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The combined influence of two agricultural contaminants on natural communities of phytoplankton and zooplankton

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Abstract Concentrations of glyphosate observed in the environment are generally lower than those found to exert toxicity on aquatic organisms in the laboratory. Toxicity is often tested in the absence of other expected co-occurring contaminants. By examining changes in the phytoplankton and zooplankton communities of shallow, partitioned wetlands over a 5 month period, we assessed the potential for direct and indirect effects of the glyphosate-based herbicide, Roundup WeatherMax© applied at the maximum label rate, both in isolation and in a mixture with nutrients (from fertilizers). The co-application of herbicide and nutrients resulted in an immediate but transient decline in dietary quality of phytoplankton (8.3 % decline in edible carbon content/L) and zooplankton community similarity (27 % decline in similarity and loss of three taxa), whereas these effects were not evident in wetlands treated only with the herbicide. Thus, even at a worst-case exposure, this herbicide in isolation, did not produce the acutely toxic effects on plankton communities suggested by laboratory or mesocosm studies. Indirect effects of the herbicide-nutrient mixture were evident in mid-summer, when glyphosate residues were no longer detectable in surface water. Zooplankton abundance tripled, and zooplankton taxa richness increased by an average of four taxa in the herbicide and nutrient treated wetlands. The lack of significant toxicity of Roundup WeatherMax alone, as well as the observation of delayed interactive or indirect effects of the mixture of herbicide and nutrients attest to the value of manipulative field experiments as part of a comprehensive, tiered approach to risk assessments in ecotoxicology.

Keywords Zooplankton · Phytoplankton · Glyphosate · Fertilizers · Mixture effects · Indirect effects

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Introduction

Habitat degradation in wetlands adjacent to agricultural crops can occur as a result of receiving agricultural drainage, runoff and spray drift containing pesticides and fertilizers (Maillard et al. 2011). More recently, there has been concern about the potential for effects on non-target aquatic organisms from glyphosate-based herbicides (e.g. Roundup®) which are among the most-widely used herbicides in the world for control of weeds in agriculture (Giesy et al. 2000). While over-water application of most glyphosate-based formulations is prohibited, monitoring studies (Battaglin et al. 2014, 2005; Byer et al. 2008; Scribner et al. 2007; Struger et al. 2008) demonstrate that glyphosate residues are often detected in agricultural surface waters at concentrations generally in the 0.5–100 µg/L range, which is well below the current glyphosate Canadian water quality guideline level for the protection of aquatic life at 27 mg acid equivalents (a.e.)/L for acute exposures (CCME 2012), where acid equivalents is the most accurate method to compare the yield of glyphosate acid (the active ingredient) that can be derived from glyphosate salts

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(having variable masses) typically used in herbicide formulations.

Observed environmental concentrations of glyphosate are much lower than those shown to result in significant toxicity to freshwater zooplankton and phytoplankton test species. Glyphosate-based herbicides demonstrate toxicity (as LC_{50}) to some zooplankton species at concentrations of 1.5 mg a.e./L (*Simocephalus vetulus*-Vision®; Chen et al. 2004) up to as much as 11 mg a.e./L (*Daphnia* spp.-Roundup®; Monsanto Company 2009). Mixtures of multiple herbicides, including glyphosate, have been shown to decrease phytoplankton growth (Relyea 2009). Glyphosate acid is toxic (72 h-EC_{50}) to freshwater algal species (*Scenedesmus acutus*, *Chlorella vulgaris*) at concentrations between 24.5 and 41.7 mg a.e./L (Vendrell et al. 2009). Conversely, many freshwater cyanobacterial species appear to be relatively tolerant of glyphosate (Forlani et al. 2008; Powell et al. 1991). Some, but not all, of these experiments tested the effects of commercial glyphosate-based formulations, which contain a known quantity of glyphosate but also contain other chemical additives, such that causal links should not be made only to the active ingredient. In fact, toxicity of formulated glyphosate-based herbicides to animals has been attributed mainly to the presence of surfactants in the formulation (Henry et al. 1994; Tsui and Chu 2004). A common surfactant used in Roundup formulations is the non-ionic surfactant polyethoxylated tallow amine (POEA) (Giesy et al. 2000), and it is toxic to algae and zooplankton species at concentrations an order of magnitude lower than glyphosate acid (Tsui and Chu 2003).

Both glyphosate and POEA readily bind to sediment in wetland ecosystems where they are degraded through microbial processing, resulting in their limited environmental persistence (Giesy et al. 2000; Zaranyika and Nyandoro 1993). However, glyphosate-based herbicides are unlikely to occur in isolation in wetlands in agricultural areas (Maillard et al. 2011), where coincident presence of nutrients and herbicides from surface runoff or drainage tile flow is possible. The interactions of multiple stressors are poorly understood and effects may occur through a multitude of direct and indirect pathways in natural ecosystems (Chen et al. 2004; Schindler 2001), which are challenging to replicate in a laboratory or mesocosm. Thus, we expect that the results of the present experiment may differ somewhat from what has been observed in more controlled studies, however we intend this research not as a criticism or replacement of existing literature, but as complimentary. The marriage of laboratory studies and with field-based experiments in a tiered approach to risk assessment delivers an understanding of the broader, long-term ecotoxicological consequences which are also needed for effective risk assessment and ecological management efforts (Boone et al. 2005; Cuppen et al. 1995; Zrum and Hann 2002).

