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The response of amphibian larvae to exposure to a glyphosate-based herbicide (Roundup WeatherMax) and nutrient enrichment in an ecosystem experiment

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ABSTRACT

Herbicides and fertilizers are widely used throughout the world and pose a threat to aquatic ecosystems. Using a replicated, whole ecosystem experiment in which 24 small wetlands were split in half with an impermeable barrier we tested whether exposure to a glyphosate-based herbicide, Roundup WeatherMax™, alone or in combination with nutrient enrichment has an effect on the survival, growth or development of amphibians. The herbicide was applied at one of two concentrations (low = 210 μg a.e./L, high = 2880 μg a.e./L) alone and in combination with nutrient enrichment to one side of wetlands and the other was left as an untreated control. Each treatment was replicated with six wetlands, and the experiment was repeated over two years. In the high glyphosate and nutrient enrichment treatment the survival of wood frog (*Lithobates sylvaticus*) larvae was lower in enclosures placed *in situ* on the treated sides than the control sides of wetlands. However, these results were not replicated in the second year of study and they were not observed in free swimming wood frog larvae in the wetlands. In all treatments, wood frog larvae on the treated sides of wetlands were slightly larger (< 10%) than those on the control side, but no effect on development was observed. The most dramatic finding was that the abundance of green frog larvae (*Lithobates clamitans*) was higher on the treated sides than the control sides of wetlands in the herbicide and nutrient treatments during the second year of the study. The results observed in this field study indicate that caution is necessary when extrapolating results from artificial systems to predict effects in natural systems. In this experiment, the lack of toxicity to amphibian larvae was probably due to the fact the pH of the wetlands was relatively low and the presence of sediments and organic surfaces which would have mitigated the exposure duration.

1. Introduction

Worldwide, herbicides are used extensively in a variety of sectors including agriculture and silviculture. The most commonly used herbicides in the world are glyphosate-based, likely due to the development of glyphosate-tolerant crop species, and that risk assessments determined they are relatively non-toxic to non-target species (Giesy et al., 2000). Increasing fertilizer use has led to an increase in the two major fertilizer components, nitrogen and phosphorous, in the soil and wetlands of agricultural regions (Bennett et al., 2004; Puckett, 1995). Detrimental effects of both these anthropogenic contaminants have been observed in studies on various non-target aquatic organisms, most notably amphibians (Edginton et al. 2004; Edwards et al., 2006; Griffis-Kyle, 2006; Relyea, 2004). However, the effects of concurrent exposure in natural systems are largely unknown, of concern, and in need of investigation.

There are many commercially available glyphosate-based products, all of which typically contain glyphosate, water, and a surfactant. Glyphosate, the active ingredient of these formulations, is considered to pose a relatively low risk to aquatic animals because it targets an enzyme (enolpyruvylshikimate phosphate synthase) which is only found in plants and some micro-organisms (Rubin et al., 1982). Surfactants vary among formulations and the Roundup family of glyphosate products may contain, to varying degree, the polyoxyethylene tallowamine surfactant (POEA) that is thought to be primary putative toxicant in these formulations (Edginton et al., 2004; Mann and Bidwell, 1999). Environmental monitoring of glyphosate in agricultural wetlands

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and surface waters indicate that maximum background aqueous concentrations range between 10 and 40.8 μg acid equivalents (a.e.)/L (Battaglin et al., 2009; Byer et al., 2008; Struger et al., 2008). Concentrations which are lower than those predicted under worst case exposure situations such as the Canadian model for Tier 1 assessment (concentrations between 1430 (Thompson et al., 2004) and 4486 μg a.e./L (Giesy et al., 2000)). The discrepancy between measured an expected concentrations is likely due to glyphosate having a relatively short half life in aquatic systems (Goldsborough and Brown, 1993; Wojtaszek et al., 2004).

Laboratory and mesocosm studies have demonstrated that exposure to some glyphosate-based herbicides results in increased mortality or effects on the growth and development of amphibian larvae at concentrations in the range predicted by worst case exposures (*e.g.*, Edginton et al., 2004; Fuentes et al., 2011; Howe et al., 2004; Mann and Bidwell, 1999; Relyea, 2005; Williams and Semlitsch, 2010) indicating these herbicides pose a high risk to amphibian larvae in natural systems. However, negative effects have not been observed in the limited number of field experiments examining impacts of on either larval (Edge et al., 2012; Lanctôt et al., 2013; Thompson et al., 2004; Wojtaszek et al., 2004) or terrestrial life stages (Edge et al., 2011, 2013). This inconsistency indicates that our current understanding of the potential effects of these herbicides in natural systems is poor and in need of further investigation.

