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THE DIRECT AND INDIRECT EFFECTS OF A GLYPHOSATE-BASED HERBICIDE AND NUTRIENTS ON CHIRONOMIDAE (DIPTERA) EMERGING FROM SMALL WETLANDS

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Abstract: Laboratory and mesocosm experiments have demonstrated that some glyphosate-based herbicides can have negative effects on benthic invertebrate species. Although these herbicides are among the most widely used in agriculture, there have been few multiple-stressor, natural system–based investigations of the impacts of glyphosate-based herbicides in combination with fertilizers on the emergence patterns of chironomids from wetlands. Using a replicated, split-wetland experiment, the authors examined the effects of 2 nominal concentrations (2.88 mg acid equivalents/L and 0.21 mg acid equivalents/L) of the glyphosate herbicide Roundup WeatherMax, alone or in combination with nutrient additions, on the emergence of Chironomidae (Diptera) before and after herbicide-induced damage to macrophytes. There were no direct effects of treatment on the structure of the Chironomidae community or on the overall emergence rates. However, after macrophyte cover declined as a result of herbicide application, there were statistically significant increases in emergence in all but the highest herbicide treatment, which had also received no nutrients. There was a negative relationship between chironomid abundance and macrophyte cover on the treated sides of wetlands. Fertilizer application did not appear to compound the effects of the herbicide treatments. Although direct toxicity of Roundup WeatherMax was not apparent, the authors observed longer-term impacts, suggesting that the indirect effects of this herbicide deserve more consideration when assessing the ecological risk of using herbicides in proximity to wetlands. *Environ Toxicol Chem* 2014;33:2076–2085. © 2014 SETAC

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INTRODUCTION

Wetlands support diverse insect communities, of which chironomids (Diptera: Chironomidae) are a large component [1]. Chironomids are a vital food source for terrestrial birds [2], and the emergence of adult chironomids represents a major flow of energy and nutrients from aquatic to terrestrial systems [3]. Worldwide wetland loss has been estimated at approximately 50%, the majority of which is the result of conversion of land to agriculture [4]. Wetland restoration has been specifically targeted in agricultural areas [5] because wetlands have been shown to reduce nutrients [6] and increase diversity [7]; and in regions where wetlands have become scarce, they can serve as breeding hot spots to support our declining amphibian populations [8]. Wetlands in agricultural regions are susceptible to contamination from herbicide applications. Glyphosate-based herbicides are among the most popular herbicides used globally for control of broad-leafed weeds [9]. Herbicides can make their way into wetlands through agricultural runoff, spray drift, or accidental overspray. Early laboratory studies of the toxicity of glyphosate-based herbicides to aquatic species demonstrated some negative effects on fish and aquatic invertebrates [10]. For several animal species, the toxicity of these herbicides has been ascribed to the surfactants in the formulation [11]. Despite the crucial need for natural system–based studies of the impacts of glyphosate-based herbicides on wetland insects, very few have been undertaken.

Extensive monitoring programs in Canadian agricultural wetlands have found that glyphosate may reach maximum concentrations of 40.8 μg acid equivalents (a.e.)/L in wetlands typical of agricultural drainage systems [12]. Although laboratory studies have demonstrated that formulated glyphosate products are acutely toxic to larval dipteran species, this only occurs at concentrations that are orders of magnitude higher than those measured in wetlands. More specifically, acute toxicity of formulated glyphosate herbicides has been reported between 8 mg a.e./L (48-h median lethal concentration [LC50]; Rodeo) and 18 mg a.e./L (48-h median effective concentration [EC50]; Roundup) [10,13,14] for larval *Chironomus* spp. (Diptera: Chironomidae). Without surfactants, the LC50 of technical-grade glyphosate to chironomids has been observed at 55 mg a.e./L [13].

Direct toxicity of glyphosate herbicides to invertebrates is unlikely to occur in natural systems. However, indirect effects caused by changes in the food sources or physical structure of the system, as well as interactions with other co-occurring contaminants, are possible and more readily addressed using large field experiments than mesocosms or laboratory research. Herbicides are designed to reduce vegetation. If the macrophyte structure is impacted by these herbicides, then this is likely to indirectly affect wetland invertebrates [15]. Aquatic macrophytes provide attachment sites and materials for protective retreats, affect rates of predation and food availability, and even provide cues for colonization to ovipositing insects [16–18]. Larval chironomid communities become more abundant and less diverse in wetlands that have been experimentally manipulated to remove emergent vegetation [16].

In aquatic ecosystems affected by anthropogenic contamination, glyphosate-based herbicides are likely to be present in combination with other agricultural chemicals such as nutrients from fertilizers. "Eutrophication" is the term used to describe the ecological effects of excess nutrients in aquatic ecosystems and leads to degradation of the habitat and water quality [19].

All Supplemental Data may be found in the online version of this article.
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Chironomids tend to dominate benthic invertebrate communities in wetlands degraded by eutrophication [20]. Periphyton mats that may dominate in eutrophic conditions provide abundant attachment sites and food resources for tube-dwelling chironomids [21]. Additionally, because both nutrients and herbicides affect the food sources and habitat of Chironomidae [15,20], the net effect on these organisms should be the sum of both direct and indirect effects.

In the present study, a replicated, split-wetland experiment was designed to determine the impact of an agricultural-use glyphosate-based herbicide, Roundup WeatherMax, alone and in combination with nutrients, on the abundance and the composition of the Chironomidae assemblage as it emerged from small wetlands. We examined the predictions of 3 hypotheses. First, if Roundup WeatherMax has a direct toxic effect on the chironomids, this would be expected to produce a decline in abundance or an alteration in the composition of the chironomid assemblage in samples collected immediately following treatment application but before there is a significant change in the emergent plant cover. Second, if there are indirect effects of glyphosate-based herbicides on Chironomidae resulting from the reduction of emergent plant structure, we would expect to observe an increase in the abundance of emerging chironomids, a negative correlation between macrophyte cover and chironomid emergence from treated wetlands, and a shift in the assemblage, because both herbicides and nutrients should produce conditions under which Chironomidae tend to dominate. Finally, if nutrient additions have an interactive effect with the glyphosate-based herbicides, it would be demonstrated as significantly different effects in wetlands receiving a combination of herbicides and nutrients from that in wetlands receiving only herbicides.