Manipulative field experiments can improve our understanding of ecosystem level effects and recovery resulting from the occurrence of multiple stressors. The combined effects of glyphosate-based herbicide residues and co-occurring nutrient contamination in wetland ecosystems could lead to complex interactive effects that would be difficult to predict from studies of individual chemicals and may result in a cascade of responses through an ecosystem. For example, the grazing rates of Daphnia have been found to be reduced by exposure to glyphosate (Bengtsson et al. 2004) and this could contribute to an overabundance of their main food source, leading to eutrophication in the wetland (Cottingham et al. 1997; Schindler 1974; Timms and Moss 1984). Increased phytoplankton biomass can, in turn, decrease light penetration resulting in a loss of submerged macrophyte communities as well as a loss of habitat for the animals that depend on these macrophytes for shelter (Cazzanelli et al. 2008; Schindler 1974). In addition, the use of herbicides near wetlands could also damage emergent wetland macrophytes (Flinn et al. 2005; Simenstad et al. 1996). Macrophytes sequester nutrients in wetlands, but their senescence reduces competition with phytoplankton for nutrients (Scheffer et al. 1993). The subsequent decomposition of senesced macrophyte tissues further releases these sequestered nutrients, which may lead to longer-term indirect effects such as blooms in phytoplankton and an increase in the abundance of zooplankton (Scheffer et al. 1993; Vandonk et al. 1990) long after the dissipation of glyphosate residues from the water.

The objective of this study was to determine both initial direct and longer-term indirect effects of the application of a common commercial agricultural formulation of a glyphosate-based herbicide, Roundup WeatherMax©, alone and in combination with chemical fertilizers on the zooplankton and phytoplankton communities of wetlands. Immediate effects (toxicity) of Roundup WeatherMax on the zooplankton or phytoplankton community should manifest as declines in abundance and/or a change in community structure shortly after application. Longer-term effects of the herbicide on macrophytes could produce delayed, indirect responses of zooplankton and phytoplankton communities, manifesting as declines in community similarity versus controls. Lastly, we predict that the addition of nutrients could increase phytoplankton productivity in the wetlands (Ghadouani et al. 2006), which could, in turn, lead to an increase in the abundance of phytoplankton-consuming zooplankton.

Materials and methods

Site description and barrier design

Wetlands used in this study were located in the Long-term Experimental Wetlands Area, a 4-km^2 site on Canadian Forces Base Gagetown, approximately 60 km northwest of Saint John, NB, Canada (66°29′59.02″W, 45°40′48.62″N). Wetlands formed in depressions and next to windrows immediately after mechanical clearing of the land in 1997–1998. The area was not treated with any chemical pesticides prior to this experiment (Ollsen and Knopper 2006). Wetlands were generally shallow (21–90 cm deep at high water periods), fishless and unstratified. Barriers were used to divide the wetlands, which were composed of 30 mil (0.76 mm) opaque black, high-density polyethylene (HDPE) geomembrane (Poly-Flex Inc., Geomembrane Lining Systems, Grand Prairie, TX, USA). The barriers were approximately 1 m in height and had sealed pockets filled with crushed gravel along the bottom to anchor them into the sediments. These barriers, installed in August 2008, stretched the entire length of the wetland and extended beyond the high water mark (Fig. 1).

Chemical application and quantification

The experimental design and quantification of chemical concentrations has been previously described (Baker et al. 2014). Briefly, in each of the six divided wetlands, one randomly selected half was hand-sprayed using a backpack sprayer with the agricultural-use herbicide Roundup WeatherMax (Monsanto Company, Creve Coeur, MO, USA). Two separate herbicide applications were conducted on May 15–16, and June 9–10, 2009, to mimic the timing of agricultural weed control applications in the region. For this study, we examined the effects of a predicted maximum environmental aqueous concentration of 2.88 mg a.e./L of glyphosate on six wetlands. This predicted concentration was calculated as the concentration that could result from direct overspray of a 15 cm deep wetland at the maximum label application rate of 4.32 kg a.e./ha with no interception by emergent macrophytes. This treatment level is termed "higher" (H) glyphosate concentration to maintain consistency with treatments applied in other experimental wetlands not described here (see Baker et al. 2014; Edge et al. 2014).

Fig. 1 HDPE barrier dividing an experimental wetland

Additionally, solutions of nutrients were applied to the herbicide-sprayed side of three of the six wetlands (referred to as "HN" treatments). The nutrient solution was generated from salts of nutrients typically used in fertilizers (technical grade ammonium nitrate and phosphoric acid, Fisher Scientific), and applied on each of May 14, May 29, July 3 and August 19, 2009. Nutrients were added to each wetland with the objective of increasing the aqueous phosphorus (measured as total phosphorus (TP)) to 0.1 mg/L (i.e. eutrophic; Wetzel 2001) over the background concentrations measured in 2008, and aqueous nitrogen (measured as total Kjeldahl Nitrogen (TKN)) was added to maintain the natural TKN:TP ratio, so as to prevent a shift in which of N or P was the limiting nutrient naturally. The full experimental design here in consisted of 3 H wetland halves and 3 HN wetland halves, each with a paired untreated half (12 experimental units in total, from six bisected wetlands).

