chlorpyrifos and dimethoate

Principal Components Analysis (PCA) of the 38 genera and 5 orders of benthic macroinvertebrates identified in this experiment were highly responsive to increasing TU treatment and responded differently to treatment with either chlorpyrifos or dimethoate (Figure 4). Factor 1, (Eigenvalue 7.08, 44.3% of variance) was composed of the combined loadings of treatment in toxic units (TU, Pearson's $r = 0.34$) as well as the action of chlorpyrifos (Pearson's $r = 0.58$) or dimethoate (Pearson's $r = -0.15$). Increased insecticide treatment in Toxic Units (TU) reduced the breadth of taxa present in the community assemblage, as indicated by the decreased variation in the distribution of taxa and guilds from left to right along the horizontal axis (Factor 1 in Figure 4). Interestingly, community responses to treatment with either chlorpyrifos or dimethoate were in opposing directions, although both insecticides were important contributors to the distribution of taxa, guilds and treatments in Factor 2 (Eigenvalue 2.36, 14.7%; TU, Pearson's $r = 0.01$; chlorpyrifos, Pearson's $r = -0.31$; dimethoate, Pearson's $r = 0.29$). In particular, chlorpyrifos was an important contributor to the removal of taxa with streams treated with 0.8 TU of chlorpyrifos (C0.8TU) occurring in the PCA quadrant with the fewest taxa (bottom right, Figure 4). By contrast, responses to treatment with dimethoate occurred in the opposite quadrant suggesting firstly, that different members of the benthic

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macroinvertebrate assemblage were responding to chlorpyrifos versus dimethoate, and that treatment with dimethoate did not decrease density and diversity of taxa as forcefully as treatment with chlorpyrifos (top left, Figure 4). Interestingly, medium dose mixture treatments (M0.4TU) are located in the same quadrant as the equivalent dimethoate treatments (e.g., D0.4TU and D0.8TU) whereas high dose mixtures (M0.8TU) were more closely associated with predictions of additive toxicity in toxic units (Factor 1).