MATERIALS AND METHODS

Site description

The 24 wetlands used in the present study were located in the Long-Term Experimental Wetlands area, a 4-km^2 area on Canadian Forces Base Gagetown, approximately 60 km northwest of Saint John, New Brunswick, Canada (66°29′59.02″W, 45°40′48.62″N). This area was mechanically cleared of most forest cover over a 2-yr period in 1997 to 1998, and windrows were created from the surface soils. Wetlands formed in depressions and next to windrows because of the disruption of drainage patterns of the area. This area had never been treated with any chemical pesticides prior to the present study.

Barrier design and chemical treatments

Wetlands were divided with 30-mil (0.76-mm) thick, opaque, high-density polyethylene (HDPE) geomembrane (Poly-Flex Geomembrane Lining Systems) sheeting. Barriers were approximately 1 m in height and had sealed pockets filled with crushed gravel along the bottom to anchor into the sediments. These barriers were installed in August 2008 in all wetlands, stretching the entire length of the wetland and extending beyond the high water mark.

Chemical treatments were applied in 2009, designed to mimic a chemical application scenario experienced by small agricultural wetlands. A randomly selected half of each of the 24 divided experimental wetlands was hand-sprayed with the agricultural-use herbicide Roundup WeatherMax. Herbicides were obtained directly from Monsanto Canada. One-half of the wetlands ($n = 12$) received a predicted maximum environmental concentration of 2.88 mg a.e./L. For simplicity, this treatment level is hereafter termed the "higher" (H) glyphosate concentration. This nominal concentration was chosen based on the predicted environmental concentration of the active chemical in a 15-cm water depth if a wetland had been directly oversprayed with no intercepting vegetation at the maximum label rate for use on perennial weeds (4.32 kg a.e./ha). The remaining 12 wetlands received an environmentally realistic concentration (0.21 mg a.e./L) of the herbicide. For simplicity this treatment level is hereafter termed the "lower" (L) glyphosate concentration. This concentration was based on the concentrations measured in wetlands near agricultural fields in south-western Ontario [22]. Two separate herbicide treatments were conducted over 2 consecutive days each (12 wetlands each day) on 15 and 16 May 2009, and 9 and 10 June 2009. The number and timing of herbicide treatments were consistent with common agricultural practices in the region. All control sides of wetlands were simultaneously sprayed with uncontaminated wetland water, using a separate sprayer, every time applications were made to the treated sides of wetlands.

One-half of each glyphosate treatment group was also hand-sprayed with nutrients commonly used in inorganic agricultural fertilizers (technical grades of ammonium nitrate and phosphoric acid; purchased from Fisher Scientific). For simplicity, these wetlands will be termed "higher glyphosate concentration plus nutrients" (HN) and "lower glyphosate concentration plus nutrients" (LN). Nutrients were added on each of 16–17 May, 29 May, 3 July, and 19 August 2009. Wetlands were generally classified as oligotrophic/mesotrophic; nutrients were added to increase the aqueous total Kjeldahl nitrogen concentrations to a higher trophic status, and phosphorus was added to maintain the ratio of total Kjeldahl nitrogen to total phosphorus, based on background concentrations and wetland volume. Wetland volumes were calculated immediately prior to nutrient additions. Nutrient additions were made by dissolving the required amount of ammonium nitrate and phosphoric acid in approximately 3 L of wetland water and applying it evenly to the surface of the wetland with a backpack sprayer (Flowmaster; Root-Lowell Manufacturing). The full experimental design consisted of 6 H wetland halves, 6 HN wetland halves, 6 L wetland halves, and 6 LN wetland halves, each with a paired untreated half (48 experimental units in total), except in August, when only the wetlands containing water could be sampled. The August numbers available for each treatment category are as follows: H ($n = 3,3$); HN ($n = 4,4$); L ($n = 3,3$); and LN ($n = 3,3$).

Quantification of nutrient and glyphosate concentrations

Water samples were collected from all experimental units every 2 wk through the course of the summer and frozen in clean HDPE bottles for nutrient analysis ($n = 1$/wetland half/date). Ammonium (NH_4^+-N) concentrations were measured by the Agriculture and Food Laboratory at the University of Guelph, Guelph, Ontario, Canada, following the US Environmental Protection Agency method, using a modified Berthelot reaction [23], on a SEAL AQ2 automated discrete nutrient analyzer (colorimeter; SEAL Analytical). Total phosphorus concentrations were measured by the Research and Productivity Council, Fredericton, New Brunswick, Canada. Method detection limits were 0.05 mg/L (NH_4^+-N) and 2 μg/L (total phosphorus).

Glyphosate concentrations were measured in water samples collected from both sides of each wetland on days 1, 3, and 7 after the first round of treatment and on days 0, 3, and 7 after the second round of treatment. Samples of the water column

from 5 sites in each wetland half were composited in ethylene oxide-sterilized 50 mL polycarbonate centrifuge tubes and stored frozen until analysis ($n = 1$/wetland half/date). Glyphosate residues were quantified using a gas chromatograph (GC; Hewlett Packard HP 5890 Series II) with a nitrogen–phosphorus detector by the Great Lakes Forestry Centre, Sault Ste. Marie, Ontario, Canada. Known amounts of glufosinate ammonium were used as an internal standard, and concentrations of glyphosate were calculated in comparison to this standard. The quality of the method was checked by running natural field water samples spiked with known amounts of glyphosate and blank matrix samples. Percent recovery of spiked samples was within acceptable limits (87.6%, $n = 18$); the detection limit was 5 μg/L, and the limit of quantitation was 17 μg/L. All values ($n = 21$ of 72) below the detection limit were replaced with the value of the detection limit/ 2, which produces no bias in a small data set when approximately 25% of the values fall below the detection limit [24]. The half-life of glyphosate in each treated wetland was calculated using the nominal concentration as the initial concentration (N_0), and the measured concentration (N_t) 3 d (t) after addition, with the formula

$$t_{1/2} = t/[\log_2(N_0/N_t)]$$

Wetland plant surveys

The percent cover of both emergent and submerged macrophyte species were estimated visually from multiple locations around the wetland. Data were standardized using a comparison chart for visual estimation of percent cover, and plants were identified to species [25,26]. Herbicide treatments were conducted early in the growing season, before sufficiently mature plant material was available to estimate percent cover. For this reason, plant surveys were conducted in late May, between the initial and final herbicide treatments, and twice more after the final herbicide treatment, in late June and in August 2009. The May plant survey was not done at the same time as the chironomid sampling, and therefore, these data were not compared statistically.