Water samples were collected from all experimental units every 2 weeks through the course of the summer for nutrient analysis. 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 spraying, and on days 0, 3 and 7 after the second round of spraying. Control sides of wetlands were verified as having glyphosate concentrations below detection limits (3.54 μg/L), except in three instances where the concentration was 8 μg/L in Ag-07 (both applications) and Ag-21 (second application), although we did not consider these a concern as these concentrations were very low, less than 0.2 % of the target concentration. Quantitation methods, quality assurance, and discussion of results are presented in detail in Baker et al. (2014) and in Edge et al. (2014). Concentrations pertinent to this study have been discussed herein.

Plankton sampling and enumeration

Depth-integrated plankton samples were collected from each side of each wetland simultaneously using a 20 μm mesh, weighted, student plankton net (15 cm diameter opening, Dynamic Aqua-Supply Ltd., Surrey, BC, Canada). A set of pre-exposure samples were collected 3–5 days before applications ("before" samples). The first to sixth sampling periods occurred on days 1, 5, 10, 16, 21, and 23 after the first herbicide treatment. The 7th–14th sampling periods occurred on days 1, 6, 10/11, 15, 20, 30, 44/45, and 90 after the second herbicide treatment. Due to temporary dry-downs samples could not be collected during the 6th and 13th sampling periods in one replicate of the herbicide and nutrient treatment and the 13th sampling period of one replicate of the herbicide alone treatment. Sub-samples collected from five, flagged, permanent sampling stations distributed within each half of each wetland were combined and preserved with 5 % Lugol's solution in the field. Volume of the sampled column of water was calculated as a cylinder using the diameter of the plankton net and the known total depth sampled on each side for each day.

Zooplankton in a sub-sample from each sample were enumerated and identified to species level where possible, or to the lowest practical taxonomic level (Ward and Whipple 1945; Thorp and Covich 2001) using a Bogorov counting chamber (Wildco™, Yulee, FL, USA) under dissecting microscope. From this, zooplankton abundance, richness and community similarity were calculated. Zooplankton abundance was a tally of all zooplankton individuals across all taxa on a particular date for each wetland side. Zooplankton richness was a tally of unique taxa on a particular date for each wetland side. We used the additive inverse of the Bray–Curtis dissimilarity index (Bray and Curtis 1957) as a semi-quantitative measure of the similarity in zooplankton community composition between sides of the wetlands. The average similarity between only the control sides of all wetlands was used as a baseline comparison of the degree of similarity among wetlands to be expected at the landscape level.

For phytoplankton endpoints, aliquots from the same samples used to enumerate zooplankton were placed in a 0.1 mL nanoplankton counting chamber (PhycoTech Inc., St. Joseph, MI, USA). A transect of eight images was taken across the middle of the plankton chamber (image dimensions: 2358 × 1768 μm each). Images were converted to grayscale and analyzed with Image J software (Rasband Rasband 1997–2012). The default thresholding tool was used to separate the dark phytoplankton particles from the lighter background. The area, width and maximum length of each phytoplankton cell was calculated using the Image J particle analyzer tool. Total cell volume was estimated by multiplying the area of each cell in the image by the smaller dimension of the smallest rectangle that could enclose the cell (or by 520 μm, the thickness of the chamber, if the smaller dimension of the smallest rectangle that could enclose the particle was greater than 520 μm). Cells were assumed to lie with the largest cross-sectional area parallel (i.e. lie flat) to the bottom surface of the counting chamber; the smaller dimension of the smallest rectangle that could enclose the cell represents the maximum possible height of each cell. This results in an estimate of the maximum possible volume of phytoplankton cells within each sample, an approximation (but likely somewhat of an overestimation) of the true phytoplankton cell volume in each sample. The dietary quality of phytoplankton was calculated as the total volume of edible carbon within only those phytoplankton cells having the largest diameter 40 μm (which is generally considered to be within the gape size of zooplankton) using the following formula: Edible carbon (pg C) = 0.1204 × (volume (μl) of <40 μm cells)$^{1.051}$ (Rocha and Duncan 1985).

Statistical analysis

In this study, experimental units were wetland halves (n = 12). To maintain the power of the paired-wetland design, all endpoints except zooplankton community similarity and richness are expressed as a baseline standardized (value of control side) difference between what was observed in the control side subtracted from the value measured in the treated sides $\frac{T_i - C_i}{C_i} \times 100$. One-tailed, paired *t*-tests were used to determine the significance of declines in zooplankton and phytoplankton endpoints in the sampling periods immediately following both applications of herbicides (May 16/17 and June 10/11), as we expected that herbicide toxicity would manifest as a loss in the relative amounts of phytoplankton and zooplankton. Two tailed, paired *t*-tests were used to determine if there significant differences in phytoplankton and zooplankton endpoints in response to visible damage to macrophytes resulting from herbicide applications in the first sampling time point beyond which such damage had been observed (July 9/10; see Baker et al. 2014 for details) as well as at the end of the summer (September 7/8). All tests for differences in the Bray–Curtis index of similarity were conducted as one-tailed tests in comparison to the pre-treatment levels of similarity (baseline) where all expected changes in community composition should present as the sides of wetlands becoming less similar. All data are presented as the mean ± 90 % confidence intervals in the text and graphs.

To determine time-dependent effects of each treatment (H and HN) through the summer on the zooplankton community structure, the matrix of $\log_{10}(x + 1)$ transformed absolute abundance of zooplankton taxa was analyzed using the constrained multivariate ordination technique of partial redundancy analysis (pRDA) in the vegan package (Oksanen et al. 2015) in R (R Development Core Team 2012). Explanatory variables were treatment, time, and treatment regime (treatment x sampling time), where the effects of wetland and time were coded as dummy covariates such that these were "partialled out" similar to a repeated measures ANOVA with wetland and time as blocking factors. Significance of the model was tested by permutation (n = 199). This technique emphasizes the percentage change in abundance of taxa in the treatments relative to controls, independent from absolute abundance (van den Brink and ter Braak 1999).