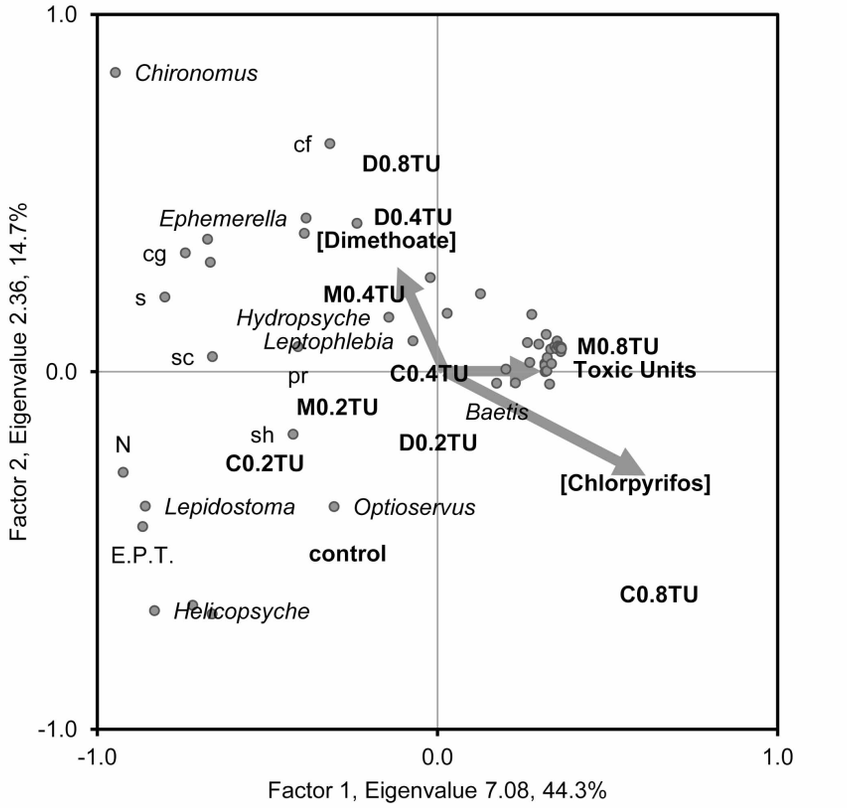

Figure 4. Principal Components Analysis (PCA) of differences in responses of 38 genera and 5 orders of benthic macroinvertebrates (each indicated, •) associated with chlorpyrifos or dimethoate insecticide treatment in Toxic Units (as vectors, above). Each treatment level is indicated (e.g., C0.2 TU, Chlorpyrifos at 0.2 TU). Factor 1 explained 44.3 % of the variance in the assemblages and was primarily driven by increased insecticide treatment in Toxic Units and secondarily by chlorpyrifos treatment. Dimethoate treatment was associated with different assemblages predominantly contributing to pattern in Factor 2 which explained an additional 14.7 % of the variance. Additional notes: guilds are indicated by codes cf = collector-filterers; cg = collector-gatherers; sc = scrapers; sh = shredders; pr = predators; total abundance per cm^2 = N; total richness per cm^2 = s; E.P.T. = sum density of Ephemeroptera, Plecoptera and Trichoptera orders. Remaining labels indicate genera of aquatic insect taxa (e.g., *Chironomus* spp.).

Significant change in measures of average total density per cm^2 and average taxa richness per cm^2 (Figure 5) were only found at the highest dose of chlorpyrifos tested (0.8 TU, abundance or richness, $P < 0.01$). The highly significant interactions (total density, TU x I, $F_{5, 69} = 68.23$, $P < 0.01$; or richness, TU x I, $F_{5, 69} = 709.03$, $P < 0.01$) were the result of total density and richness being decreased as predicted under exposure to chlorpyrifos, while dimethoate had no such effect. Throughout this study, dimethoate was non-toxic with respect to total density and richness and no negative effects of insecticides were detected irrespective of dose.

Additionally, mixture treatments were not different than control levels for either total density or richness (e.g., total density in M0.8TU, $P = 0.97$; richness in M0.8TU, $P = 0.75$). Stream communities were significantly more dense than predicted in high dose treatments containing dimethoate including the high mixture (M0.8TU, $\chi^2_{\ 7} = 20.24$, $P < 0.01$) and the high dimethoate treatment (D0.8TU, $\chi^2_{\ 7} = 16.90$, $P < 0.01$). In contrast, taxa richness was not found to be significantly different than predicted.

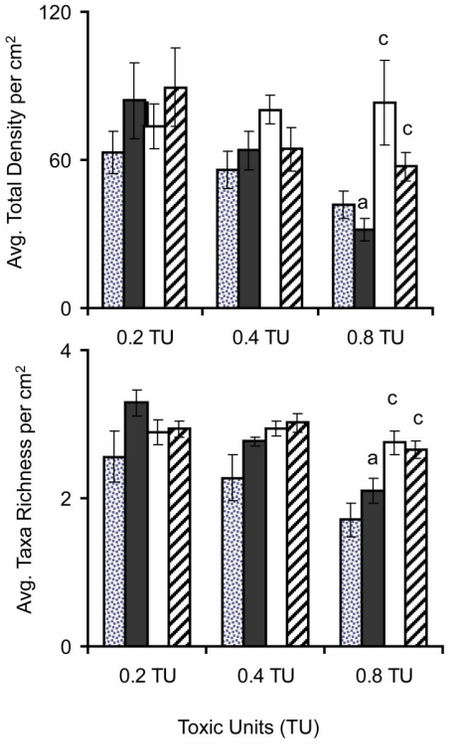

Figure 5. Total abundance and richness per cm² (± 1 SE, n = 8) of aquatic macroinvertebrates compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

Responses in the average density of E.P.T. taxa and *Chironomus* spp. per cm² (Figure 6) were only found to significantly differ from control values in the highest chlorpyrifos treatment level (0.8 TU, E.P.T. or *Chironomus*, $P < 0.01$). Highly significant interactions were evident (E.P.T., TU x I, $F_{5, 69} = 53.91$, $P < 0.01$; or *Chironomus*, TU x I, $F_{5, 69} = 50.02$, $P < 0.01$) because density of E.P.T. and *Chironomus* decreased due to chlorpyrifos but not due to dimethoate. However, *Chironomus* midges were highly negatively affected by 0.8 TU of chlorpyrifos and the mean density of larvae in this treatment level was reduced 96% compared to controls (predicted decrease at 0.8 TU = 40%; C0.8TU, $\chi^2_{\ 7} = 31.45$, $P < 0.01$). E.P.T. taxa were highly sensitive to high dose treatment with chlorpyrifos (C0.8TU, $\chi^2_{\ 7} = 12.75$, $P < 0.01$), however, treatments containing dimethoate (e.g., dimethoate and mixture) were much less toxic than predicted (e.g., mean E.P.T. density in 0.8TU mixture, 37 % greater than predicted).