Chironomidae emergence collection

Emerging adult chironomids were collected from wetlands with preservative-free emergence traps. Each emergence trap consisted of a cone constructed of 200-μm mesh size, sheer, white, polyester fabric. A bamboo stake was installed in the sediment of the wetland, and the trap was placed over the stake so that the open end of the trap was resting against the water surface, enclosing an area of 706 cm^2. Traps were deployed for 4 d to 7 d and frozen for at least 24 h to kill chironomids. Whole chironomids were removed, preserved in 70% ethanol, enumerated, and identified to the subfamily/tribe level using a dissecting microscope [27]. On each side of each wetland, 3 to 5 traps were deployed.

Sets of traps were deployed in each experimental unit (each wetland half) once before herbicide treatment, in spring 2009 (6–12 May 2009), and 3 times after both herbicide treatments (25 June–4 July 2009, 29 July–14 Aug 2009, and 13–23 April 2010). Spring arrived earlier in 2010 than in 2009; thus, the timing of temporally appropriate replicates was based on the comparative phenology of wood frogs (*Lithobates sylvaticus*), an important indicator species in the wetlands. The abundance of emerging chironomids from each experimental unit from each deployment was standardized to the average number per square meter per day.

Statistical analysis

Whole-system experiments with practical/logistical constraints on replication and high levels of natural variability will often have high probabilities of type II error relative to the 5% level of type I error resulting from use of the standard α level, 0.05. Because there is no strong rationale for why type II errors should be considered less serious than type I errors in the present study, holding α at 0.05 and causing low statistical power constitutes a bias against detecting biologically meaningful effects if they exist, in favor of increasing the likelihood that the effects are real if they are detected [28]. To guard against the unnecessarily high levels of type II errors, we used test-specific optimal α levels that minimize the combined probabilities of type I and type II errors [29] throughout the present study.

For all analyses, the optimal α level was calculated based on a method developed by Mudge et al. [29], which balances the relative probabilities of type I and II errors to minimize the overall probability of making a wrong conclusion (the smallest average of α and $\beta = \omega$) at the designated critical effect size. The critical effect size for 2-tailed tests was set as a difference between treated and control means that is greater than 1.64 standard deviations (SD) of the control data. In other words, we were interested in detecting a treated mean falling outside of 90% of the predicted control distribution centered on the control mean. Similarly, the critical effect size for 1-tailed tests was set as a difference between treated and control means of 1.28 SD of the control data, which gives us the ability to detect a treated mean falling outside 90% of one side of the predicted control distribution. When there was no clear directional hypothesis, the critical effect size associated with a 2-tailed test was used. For each test, equal prior probabilities of the null or alternative hypotheses being true were assumed. Optimal α and associated β and ω values (ω = average chance of making a wrong conclusion) for each sample size were calculated using the statistical program R [30] with code from Mudge et al. [29]. The optimal α is presented for each statistical test. The corresponding β and ω for each statistical test of a given sample size and critical effect size are given in Supplemental Data, Table S1 (to simplify reporting of results).

In the present study, experimental units were wetland halves ($n = 48$). To maintain the power of the paired experimental design, the abundance of emerging chironomids was expressed as the difference between treated and control sides, relative to the value on the control side for each wetland. Similarity of Chironomidae assemblage composition between sides of each wetland was expressed using a semiquantitative measure of community composition, the additive inverse of the Bray-Curtis dissimilarity index, which examines the differences in the relative abundances of mutually present taxa and ignores mutual absences. The formula was

$$\text{Bray}-\text{Curtis} = 100 \times (1 - [2C_{ij}/\{S_i + S_j\}])$$

where C_{ij} is the lesser sum of the abundance of species shared between 2 sites, S_i is the total abundance of species at site i, and S_j is the total abundance of species at site j. The average similarity, within sampling periods, between the control sides of independent wetlands was used as a baseline comparison at the landscape level. Two-tailed, 1-sample t tests were used to assess significant differences in relative chironomid abundance between treated and control sides of wetlands. Only declines were expected in similarity of the chironomid assemblage and in

macrophyte cover as a result of chemical treatments; therefore, 1-tailed paired *t* tests were used to check for significant declines in chironomid assemblage similarity between the pretreatment and posttreatment sampling periods and to check for significant reduction in percent macrophyte cover on treated sides of ponds relative to control sides. Two-tailed, 2-sample *t* tests were used to examine differences in chironomid assemblage similarity between the herbicide-only and herbicide plus nutrients treatments in the June and August sampling periods.

Linear regressions were used to determine the relationship between the abundance of emerging chironomids and the percent cover of macrophytes for all treated sides of experimental wetlands compared with the untreated sides. The critical effect size $R^2 = 0.40$ was used, which is the equivalent of a critical effect size of 1.64 SD, and was calculated using the conversion formula

$$R^2 = d^2/(d^2 + 4)$$

where d is the critical effect size of 1.64.

RESULTS

Nutrient and glyphosate concentrations

Measured nutrient concentrations in wetland water were lower than anticipated during the treatment period (Figure 1A). On average, NH_4^+ in treated wetland halves was below detection limits prior to treatment. Nutrient additions led to transient increases in NH_4^+, and nutrient-treated sides of wetlands had, on average, 0.006 ± 0.015 mg/L higher NH_4^+ than the paired control sides. There was negligible difference in total phosphorus between wetland sides prior to application of nutrients. During the treatment period, the concentration of total phosphorus increased on the treated sides of wetlands by an average 0.023 ± 0.014 mg/L when compared with paired controls.

The concentration of glyphosate in the water column of the treated sides of experimental wetlands declined rapidly and was mostly nondetectable 7 d after either herbicide addition (Figure 1B). The half-life of glyphosate in wetland water was approximately 0.56 d and 0.69 d following the first and second treatment applications, respectively. One day after the first treatment, there was significantly more glyphosate in all of the H-treated sides than in the L-treated sides of wetlands (all Hs 835.57 ± 390.68 μg a.e./L; all Ls 26.69 ± 12.62 μg a.e./L; $p = 0.001$, $n = 12$, 12, $\alpha = 0.068$, 2-sample, 1-tailed, independent *t* test). At 3 d after the first treatment, only the H-treated wetlands had a detectable concentration of glyphosate of 75.88 ± 56.59 μg a.e./L, and at 7 d after treatment, neither treatment category had an average concentration of glyphosate above the limit of detection. After the second spray, only H-treated wetlands had an average concentration of glyphosate above the limit of detection at both 3 d and 7 d posttreatment (3 d 120.56 ± 123.01 μg a.e./L, 7 d 28.66 ± 19.55 μg a.e./L).