For all analyses, a compromise between Type I and II errors was reached by choosing the α level that minimizes the average of α and β at a given critical effect size, accomplished by an iterative examination of β (through power analysis) over a range of α-levels (Mudge et al. 2012). Type I and II errors were considered to have equal costs, because we had no substantive evidence to indicate that either type of error was more serious/costly in these circumstances. The mean of a treatment variable falling outside 90 % of the predicted control distribution centered on the control mean was deemed to be a critical effect size (CES) to be detected, should it exist (Munkittrick et al. 2009). For a two-tailed test this corresponded to a CES of 1.64 standard deviations (SD) of the data, and 1.28 SD of the data was used for one-tailed tests. When there was no clear directional hypothesis we used the CES associated with a two-tailed test. For each test, equal prior probabilities of the null or alternative hypotheses being true were assumed. Optimal α and associated betas and omegas (ω = average chance of making a wrong conclusion) for each sample size were calculated using R with code from Mudge et al. (2012). Other combinations of the relative costs of Type I and II errors and CES may be used by the reader to re-evaluate the results using the data provided in Online Resource 1.

Results

Nutrient and glyphosate concentrations

Graphical presentation and extensive discussion of both nutrient and glyphosate concentration data have been published elsewhere (Baker et al. 2014; Edge et al. 2014), however we have briefly reviewed relevant concentrations here. Measures of nutrient concentrations in wetland water were lower than intended target concentrations after applications of nutrients began. Ammonium (NH_4^+) concentrations varied substantially throughout the course of the experiment, whereas TP concentrations remained relatively constant following the first nutrient addition. On average, the NH_4^+ concentration in treated wetland halves was 0.02 ± 0.03 mg/L higher than controls, and TP was 0.05 ± 0.01 mg/L higher than controls.

Glyphosate residues in the water column of the treated sides of experimental wetlands declined rapidly and were undetectable beyond 7 days post initial herbicide application. After this first herbicide application, the glyphosate concentration in the treated sides of all wetlands in the present study (n = 6) was 799 ± 644 µg a.e./L at 1 day post-application, 108 ± 112 µg a.e./L at 3 days post-application and 13 ± 11 µg a.e./L at 7 days post application. Despite having the same target glyphosate concentrations, glyphosate residues appeared (not statistically significant) to decline more slowly in the herbicide with nutrients-treated wetlands than the herbicide alone-treated wetlands, where the herbicide and nutrient wetlands had higher glyphosate concentrations of 1173.1 ± 1256.5 µg a.e./L on Day 1 ($p = 0.398$, n = 3,3, α = 0.244, paired two-tailed *t*-test) and 195.1 ± 205.1 µg a.e./L on Day 3 ($p = 0.239$, n = 3,3, α = 0.244, paired two-tailed *t*-test) than was observed in the herbicide alone-treated wetlands had glyphosate concentrations of 424.5 ± 343 µg a.e./L on Day 1 and 22.3 ± 18.3 µg a.e./L on Day 3. We examined whether dissipation rates of the two treatments could be related to differences in natural features of the wetlands including water depth and volume (of the treated half of wetlands only), or due to microbial decomposition capacity (as background microbial respiration rates; data and methods available in Online Resource 2). Post hoc linear regression analysis demonstrated that the maximum estimated water volume explained only a small portion of the variation in glyphosate concentrations ($R^2 = 0.14$, $p = 0.050$, n = 12) where larger wetlands halves had slower glyphosate removal rates. Microbial respiration rates also explained a very small amount of dissipation rates ($R^2 = 0.043$, $p = 0.0002$, n = 11), with higher respiration rates leading to more rapid glyphosate removal. Ultimately, glyphosate removal rates were not well described by these factors.

In water samples collected after the second herbicide application, glyphosate residues in treated sides of all wetlands (n = 6) were measured at 215 ± 244 µg a.e./L and 40 ± 38 µg a.e./L, at 3 and 7 days post-application, respectively. Based on these concentrations the half-life of glyphosate in the water of the treated wetlands was calculated to be approximately 1 day for each application period.

Pre-treatment characteristics of wetland plankton communities

Phytoplankton cell volumes ranged from 4.7 to 2608.0 µL/L and averaged 94.7 ± 0.06 µL/L during the pre-treatment period. Phytoplankton dietary quality (measured as edible carbon) ranged from 0.003 to 0.528 pg/L of wetland water, with an average of 0.090 pg/L. Generally phytoplankton amounts and quality (edible carbon content) were highly variable over the summer. Zooplankton abundance ranged from 3 to 331 organisms/L, with an average 44.2 organisms/L prior to chemical treatments across all wetland halves. Samples contained between 3 and 12 zooplankton taxa each with a total of 18 taxa found. *Cyclopoida* sp. adults and Copepoda nauplii dominated, followed by the cladoceran, *Chydorus sphaericus*. The similarity of zooplankton community composition was on average 59.0 ± 1 % (n = 6) between control and treated sides prior to chemical applications, which was similar to the natural levels of Bray–Curtis similarity measured in other studies (Cottenie et al. 2003; Hunt et al. 2008). The average community similarity of zooplankton between the paired halves of a wetland was 22.6 % higher than the average of the similarity found between independent wetlands (36.4 ± 11 %), suggesting that the paired wetland half design of this experiment significantly improved the overall power of the experimental design.