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Scraper density was not different than the control, although predators were highly responsive to all high dose insecticide treatments ($P < 0.01$, Figure 6). Once again, significant interactions were found for both guilds (scrapers, TU x I, $F_{5, 69} = 12.46$, $P < 0.01$; predators, TU x I, $F_{5, 69} = 26.35$, $P < 0.01$). However, the extent of significant interactions in scraper genera appeared to be largely due to the high variation in the density of the guild in the low dose, chlorpyrifos treatment (0.2 TU). Doses of 0.2 to 0.4 TU of chlorpyrifos and 0.2 TU of dimethoate all contained more scrapers than predicted (e.g., 74 % greater than predicted scraper density in chlorpyrifos 0.2 TU, $\chi^2_{7} = 50.03$, $P < 0.01$). In contrast, responses in predators were unique in that they responded to high insecticide doses (0.8 TU) by significantly decreasing abundance in these treatments, irrespective of the insecticide applied (e.g., 0.8TU mixture, 46 % less than predicted, $\chi^2_{7} = 28.38$, $P < 0.01$). Finally, the bell-shaped abundance pattern in predators with increased dimethoate treatment, compared with the linear decrease in abundance of the chlorpyrifos treatment, suggests that responses in predators were more complex than in other groups, potentially as a result of indirect effects due to reduced prey density.