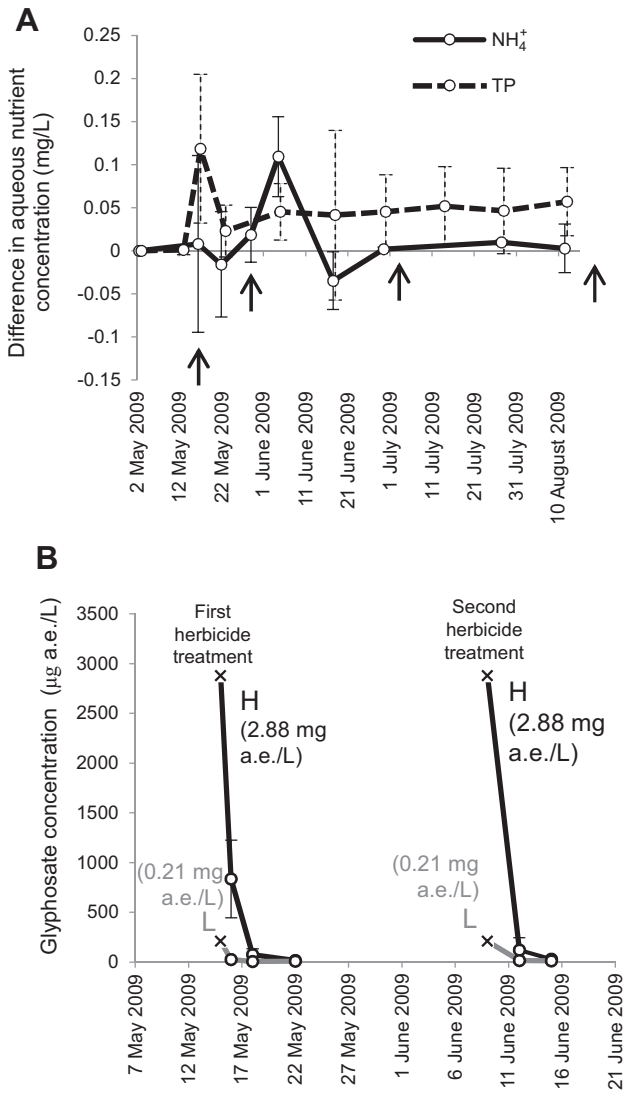

Figure 1. (**A**) The difference (treated vs control) in aqueous ammonia (NH_4^+-N) and total phosphorus (TP) concentrations between treated and control sides of all nutrient-treated (HN [black] and LN [gray] treatments) split-wetlands ($n = 12$; ± 90% confidence interval). Arrows indicate the dates where nutrients were added to the treated sides of wetlands. (**B**) Aqueous glyphosate concentrations on the treated sides of wetlands (± 90% confidence interval; $n = 12$ for each treatment). Initial nominal concentrations are indicated by an *x* on each curve; analytically measured concentrations are indicated with open circles. a.e. = acid equivalents; HN = higher glyphosate concentration plus nutrients; LN = lower glyphosate concentration plus nutrients.

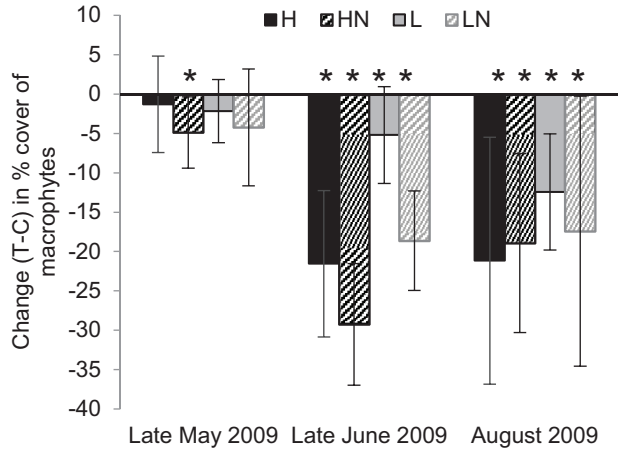

Figure 2. Average differences in the percent cover of macrophytes ± 90% confidence interval; $n = 6$ control and 6 treated experimental units for each treatment category, except in August when only the wetlands containing water could be sampled and H is $n = 3, 3$; HN is $n = 4, 4$; L is $n = 3, 3$; and LN is $n = 3, 3$. H = higher herbicide concentration (2.88 mg acid equivalents/L), HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients. *Significant differences.

Effects on macrophyte cover

In late May 2009, mean differences in the percent cover of macrophytes between the treated and control sides of wetlands were small (no greater than 5% for any treatment), and only the HN treatment sides differed significantly from 0 (H: $p = 0.371$, $n = 6$, $\alpha = 0.097$; HN: $p = 0.068$, $n = 6$, $\alpha = 0.097$; L: $p = 0.207$, $n = 6$, $\alpha = 0.097$; LN: $p = 0.208$, $n = 4$, $\alpha = 0.160$; all by paired, 1-tailed t tests; Figure 2). The minor differences between the treated and control sides in late May 2009 (7–13 d after herbicide treatment) were consistent with the expected 3-wk delay in plant damage after glyphosate application [31]. Following the second herbicide application, macrophyte cover declined in every treatment category (H: $p = 0.009$, $n = 5$, $\alpha = 0.124$; HN: $p = 0.002$, $n = 5$, $\alpha = 0.124$; L: $p = 0.118$, $n = 5$, $\alpha = 0.124$; LN: $p = 0.002$, $n = 6$, $\alpha = 0.097$; all by paired, 1-tailed t tests). Similarly, in August 2009, macrophyte cover was significantly lower on the treated than the paired control sides of the wetlands that did not go dry (H: $p = 0.078$, $n = 3$, $\alpha = 0.212$; HN: $p = 0.036$, $n = 4$, $\alpha = 0.160$: L: $p = 0.055$, $n = 3$, $\alpha = 0.212$; LN: $p = 0.118$, $n = 3$, $\alpha = 0.212$; all by paired, 1-tailed t tests).

Effects on emerging chironomids

A total of 10 459 chironomids were collected and identified in the present study. Prior to the treatment of wetlands, there was no significant difference in the number of chironomids emerging from the treated sides of wetlands when compared with control sides ($+58.1 \pm 67.9\%$, $\alpha = 0.0005$, $p = 0.174$, $n = 24$, 2-tailed, 1-sample t test; Figure 3). Average community similarity between wetland halves of all wetlands prior to treatment application was $50.0 \pm 9.9\%$. While the average similarity between the sides of each wetland was not particularly high at this time, it was greater than the average similarity among all control sides of wetlands ($24.3 \pm 2.5\%$; Figure 4).