Short-term effects on plankton communities

One day after the first application of the herbicide to the treated sides of the nutrient-treated wetlands (HN treatment) there was a significant decline in phytoplankton quality (−8.3 ± 12.4 %, $p = 0.154$, $n = 3$, $\alpha = 0.212$ one-tailed paired *t*-test, Fig. 2b) and in zooplankton abundance of 37.7 ± 23.5 % (Fig. 3a), or an average loss of approximately 14 zooplankters/L ($p = 0.060$, $n = 3$, $\alpha = 0.212$, one-tailed paired *t*-test). This same treatment group also experienced a significant decline in zooplankton taxa richness, with an average loss of 2.7 ± 2.9 taxa (Fig. 3b), ($p = 0.135$, $n = 3$, $\alpha = 0.212$, one-tailed paired *t*-test). This also led to a decline in the overall similarity of the herbicide and nutrient treated zooplankton communities of 27.8 ± 29.2 % ($p = 0.129$, $n = 3$, $\alpha = 0.212$, paired one-tailed *t*-test) (Fig. 3c). In contrast, during this initial response period for the herbicide alone treatment there was only a small, but significant decline in zooplankton richness of 1.7 ± 0.55 taxa ($p = 0.019$, $n = 3$, $\alpha = 0.212$ paired one-tailed *t*-test), with no attendant declines in zooplankton abundance or community similarity, nor in phytoplankton quality or amounts (Fig. 2 and 3, Online Resource 1). As shown in Fig. 3, one day prior to the second herbicide application the zooplankton communities of all treated wetlands appeared to have recovered to approximately pre-treatment levels of abundance (H $p = 0.519$, HN $p = 0.821$), richness (H $p = 0.500$, HN $p = 0.250$) and community similarity (H $p = 0.243$, HN $p = 0.327$).

Unlike after the first application, one day after the second application of herbicide, there were no significant changes observed in phytoplankton or zooplankton endpoints in the herbicide and nutrient treated wetlands (Fig. 2 and 3, and Online Resource 1). For the herbicide only treated wetlands, phytoplankton abundance and quality appeared to decline, but not significantly. However, the richness of zooplankton in the herbicide alone treated wetlands was reduced by an average of 2.7 ± 0.6 taxa compared to controls ($p = 0.008$, $n = 3$, $\alpha = 0.212$, one-