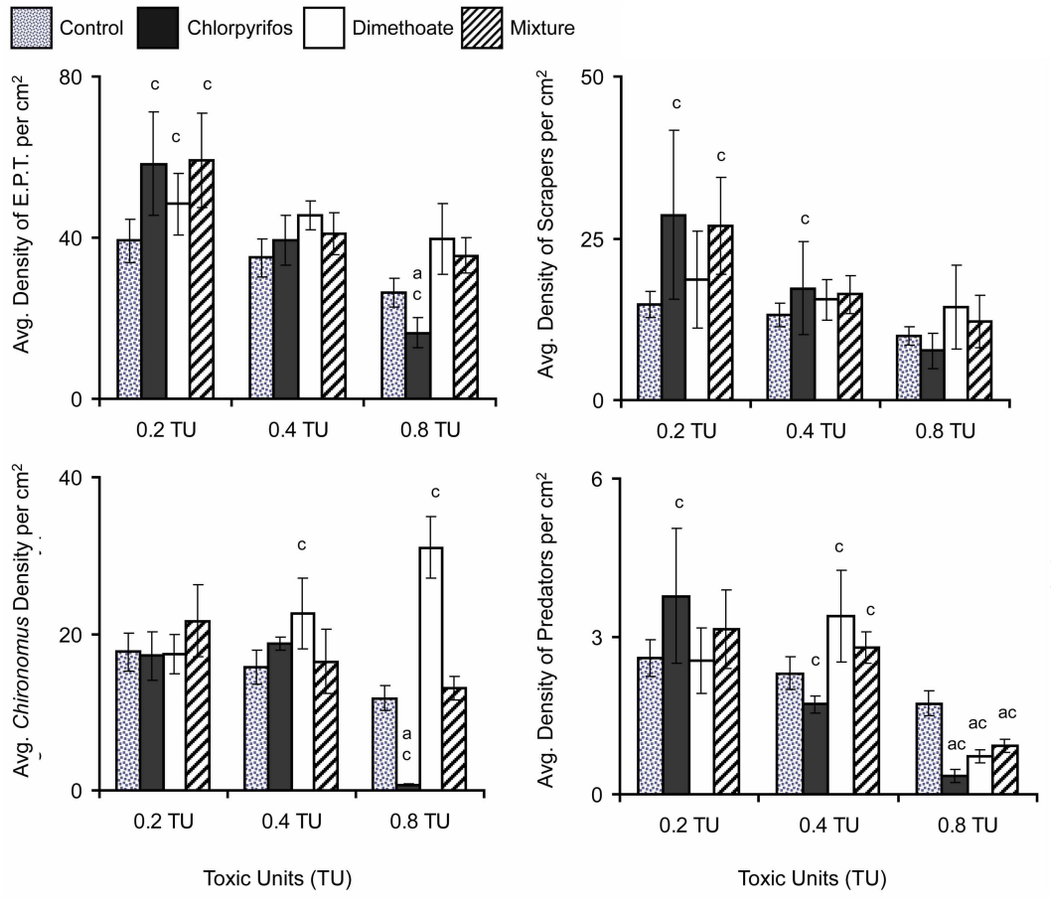

Figure 6. Density of E.P.T., Chironomus spp., scrapers and predators per cm² (± 1 SE, n = 8) compared to treatment with the insecticides chlorpyrifos (black bars), dimethoate (white bars) or a 1:1 mixture of both insecticides (patterned bars). Letters indicate: 'a' significant differences compared to control (ANOVA approach), and 'c' differences in specific treatments (χ2 approach).

3.2. Preliminary findings comparing binary and ternary mixtures

Statistical comparisons of the differences in density between binary (0.1 TU x 2) and ternary (0.1 TU x 3) mixtures of insecticides determined that the average total density (*P = 0.02*), taxa richness (*P < 0.01*) and *Chironomus* spp. (*P < 0.01*) were all significantly reduced due to the addition of imidacloprid to the mixture (Figure 7). In contrast, the average density of E.P.T. genera, scrapers and predators were not found to be significantly reduced in the presence of imidacloprid (*P > 0.06*, all cases). On average, the addition of a third insecticide resulted in a 62.9 ± 13.0 % reduction in average density. Density was more greatly reduced in some groups than others with scrapers the most affected (-111.6 ± 16.9 %) and taxa richness the least affected (-18.2 ± 16.5 %).

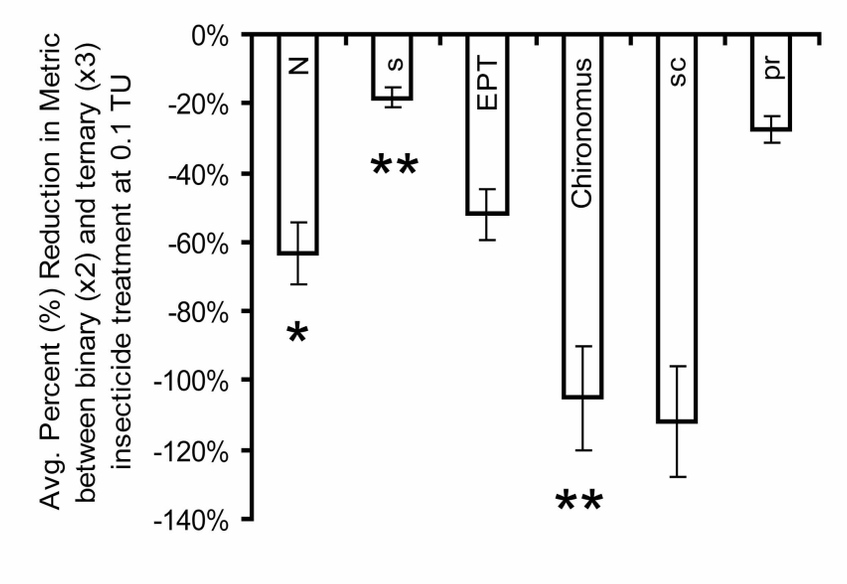

Figure 7. Comparison of % reduction in metrics due to treatment with the ternary mixture of 0.1 TU versus the binary mixture with the same doses. Each 0.1 TU dose should reduce the density of sensitive taxa by 5% because 1 TU = LC50. Therefore, reductions greater than 5% in the density of aquatic taxa is of biological interest even if differences in the density of organisms were not found to be statistically significant.