We found little evidence of direct toxic effects of herbicides or herbicides plus nutrients on the abundance or assemblage composition of emerging chironomids collected immediately posttreatment in June 2009 (Supplemental Data, Table S1). Only LN ponds showed a significant difference in chironomid emergence on treated sides relative to control sides, with $38 \pm 27.3\%$ greater average emergence of chironomids on treated versus control sides (2-tailed, 1-sample t test, $\alpha = 0.0845$, $p = 0.0697$; Figure 3). Chironomid assemblage similarity between halves was an average of 19.0% higher than was observed in May 2009, with an average similarity of $64.0 \pm 5.4\%$ (Figure 4). The average similarity between the control sides of independent wetlands also increased by 18% over the initial pretreatment sampling, to $43.0 \pm 2.2\%$. There was a decrease in the proportion of Orthocladiinae chironomids

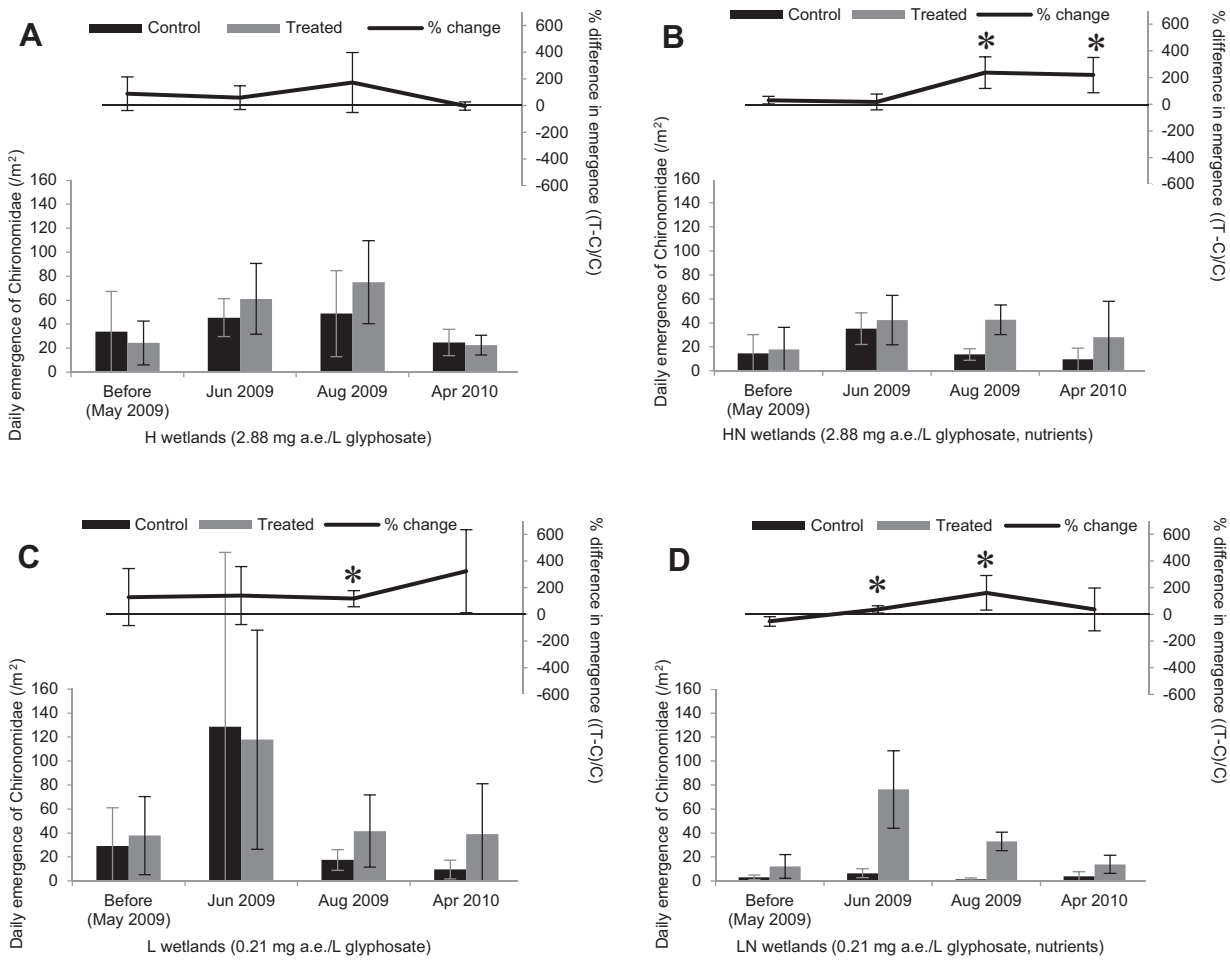

Figure 3. Average daily emergence of Chironomidae from wetlands expressed as average number per square meter (left axis) and the average percent difference (right axis) between sides of wetlands ($[(T - C)/C] \times 100 \pm 90\%$ confidence intervals) in total abundance of all emerging Chironomidae. For each treatment category $n = 6$ control and 6 treated experimental units, except in August where only the wetlands containing water could be sampled and H is $n = 3, 3$; HN is $n = 4, 4$; L is $n = 3, 3$; and LN is $n = 3, 3$. Emergence samples were collected in early May were prior to any herbicide application. H = higher herbicide concentration (2.88 mg acid equivalents/L); HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients. * Indicates significant differences.