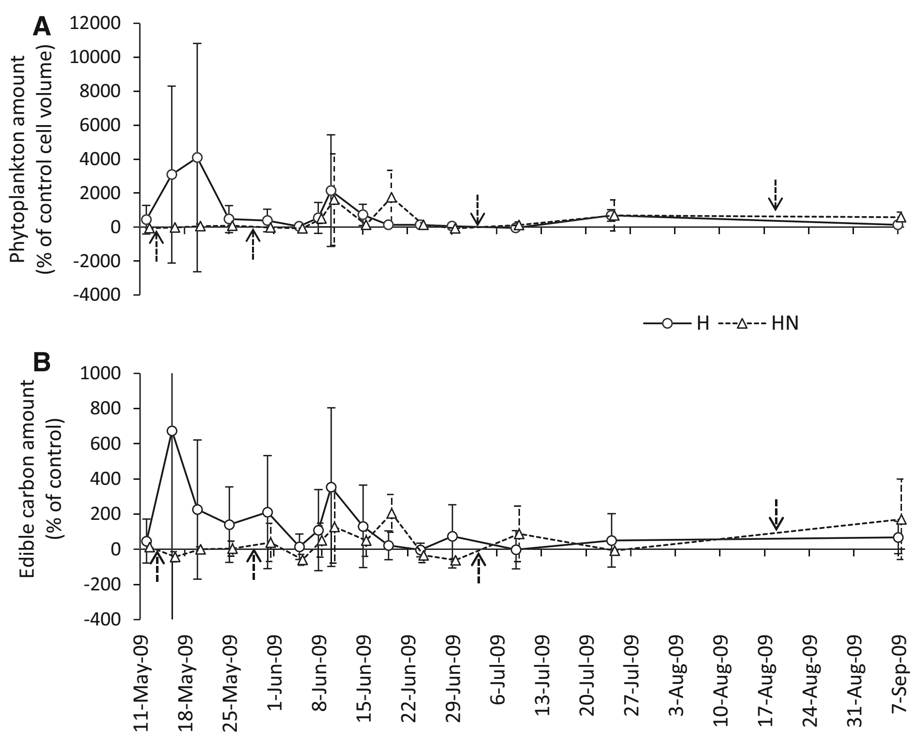
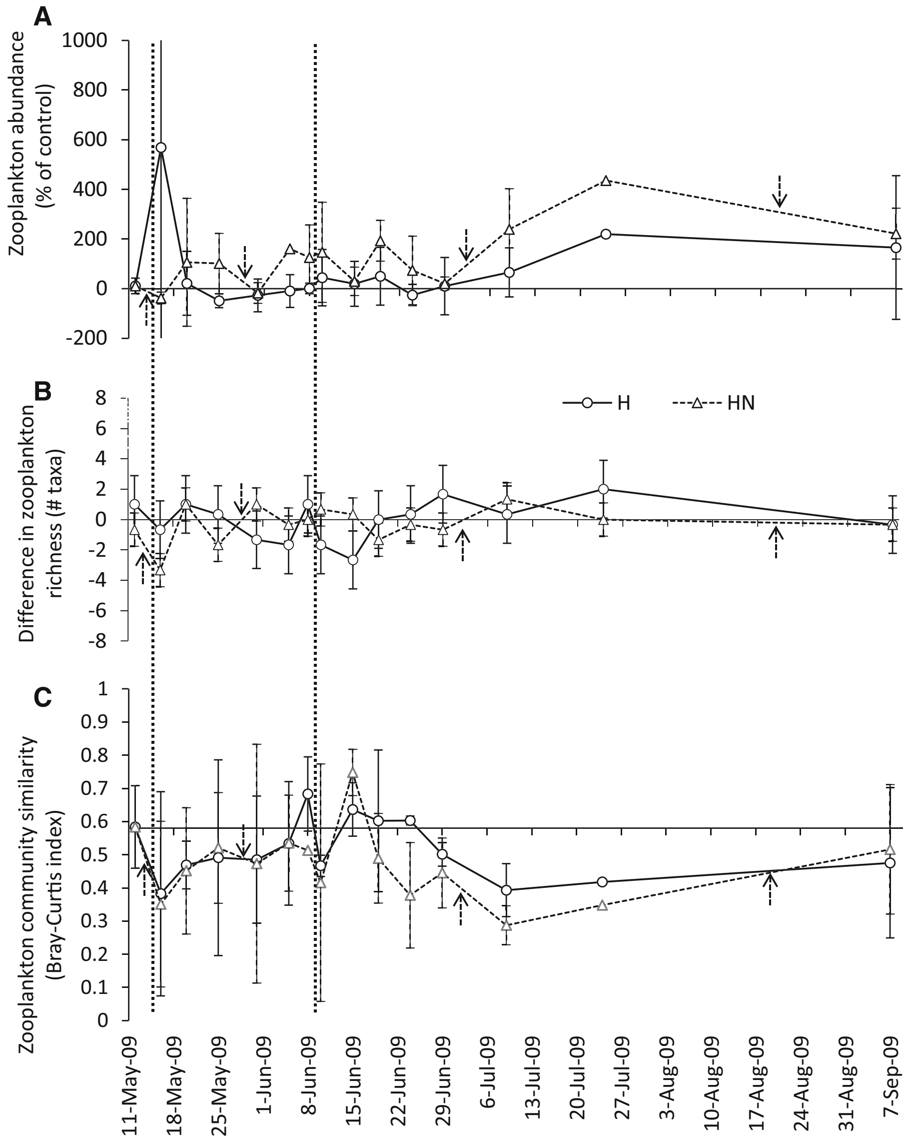


Fig. 2 Change (±90 % CI), as percentage of the control, in phytoplankton **a** cell volume and **b** edible carbon in the treated sides of wetlands, (H: n = 3, HN: n = 3). H = treatments with 2.88 mg a.e./L of Roundup WeatherMax© on the dates indicated by the *vertical lines*. HN = identical herbicide treatment regime with the addition of nutrients to wetlands on dates indicated by *arrows*

Fig. 3 Differences (±90 % CI) in zooplankton **a** abundance, as a percentage of control; **b** richness (treatment–control) and **c** the average community similarity between the treated sides of wetlands compared to respective control sides (H: n = 3, HN: n = 3). H = treatments with 2.88 mg a.e./L of Roundup WeatherMax© on the dates indicated by the *vertical lines*. HN = identical herbicide treatment regime with the addition of nutrients to wetlands on dates indicated by the *arrows*



tailed paired *t*-test). Zooplankton abundance and community similarity were not significantly different between treatment and control halves as a result of the second herbicide application for either treatment.

Longer-term effects on plankton communities

After macrophyte declines were first observed on June 24–25 (Baker et al. 2014), which was 10 days beyond when glyphosate was no longer measurable in the water column, changes in the zooplankton (Fig. 3a)—but not phytoplankton (Fig. 2)—community were observed. By July, the herbicide and nutrient treated wetlands showed no significant changes in algal abundance or quality. However, zooplankton communities in this treatment became less similar, by as much as 34.1 ± 2.0 % on July 9/10 ($p = 0.001$, $n = 3$, $\alpha = 0.212$, paired one-tailed *t*-test), compared to pre-treatment levels, an effect which persisted for over 6 weeks, past the July 24th (period 11) sampling date (Fig. 3a). This change was much larger than natural background variability where there was a minimal change in community composition in control sides of wetlands (similarity among the control halves of wetlands had not otherwise changed—on average 7.2 ± 7.6 % below pre-treatment levels). At this same time the abundance and richness of zooplankton in treatment versus control halves of the herbicide and nutrient treated wetlands increased by 238.6 ± 163.7 % ($p = 0.139$, $n = 3$, $\alpha = 0.244$ paired, two-tailed *t*-test) and by 1.33 ± 2.19 taxa ($p = 0.213$, $n = 3$, $\alpha = 0.244$, paired two-tailed *t*-test), respectively. Herbicide alone treated wetlands showed variable but no significant differences in phytoplankton amounts and quality. When compared to the herbicide and nutrient treated wetlands, all zooplankton endpoints in the herbicide only treated wetlands showed similar but non-significant trends.