4. Discussion

4.1. Responses to chlorpyrifos and dimethoate

All of the metrics of benthic invertebrate responses measured also had significant interaction terms (TU x I, *P* < 0.1) suggesting that not all taxa, groups or guilds were equally sensitive to insecticide treatment. Differential toxicity within the organophosphorus insecticides has been reported previously and is predominantly due to the complexity of the biochemical pathway to reach what is considered the primary target, acetylcholinesterase [8,9,10]. Specifically, the toxic potency of organophosphorus insecticides depends on the creation of an

oxygen analogue (oxon) via metabolic bioactivation, creating an excretable endproduct which is also potentially toxic [9]. It is the oxon that binds acetylcholinesterase (AChE) and prevents the capture and removal of acetylcholine in the synapses, creating a positive feedback loop whereby uncontrolled neural signalling is initiated. Therefore, increased or decreased toxicity, even from the standpoint of a single mode of action (AChE), is due to the interaction of at least five factors: firstly, in/efficient creation of the oxygen analogue (oxon), i.e., differences in basal metabolism; secondly, insufficient binding of the target esterase(s) and/or binding to alternative targets; thirdly, insufficient accumulation of acetylcholine in the synaptic gap, due to inherent neurochemical differences or deficiencies, e.g., Myasthenia gravis; fourthly, other forms of tolerance and/or resistance, e.g., species, strain or regional differences (e.g., as reported in [22]), and finally, excretion and/or uptake efficiency of the parent toxicant or its metabolites. Furthermore, organophosphates also bind other receptors (e.g., muscarinic and nicotinic receptors), which in themselves can up or down regulate the effectiveness of the insecticide dose [23].

Despite the equivalent toxic unit doses employed in this study, treatment with dimethoate was associated with increased abundance of different taxa and guilds with the exception of predators, which were found to be substantially negatively impacted by all high dose treatments. In mixture treatments, the density of taxa often fell between that of either of the two insecticides individually, or, resembled the relatively non-toxic dimethoate at 0.4 and 0.8 TU. The highly significant declines in abundance of different taxa and guilds due to chlorpyrifos treatment, and the lack of similar findings due to dimethoate treatment are troubling because this study determined the appropriate doses from standard bioassays of the same genera from public databases of the published literature. For instance, according to a Norwegian study by Baekken and Aanes [5], the 96-hr LC_{50} for *Baetis rhodani* exposed to dimethoate was ~ 7 μg/L. In this study *Baetis* not only survived but emerged as adults (37 females and 26 males, not shown) in the 0.8 TU treatment where the dimethoate concentration was in the range of 19.93 – 22.96 μg/L. Disparities such as these invite speculation. If regional differences in sensitivity are as pronounced as the above finding suggests, then modeling may be restricted to more local scales. Alternatively, regional variation in data quality also invites speculation.

This study generally found that the mixture pattern at high doses had intermediate toxicity. Specifically, invertebrate responses to the binary mixtures were between that of dimethoate or chlorpyrifos individually. LeBlanc *et al.* [14] also found mixtures of chlorpyrifos and dimethoate to exhibit dose-level dependency in concurrent laboratory studies using chlorpyrifos and dimethoate in both binary mixtures (i.e., low dose antagonism to high dose synergy). Although high dose exposures are likely less common than sublethal effects (as described in [24]), high dose synergy is a concern because isolated high-dose events (e.g., a rain event) could significantly alter the composition of aquatic communities. Additionally, in more complex mixtures where multiple modes of action may be the norm, the concentration that initiates a synergistic effect may be lower than implied from bioassay results using single-species and single compounds.

4.2. Preliminary findings for responses in binary versus ternary mixtures

In this study, the addition of a third insecticide at 0.1 TU resulted in an average reduction in invertebrate density of approximately 60% (-62.9 ± 13.0%). However, the addition of 0.1TU of imidacloprid should, in theory, only result in a reduction of 5% in the abundance of organisms because 0.1 TU equals the 5% median lethal concentration or the LC5. Therefore, average density was reduced 50% more with the addition of one more insecticide to the mixture despite the addition occurring at what would otherwise be considered a very low dose. The implication of these findings is that the presence of imidacloprid in a mixture, an insecticide with a similar mode of action to chlorpyrifos and dimethoate, may cause significantly greater than additive reductions in invertebrate density in naturally occuring assemblages such as those tested in this study. These findings are similar to those of Leblanc *et al.* [14] where the combined action of imidacloprid resulted in greater than additive toxicity of mixtures of the same insecticides used in this study.