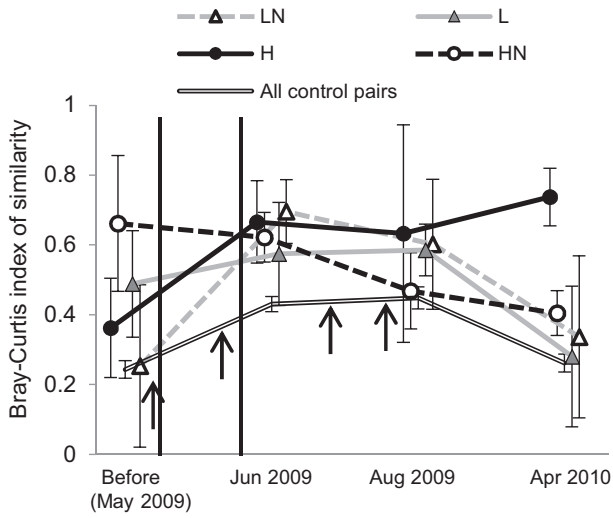

Figure 4. Bray-Curtis index of similarity ± 90% confidence interval of emerging Chironomidae taxa between the sides of divided wetlands for each treatment. The hollow double line indicates the average similarity among only the control sides of all wetlands, representing the average similarity between independent unaffected wetlands. Similarity ranges from 0 to 1, with 1 = 100% similar communities. H = higher herbicide concentration (2.88 mg acid equivalents/L); HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients.

and an increase in the proportion of all other chironomid groups across all wetlands, regardless of treatment type or side of wetland (Figure 5). Wetlands treated with both herbicide and nutrients were not significantly different from wetlands treated with herbicide only in terms of the abundance of emerging midges (H vs HN: $p = 0.889$; and L vs LN: $p = 0.744$; both with $n = 12$, 12, by 2-sample, 2-tailed, independent t tests) as well as the assemblage similarity (H vs HN: $p = 0.060$; L vs LN: $p = 0.331$, $\alpha = 0.129$, $n = 6$, 6, 2-sample, independent, 2-tailed t test).

In August 2009, well after the effects of the treatments on the plant community had occurred, there was an increase in chironomid emergence from the treated sides of wetlands relative to their control sides for all treatments except H (H: $173.1 \pm 224.1\%$, $n = 3$, $p = 0.332$; HN: $236.7 \pm 118.6\%$, $n = 4$, $p = 0.046$; L: $117.9 \pm 61.1\%$, $n = 3$, $p = 0.087$; LN: $161.5 \pm 129.7\%$, $n = 3$, $p = 0.177$; all by 1-sample, 2-tailed t tests; $\alpha = 0.244$ for H, L, and LN; $\alpha = 0.166$ for HN) (Figure 3). The mean similarity in the Chironomidae assemblages between sides of each wetland in August was $56.4 \pm 8.5\%$, 11.4% higher than before treatment and 7.5% lower than in June (Figure 4). Again, there was an increase in the assemblage similarity in wetlands over the pretreatment levels, and this was contrary to a predicted decline in similarity attributable to any indirect effects of the treatments. While the similarity between wetland halves increased somewhat compared with pretreatment levels, this increase in similarity was only half the average magnitude of the increase in similarity observed between all pairs of control sides of independent wetlands, which increased by 20.5% in August compared with pretreatment (May). Overall, wetlands treated with both herbicide and nutrients were not significantly different from wetlands treated with herbicide only in terms of the abundance of emerging chironomids (H vs HN: $p = 0.461$, $n = 3$, 4, $\alpha = 0.224$; L vs LN: $p = 0.632$, $n = 3$, 3, $\alpha = 0.251$; both by 2-sample, 2-tailed, independent t tests). However, the similarity of the chironomid assemblage in wetlands treated with both herbicide and nutrients was significantly different from that in wetlands treated with herbicide alone in both the higher and lower herbicide additions (H vs HN: $p = 0.123$, $n = 3$, 4, $\alpha = 0.224$; L vs LN: $p = 0.004$, $n = 3$, 3, $\alpha = 0.251$; both by 2-sample, 2-tailed, independent t tests).

The following spring of 2010, only HN ponds had a significantly higher abundance of emerging chironomids on treated sides relative to control sides (H: $-3.5 \pm 30.3\%$, $\alpha = 0.117$, $p = 0.859$, $n = 5$; HN: $+219.4 \pm 131.5\%$, $\alpha = 0.084$, $p = 0.041$, $n = 6$; L: $+323.9 \pm 311.4\%$, $\alpha = 0.117$, $p = 0.162$, $n = 5$; LN: $+37.3 \pm 160.7\%$, $\alpha = 0.084$, $p = 0.718$, $n = 6$; all by 2-tailed, 1-sample t tests; Figure 3). One year posttreatment in most wetlands, the assemblage similarity of Chironomidae was no different from that of the previous spring, indicating possible recovery from any perturbations induced by the treatments on these organisms (Figure 4). The exception to this was the HN-treated wetland halves, which were on average $13.2 \pm 27.3\%$ less similar than in the pretreatment data from the same time in 2009 (H: $p = 0.986$; HN: $p = 0.030$; L: $p = 0.104$; LN: $p = 0.525$; all by 1-tailed, paired t tests, $\alpha = 0.097$, $n = 6$). Although it appears that the predatory Tanypodinae had a higher overall relative abundance in spring 2010 than in spring 2009, this was consistent across all wetland halves and did not appear to be affected by treatment category (Figure 5).

Effects of macrophyte cover on chironomid emergence

In August 2009, when significant increases in chironomid emergence were observed on treated sides of wetlands relative to control sides, there was also a significant negative relationship between chironomid emergence and macrophyte cover on all treated sides of wetlands ($R^2 = 0.230$, $p = 0.092$, $n = 13$, $\alpha = 0.145$; Figure 6). No relationships between macrophyte cover and chironomid emergence were observed on the control sides of ponds where there were no declines in macrophyte cover; nor were any significant relationships observed in June 2009, when there were no differences in chironomid emergence for either the control or treated sides of wetlands (Supplemental Data, Table S1).

DISCUSSION

Mixtures of agricultural contaminants are likely to be found in wetlands adjacent to farm fields. Individually, many herbicides and fertilizers may not be particularly toxic to the multitude of nontarget wetland organisms at the concentrations expected in the environment. However, more than just direct toxicity, it is important to understand the effects that these chemicals can also have on other parts of the ecosystem, which can indirectly affect organisms of interest. The patterns of emergence of Chironomidae from these small wetlands may respond to both the short-term impacts of the chemicals themselves as well as the longer-term chemical-induced alterations, such as a reduction in macrophyte abundance as a result of herbicide contamination. In the present study, we used whole-ecosystem experiments to examine the effects of a glyphosate herbicide, alone or in combination with nutrients, on chironomids.