Late in the summer (Sep 7/8 sampling period) there was also evidence of ongoing effects of the herbicide and nutrient treatments on phytoplankton (Fig. 2). There was an increase in phytoplankton amounts by 640.7 ± 295.2 % compared to controls ($p = 0.182$, $n = 3$, $\alpha = 0.244$, paired two-tailed *t*-test). The trend of significantly higher abundance of zooplankton in herbicide and nutrient treated wetlands continued through to the end of the summer, where there was, on average, 221.4 ± 102.7 % more zooplankton in the affected sides of herbicide and nutrient treated wetlands than the controls ($p = 0.071$, $n = 3$, $\alpha = 0.244$, paired two-tailed *t*-tests). However, the richness and similarity of zooplankton communities had returned to approximately pre-treatment levels by this date. Phytoplankton amounts and quality in herbicide alone treated wetlands were variable, but ultimately were not significantly affected. Zooplankton communities of wetlands that had been treated with only herbicides showed responses similar in direction, but not magnitude to those of the herbicide and nutrient treated wetlands.

An examination of the cumulative effects of treatments on zooplankton community composition in the wetlands over the summer showed that treatment regime (treatment x sampling date) explained 8.7 % of the variability in the zooplankton community composition data, and treatment category explained 0.7 %. In contrast the partialled covariates of sampling date and wetland explained 35.7 % of the variability where 54.9 % of the variation in zooplankton community was not explained at all by this model pRDA. Zooplankton taxa did not strongly load on the first two axes of the model (species weight of <0.5), which is logical considering the lack of significant overlap in species composition among all wetlands and, as such, we cannot comment on effects on individual taxa. The model was not statistically significant ($p = 0.680$, by permutation $n = 199$). The effects (across wetlands) of treatment regime (treatment × sampling time) of herbicides alone and herbicide + nutrient treatments had inconsistent effects on the zooplankton community, which is consistent with the data described herein showing different effects in the short term versus long term. This result suggests that experimental treatments on the whole were less important in determining zooplankton community composition than wetland-specific factors or time of year.

Discussion

The over-spray of small, shallow wetlands in NB, Canada with a maximum label-rate application of Roundup WeatherMax by itself resulted in only minor and transient effects on freshwater zooplankton and phytoplankton communities. These findings are in agreement with prevailing literature; under normal-use circumstances including the observation of appropriate buffer zones for aquatic ecosystems and recommended application rates, glyphosate-based herbicides seem unlikely to cause significant acute toxicity to non-target organisms in freshwater ecosystems (Folmar et al. 1979; Perez et al. 2007; Solomon and Thompson 2003; Thompson 2004; Tsui and Chu 2003, 2004). Our results contrast sharply with the findings of Relyea (2005a, b) who suggested that the use of these herbicides in and around wetlands may result in severe impacts on amphibians, and that this could lead indirectly to effects on primary productivity (periphyton biomass) from the loss of grazing pressure. The results of our study, taken in context with those of a simultaneous study (Edge et al. 2014) showing no significant impacts on amphibian growth, development or survival, implies that ecosystem-level effects under realistic conditions are unlikely. Our results are in line with a recent study indicating that glyphosate-based herbicides can be more toxic to aquatic organisms under laboratory than field conditions, with paradoxically opposite directions of effects depending on the experimental venue (Mikó et al. 2015). These concepts are certainly not new to ecotoxicology and echo long-standing calls and needs for the inclusion of more complex multispecies and mesocosm testing approaches, as well as operational monitoring, to inform comprehensive ecotoxicological risk assessments (e.g. Bartell et al. 1992; Cairns 1988; Draggan and Giddings 1978; Thompson 2004).

In contrast to the lack of significant negative effects in the glyphosate only treatment, we observed acute effects on the plankton communities exposed to a mixture of Roundup WeatherMax and nutrients from fertilizers which is suggestive of the possibility of additive or interactive effects of this mixture. Aqueous glyphosate concentrations dropped more gradually in the wetlands treated with both herbicides and nutrients compared to the herbicide alone treatment. Although the mechanisms are not known, it is possible that this could have been mediated through the microbial community. Glyphosate is metabolized in freshwater ecosystems primarily by heterotrophic bacteria and cyanobacteria (Zaranyika and Nyandoro 1993), which will use the glyphosate molecule as a source of phosphorus, particularly in conditions of phosphorus limitation in oligotrophic systems (Kirchman 1994). In the present study, the microbial communities of wetlands which had been treated with nutrients (phosphoric acid and ammonium nitrate) along with herbicides may have initially utilized the supplemented labile phosphorus in favour of a more complex extraction of phosphorus from the glyphosate molecule. However, in the herbicide alone treated wetlands, which were lacking an experimentally augmented phosphorus source, the microbial communities would be more likely to resort to metabolism of glyphosate molecules as a source of phosphorus, resulting in a more rapid degradation process for glyphosate residues in these wetlands. This may have been responsible for the lower concentration of glyphosate in the water column of herbicide alone treated wetlands, which in turn resulted in relatively smaller effects observed on the plankton community. These findings emphasize the need for further studies on multiple stressors.