Although we did not detect significant differences when comparing the density of predators in low dose binary versus ternary mixtures, responses in groups such as predators continue to be of interest because of the importance of certain feeding groups in food webs (e.g., see [25]). For predators, the average percent reduction in density was -27.4 ± 9.9% at a dose that in theory will cause a 15% reduction in density (0.3 TU = LC15). However, if the addition of one insecticide can cause (at best) a 30% reduction in density, then what effects are likely for more complex mixtures acting on highly interconnected aquatic communities? Gilliom has previously reported that mixtures of up to 5 insecticides are routinely found in the environment [1]. If the patterns found in this study are true of more complex mixtures, then 5 insecticides at 0.1 TU could remove more than half the invertebrate population (> LC50) at individual doses that are thought to cause a mere 5% reduction in density. Clearly, further study of the effects of mixtures on keystone species, such as predators, will be important for untangling community responses to multiple stressors.

4.3. Implications to additive models: a biological argument

It is questionable whether additive predictions of responses can be made for these insecticides despite having the same (or similar) primary modes of action. Clearly, chlorpyrifos and dimethoate were not sufficiently similar in their actions on organisms in the community assemblage studied here to warrant additive treatment, even though their effects may be similar *in vitro*. In this study, dose-level dependency and genus or guild specific differences were the norm. Therefore, although the use of additivity to predict effects of insecticide mixtures has the appeal of simplicity, pest managers and regulators may be better informed by focused study of common mixtures of multiple compounds on relevant assemblages of organisms. Differences in sensitivity and tolerance may be region or system specific due to the predisposition of different populations to up or down-regulate the production of alternative substrates to which these insecticides can bind [9,26,27].

Thus, arbitrary grouping of two similar insecticides based on their primary mode of action, is inappropriate, particularly in an ecological context. Although grouping organophosphorus insecticides to model responses additively has been demonstrated to be appropriate

chemically (as in [28,29]), there appears to be little empirical evidence to support the uniform toxicity, or activity of organophosphorus compounds in biota (see [9]). Rather, non-additive responses appear to be the norm in real systems, perhaps because effects in real systems are mediated by biotic filters such as trait-mediated indirect effects [30,31]. We suggest that grouping these compounds into potency subclasses, as first suggested by Mileson *et al.* [23] will aid modelling efforts to overcome dose dependent effects of similar mixtures with variable potency. This is particularly warranted because dose-dependency appears to be a common mixture pattern [32]. Although concentration addition is widely thought to be a conservative approach to modelling impacts in streams (as in [33]), regional differences in sensitivity, or alternatively data quality, will reduce the usefulness of additive models. Finally, current toxicological models such as concentration addition and independent action, do not consider biological interactions between species. Interactions between species in a community can increase or mask organismal responses to stress and may be more important than isolated laboratory responses for the prediction of community level patterns.

5. Conclusions

In this study, when chlorpyrifos and dimethoate were both applied these mixtures were often intermediately toxic to aquatic invertebrates with the exception of predators that were severely impacted by all elevated insecticide treatments. In contrast, ternary mixtures were generally more toxic than expected and predators were highly affected even at the very low doses tested. Although only an additional 0.1 TU (= LC5) was added of a third insecticide, imidacloprid, responses in the density of different benthic macroinvertebrate metrics were reduced on average by more than 20%. From a community standpoint, it is apparent that different taxa and guilds within the macroinvertebrate community tested were not equally sensitive to treatment with different insecticides despite the use of equivalent toxic unit doses drawn from published bioassays on the same genera of aquatic insects as those examined in this study. As such, additive assumptions of toxicity in a community context are questionable. This is particularly true given that the interactions between species are rarely measured in ecotoxicology and thus, significant biological effects are likely ignored. Pest managers and regulators concerned with the impact of complex mixtures on naturally occurring communities may be better informed by focused study of common mixtures of multiple compounds on locally and regionally relevant assemblages of organisms than predictions derived from laboratory based mode of action models.

Acknowledgements

Many thanks to Kristie Heard (Environment Canada, Fredericton) for her assistance with the taxonomy and subsampling procedure, to Dave Hryn (Environment Canada, Fredericton) for his technical expertise and assistance in conducting the artificial stream experiment, and to Jon Bailey (Environment Canada, Saskatoon) who conducted the chemical analyses.

This work was made possible by the support of Environment Canada and a grant from Health Canada's Pesticide Science Fund. Further support was provided by a National Sciences and Engineering Research Council (NSERC) Discovery Grant to JMC and a NSERC (PGS-D3) to A.C. Alexander.

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