The abundance and similarity of the Chironomidae assemblage emerging from experimental wetlands generally did not demonstrate a direct toxic response to the glyphosate-herbicide Roundup WeatherMax alone or in combination with inorganic nutrients. Nominal and measured concentrations of herbicides in the present study were lower than the LC50 values for other types of glyphosate herbicides of 8 mg/L to 18 mg/L on

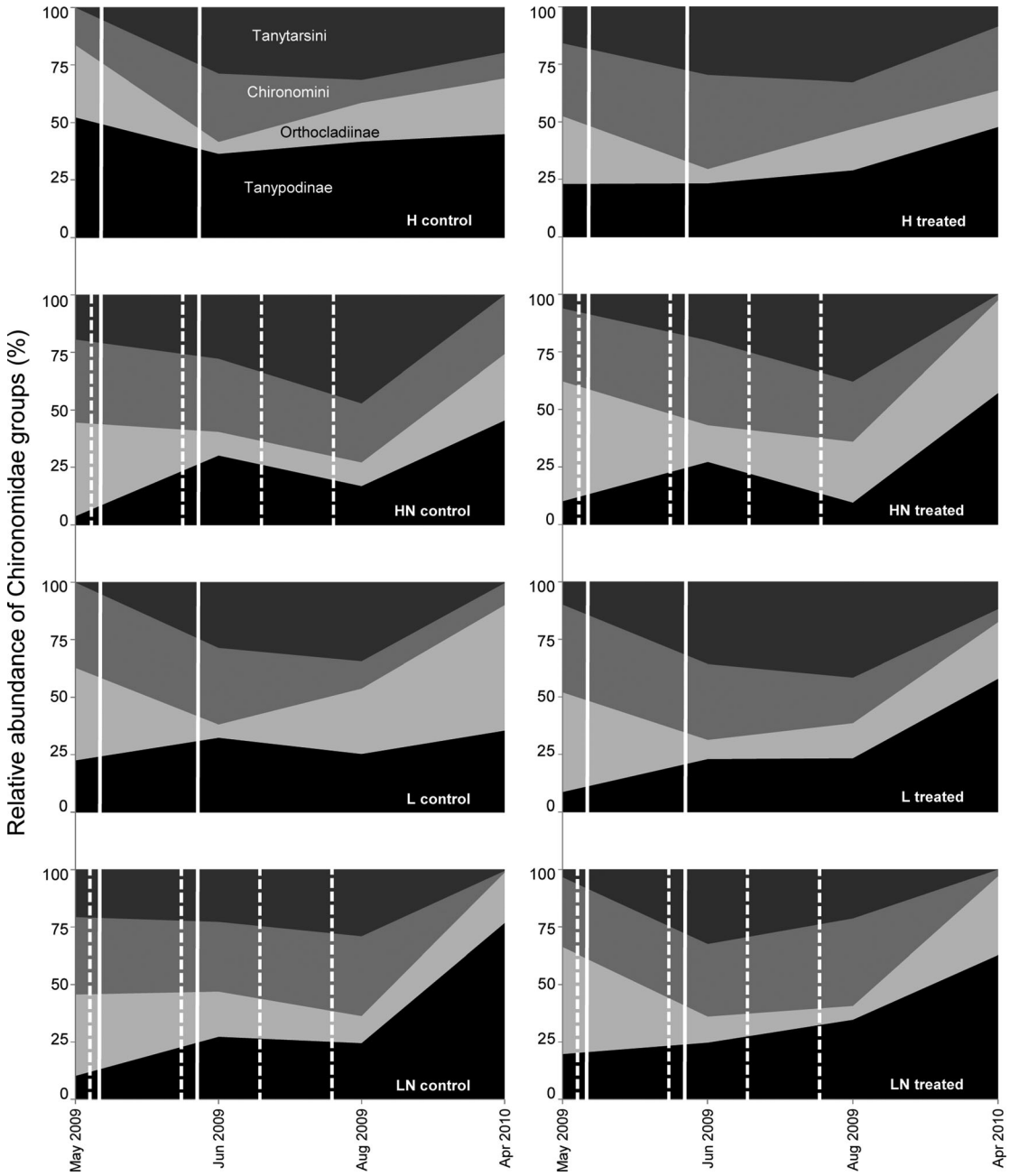

Figure 5. Average relative abundance of major Chironomidae taxa in treated and control sides of wetlands at each sampling time. Vertical solid white lines indicate approximate time of each herbicide application; vertical dashed white lines indicate approximate timing of each nutrient application, relative to sample collection dates. H = higher herbicide concentration (2.88 mg acid equivalents/L); HN = higher herbicide and nutrients; L = lower herbicide concentration (0.21 mg acid equivalents/L); LN = lower herbicide and nutrients.

chironomids reported in the literature [10,13,14]; thus, these results are consistent with the postulates derived from these prior laboratory studies.

In addition, the assemblage similarity of emerging Chironomidae increased by 19.8% between treated and control sides from May 2009 (pretreatment) to June 2009 (posttreatment). Although the low numbers of chironomids collected in the pretreatment sampling (May 2009) likely obscured our ability to detect more subtle changes in assemblage composition, Bray-Curtis similarity indices are often low among natural Chironomidae assemblages (average of approximately 20% similarity in many studies; e.g., Alvarez et al. [32]). In addition, the similarity among the control sides of independent wetlands demonstrated a nearly identical pattern, in which similarity increased by 18.7% when compared with pretreatment levels. This concurrence could be a response to natural drivers of midge emergence, such as temperature, day length, hydroperiod, and species phenology [1,33–36], rather than the effects of the experimental treatments. The results indicate that Roundup WeatherMax applied at the maximum label-rate concentration (2.88 mg a.e./L of glyphosate) or at environmentally realistic concentrations (0.21 mg a.e./L of glyphosate) would not likely pose an immediate toxicological concern for the Chironomidae assemblages of these small, shallow, Acadian forest wetlands.

However, the treatment of wetlands with Roundup WeatherMax indirectly increased the total abundance of emerging chironomids 3 mo after treatment in all treatment categories with the exception of the H treatment. There was a concurrent trend of