Immediately following the second application of herbicides there was a small increase in zooplankton and an even greater increase (2000–3000 %) in phytoplankton abundance in all treated wetland halves. Our hypothesis was for reductions in plankton communities directly resulting from toxicity due to herbicide applications and, as such, we intentionally used one-sided statistical tests that would ignore such increases. Increased abundance of plankton was unlikely to have been a direct stimulatory effect of the herbicide, but it may have been a result of the combination of indirect (from the first application of herbicide) and direct (from the second application of herbicide) effects. The loss of some zooplankton taxa during the initial herbicide application might have released the remaining zooplankton and phytoplankton assemblages from competition and predation. It is also possible that this observation represents an indirect effect of the reduction of zooplankton grazing pressure on some phytoplankton taxa in the community, resulting from the significant loss of zooplankton abundance after the first herbicide application. In a similar study of the effects of another glyphosate herbicide formulation, Vision, in a shallow Canadian wetland, Wojtaszek (unpublished data, 2004) observed an increase in the small phytoplankton taxa (Cyanophyta and Crysophyceae) approximately 7 days after herbicide application. There are many potential mechanisms for the increase in phytoplankton observed herein including greater nutrient availability to phytoplankton resulting from reduced competition from injured macrophytes or from the release of dissolved organic exudates from macrophytes as a result of shock or stress (Wetzel 2001) which could serve as a further nutrient source for phytoplankton.

The main source of toxicity of formulated glyphosate herbicides to zooplankton taxa is known to be the surfactants (generally POEA-type surfactants, Folmar et al. 1979), but their exact composition can be proprietary information of the manufacturers. Surfactants are added to most formulations to increase the penetration of the active ingredient, the glyphosate acid, through the plant cuticle. Comparison of the mechanistic role of surfactants in of different formulations of glyphosate-based herbicides is challenging because of the lack of publically-available information on the chemical composition of surfactants available.

A key finding in this experiment was the significant, indirect effects of Roundup WeatherMax in combination with nutrient applications on plankton communities, where these effects were more persistent than any direct effects. The late-summer effects on the plankton community were unlikely to be the result of direct toxicity, as any glyphosate residues in the surface water of wetlands were undetectable beyond 7 days post application in the spring. The timing of the increasing abundance and richness of zooplankton occurred approximately in parallel to the reduction of emergent vegetation; by June 24 the treated sides of all wetlands had visibly reduced macrophyte cover (average of 19 % reduction; see Baker et al. 2014). Other studies of fishless wetlands suggest that predacious littoral macroinvertebrates use macrophytes as a platform from which to hunt zooplankton (Sagrario et al. 2009). Thus, zooplankton in the current study may have had lower predation pressure as macrophytes cover was reduced. Increased zooplankton abundance or richness should not necessarily be interpreted as a positive effect, as this was a significant departure from the normal conditions of the wetlands. This increase in zooplankton could have important consequences for the food web structure of the wetland as they are an important food source for many benthic invertebrates (Bollens et al. 1992) and they can exert top-down control over the phytoplankton community (Kerfoot and Deangelis 1989).

The absence of consistent or prolonged toxicity is not surprising given that glyphosate concentrations in the water of our experimental wetlands degraded more rapidly than has been observed in most studies. The half-life of slightly less than one day was on the shorter end of the reported half-lives in natural waters of between 1.3 and 14 days (Degenhardt et al. 2012; Giesy et al. 2000; Goldsborough and Beck 1989; Goldsborough and D. J. 1993; Perez et al. 2007). These studies have suggested that shallower, warmer wetlands having a higher sediment surface area to water ratio are more likely to have rapid glyphosate dissipation from the water column. Wetlands in the current study were generally shallow with clay/loam-based sediments and these characteristics, along with the suspension of some sediments during herbicide application, likely led to a rapid binding of the glyphosate to sediment particles (Glass 1987), and would be more representative of the typical mode of entry into wetlands as a result of sediment run-off from rain events. Thus, the potential reduction of bioavailability through sorption to suspended sediments, as may have occurred in this experiment, is likely representative of the fate of glyphosate in shallow wetlands. Plankton communities in these wetlands may have had a shorter exposure to glyphosate than in previous laboratory or microcosm studies [e.g. 4 days: (Bengtsson et al. 2004); 8 days: (Chen et al. 2004); 13 days: (Relyea 2005a)], which may partially explain why the direct effects are smaller and more transient in this ecosystem-scale study than might be predicted based on standard laboratory toxicity tests. These findings suggest that studies with longer exposure times or those lacking natural sediments may be overestimating the effects of glyphosate herbicides on zooplankton in wetlands in agricultural crop areas.

Conclusions

We demonstrated that worst-case contamination of wetlands with the herbicide Roundup WeatherMax in combination with fertilizer nutrients resulted in transient and relatively minor disruptions of plankton community structure. We emphasize two important points as they relate to risk assessment of this chemical. First, there is a greater need to incorporate field testing as an integral component of a tiered risk assessment process. Despite the identification of longer-term, indirect impacts on the zooplankton community, it would appear that the regulated use of this glyphosate-based herbicide, which prohibits direct application to wetlands such as those used in this study, is unlikely to result in the serious impairment of wetland plankton communities, as might have been predicted from the findings of laboratory-based studies of similar glyphosate-based herbicides. Secondly, the findings of significant effects only in the treatment containing both the herbicide and fertilizers implies that effective ecotoxicological risk assessments should also consider scenarios in which other contaminants or stressors may co-occur in the receiving system, as the possibility exists for joint activity. Addressing the significance of complex ecosystem-level responses to complex mixtures of contaminants, as was done in this study, will contribute to more ecologically-relevant ecotoxicological risk assessments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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