Many wetlands in agricultural regions may also experience nutrient enrichment (nitrogen and phosphorus) due to fertilizer runoff from adjacent fields (Carpenter, 2005; Galloway et al., 2002). All three major forms of nitrogen (ammonium, nitrite, and nitrate) can be toxic at high concentrations, but at environmentally relevant concentrations they are more likely to result in sublethal impacts on the growth and development of amphibian larvae (Edwards et al., 2006; Egea-Serrano et al., 2009; Griffis-Kyle, 2006; Ortiz-Santaliestra and Sparling, 2007) For phosphate, no direct toxicity has been detected (Earl and Whiteman, 2009, 2010). In aquatic systems that are nitrogen or phosphorus limited nutrient additions are quickly assimilated by phytoplankton and macrophytes and result in eutrophication (Carpenter, 2005), which is a risk to aquatic life (Schindler, 2006; Smith et al., 2006; Vadeboncoeur et al., 2001). Increasing primary productivity can result in changes to the structure and function of aquatic communities by changing abiotic (*e.g.*, oxygen depletion or reduced light penetration) and/or biotic (*i.e.*, bottom-up trophic cascades) conditions (Carpenter, 2005; Schindler, 2006). Thus, any observed changes to amphibian growth and development rates are likely due to indirect effects mediated through changes to wetland productivity and not direct toxicity (Edwards et al., 2006; Rouse et al., 1999).

This field study was undertaken with the general goal of determining whether the commercial, glyphosate-based, herbicide Roundup WeatherMax™ when applied alone or in combination with nutrient enrichment has significant direct effects on amphibian larvae in natural wetlands. This goal tests the findings of prior laboratory and mesocosm studies that found glyphosate-based herbicides are toxic to amphibian larvae at environmentally realistic concentrations. This is an extremely important test because laboratory and mesocosm studies predict that effects should occur in nature, a prediction that must be tested. To address this goal, we utilized a suite of natural wetlands split in half as experimental units and replication through time (two years of study) to examine:

(1) Whether exposure to Roundup WeatherMax at one of two test concentrations, alone or in combination with nutrient additions, has an effect on the abundance, growth, and development of larval wood frogs (*Lithobates sylvaticus*) in natural wetlands or caged *in situ*.

(2) Whether exposure to Roundup WeatherMax at one of two test concentrations, alone or in combination with nutrient additions, has an effect on the abundance of larval green frogs (*L. clamitans*) or spring peepers (*Pseaudacris crucifer*) in natural wetlands.

2. Materials and methods

2.1. Study species

The primary focal species for this study was the wood frog. Wood frogs breed over a two week period immediately after wetlands become ice-free in the spring (Late April/early May at the study site), but the majority of eggs are often laid over 3–5 days. The embryonic period for wood frogs lasts between 7 and 14 days and larvae complete development 45–75 days later.

There were two secondary focal species: the spring peeper and the green frog. Spring peepers begin to breed shortly after wetlands become ice free and larvae metamorphosis after 50–70 days of development (Skelly, 1995). Green frogs begin to breed eight weeks after wetlands become ice free and larvae require two years to complete development in northern populations such as the one studied here (Patton and Crouch, 2002).

2.2. Site description

The field site was located at the Long Term Experimental Wetlands Area (LEWA) on Canadian Forces Base Gagetown (CFB Gagetown) in New Brunswick, Canada (45°40′N, 66°29′W). Within the 6 km^2 area, we chose 24 wetlands that were relatively small (< 1 ha), had no permanent inflow or outflow, had relatively homogenous macrophyte cover, and were known wood frog breeding sites.

Each wetland was split in half using an impermeable plastic barrier constructed from 0.76 mm black high density polyethylene (HDPE) (Poly-Flex Inc. Geomembrane Lining Systems, Grand Prairie, Texas). A permanent staff gauge was installed at the deepest point in the each wetland half to monitor water depth and to calculate wetland volumes throughout the summer. See Online Resource for additional details and the physical and chemical characteristics of all wetlands (Online Resources Tables 1 and 2).

2.3. Egg mass manipulation

Before chemical applications each year all wood frog egg masses in each wetland were counted, collected, and then randomly re-deployed back to the wetlands. Egg masses were placed in hair nets (10 mm netting) with a plastic float and held in place with bamboo poles. Every 3 days egg masses were counted and those that were preyed upon by green frog larvae were counted. Larvae could swim out of the gaps in the netting and into the wetland after hatching. Hatching success was high (~90%), unless egg masses were preyed upon by green frog larvae which typically resulted in 100% mortality.

In 2009 the number of wood frog egg masses each wetland received was determined by grouping the 24 wetlands into three categories (9 small, 10 medium, and 5 large) based on estimated surface areas. Small wetlands received 13 egg masses per side, medium wetlands received 25 egg masses per side and large wetlands received 50 egg masses per side (Online Resources Table 1). In 2010, 543 wood frog egg masses were laid in the 24 experimental wetlands, substantially fewer than the 813 that were laid in 2009. Due to the reduction in egg masses available 13 egg masses were placed on each side of 21 wetlands (Online Resources Table 1).

2.4. Experimental design and treatment applications

The 24 wetlands were assigned to one of four experimental treatment groups to minimize differences among treatments in water chemistry, location and wetland size. A split-plot (wetland) experimental design was employed; one side of each wetland was randomly assigned as the control (received no treatment) and the other as treatment (received the herbicide or herbicide and nutrient treatment). This design controls for among wetland differences (such as pH, size, and density) because comparisons are made between the control and treatment sides within each wetland.

Treatments were achieved by direct application (See below) of the glyphosate-based herbicide Roundup WeatherMax (Monsanto, Winnipeg, MB, CAN) at one of two concentrations, alone and in the presence of nutrient additions to the surface of the wetlands. Nutrient treatments were designed to increase the trophic status of each wetland one level (*e.g.*, oligotrophic to mestrophic) above background levels (calculated in 2008 – one year prior to chemical treatment). Nitrogen and