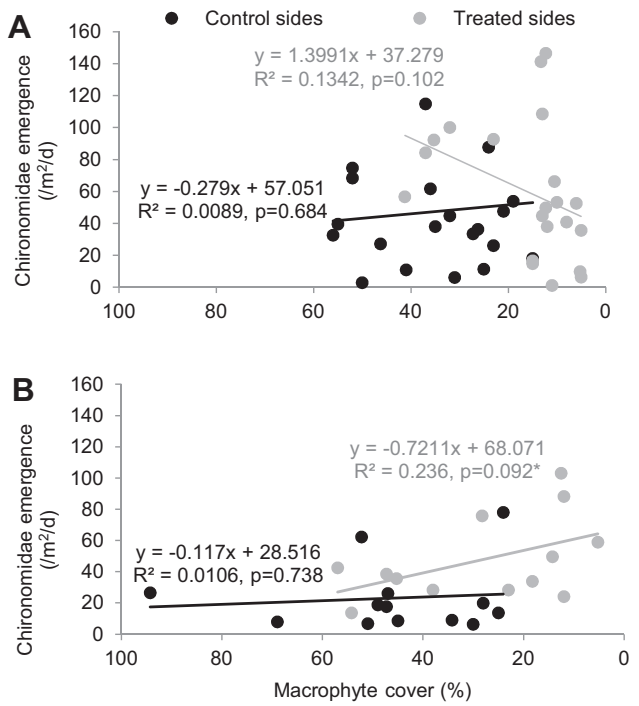

Figure 6. Linear regressions of the emergence of all Chironomidae and the cover of macrophytes for (**A**) after treatment in late June 2009 ($n = 22, 22$; $\alpha = 0.058$), and (**B**) after treatment in August 2009 ($n = 13, 13$; $\alpha = 0.145$). Macrophyte survey data were not available for the spring of 2009 or 2010, to correspond to the first and last sampling of Chironomidae, as the plant community was too immature for identification. The critical effect size for all tests was $r^2 = 0.40$. * Indicates significant difference.

increased emergence from the treated sides of H-treated wetlands, but this was not statistically significant. These effects were observed long after the glyphosate was no longer detectable in the water column but when macrophyte cover was significantly affected by the herbicide. The paired design of the present study allowed us to observe the statistically significant increase in the abundance of emerging midges in all experimental units (treated or untreated), even with considerable variability among wetlands.

Prior to treatment of the wetlands with any chemicals, the similarity in chironomid assemblages across all control halves was much lower than the average similarity observed between halves of the same wetland. In August 2009 after treatment, similarity of the chironomid assemblage between wetland halves was higher than pretreatment levels (11.4% increase), but this increased similarity was only about half as large as was observed between the control sides of independent wetlands (20.5% increase). If treatments had had no effect on the chironomid assemblage, it would seem reasonable to expect that the sides of the same wetland should have increased in their similarity at least as much as the similarity between independent wetlands, as had occurred in the June 2009 samples. Hence, the lack of a similar-sized increase in similarity between the treated and untreated pairs of wetland sides might be suggestive of an indirect effect of the treatments on chironomid assemblage similarity.

The colonization of chironomids in wetlands has been shown to be negatively correlated with plant cover [17], where open water is correlated with increased colonization and/or decreased predation rates. However, we were unable to find a relationship between the total abundance of chironomids emerging from the untreated sides of wetlands and percent plant cover within any sampling period, as has been observed in other studies [17,37]. This may be attributable to the presence of other stronger controls of chironomid populations (such as temperature, water depth, and permanence [35,38]) that may have larger effects in small, shallow wetlands than in the deep, permanent wetlands typical of other studies. There was, however, a negative relationship between chironomid emergence rates and plant cover across all treated sides of wetlands in August 2009, suggesting that the loss of macrophytes from herbicide treatments led to increased chironomid abundance, possibly through some intermediary mechanism such as a loss of predators or increased food amounts. These results concur with another study that found higher emergence rates of chironomids after removal of macrophytes with a glyphosate-based herbicide [15].

Increased chironomid emergence might be considered a positive outcome in some contexts. For example, the management of wetland macrophytes for breeding duck habitat aims to decrease dense macrophyte cover and increase the emergence of dipteran flies, which are known to be an important food source for wetland-associated avian species [2]. However, caution is warranted in this case because the relationship between macrophyte cover and chironomid emergence may not always be a direct one. The increase in emergence may have occurred because of some other functional alteration in the wetland. In light of more recent calls to protect the world's dwindling wetland resources [39] and preserve particular functions, such as carbon sequestration and nutrient retention, it would be wise to consider how glyphosate herbicide treatments alter the overall integrity of wetland function.

On average, the statistical tests performed in the present study had an overall type I error rate (α) of approximately 0.153 (see Supplemental Data, Table S1 for a summary of error rates across tests). While an $\alpha = 0.153$ is not necessarily a powerful experiment by conventional standards, error rates higher than those typically observed in laboratory studies should be expected for ecosystem experiments as they are rarely as statistically powerful because of logistical constraints and high natural variability. Holding the present study to the traditionally accepted type I error rate ($\alpha = 0.05$) would result in comparatively large chances of type II errors for these tests; of the tests conducted in the present study, the average statistical power would have been 46.2% using $\alpha = 0.05$. Thus, we would have a very high chance of being incorrect in cases in which we have concluded that there is no significant effect. The use of the optimal α method increased statistical power in the present study to 88.2%, suggesting a high degree of confidence in the conclusion of no significant effects.

CONCLUSIONS

The present study has demonstrated that there was negligible evidence of any negative toxic effects of glyphosate herbicides on the emergence of Chironomidae taxa in our treated wetlands, similar to what has been observed in laboratory and mesocosm studies. The lack of direct toxic effects was likely a result of the lower treatment concentrations used in the present study than those that are known to cause acute toxicity, the rapid dissipation of glyphosate from the water column, and the presence of stronger natural drivers of the emergence patterns of chironomid taxa present in the wetlands (e.g., wetland size and hydroperiod, water depth, presence of predators). However, there was evidence of some indirect, longer-term effects, resulting in a higher abundance of emerging midges on treated sides of

wetlands. While an increase in the abundance of emerging chironomids may be viewed favorably by some, any change from the natural condition of an ecosystem indicates that the fundamental functioning and structure of the system has been shifted and may be cause for concern.

Emergent plant cover has been hypothesized to play an important role in structuring the chironomid assemblage in wetlands, possibly through habitat cues and physical structure of the pond [15,17,40,41]. We were unable to find a strong relationship between the abundance of emerging chironomids and the percent cover of macrophytes in these wetlands under normal conditions. It was only through the application of Roundup WeatherMax and long-term monitoring for indirect effects that we were able to find a relationship between these 2 variables. There may be a more indirect route by which emergent macrophyte structure affects the chironomid assemblage, such as through alteration of algal food sources, but these hypotheses will require investigation. There was also evidence that herbicide impacts on the macrophyte community persisted beyond the year the herbicides were applied (L. Baker, personal observation), whereas chironomid emergence patterns seemed to return to normal. The macrophyte effect on Chironomidae emergence may be present only in taxa that emerge later in the summer, when macrophyte communities are typically more developed. Thus, important areas of further study are to determine the mechanisms by which chironomid emergence is influenced by macrophyte cover and how these mechanisms are affected by herbicide treatment.

SUPPLEMENTAL DATA

Table S1. (86 KB PDF).

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