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Effects of atrazine on egg masses of the yellow-spotted salamander (*Ambystoma maculatum*) and its endosymbiotic alga (*Oophila amblystomatis*)

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ABSTRACT

Embryonic growth of the yellow-spotted salamander (*Ambystoma maculatum*) is enhanced by the presence of the green alga *Oophila amblystomatis*, in the egg capsule. To further assess potential impacts of herbicides on this relationship, *A. maculatum* egg masses were exposed to atrazine (0–338 μg/L) until hatching (up to 66 days). Exposure to atrazine reduced PSII yield of the symbiotic algae in a concentration-dependent manner, but did not significantly affect visible algal growth or any metrics associated with salamander development. Algal cells were also cultured in the laboratory for toxicity testing. In the 96-h growth inhibition test (0–680 μg/L), ECx values were generally greater than those reported for standard algal test species. Complete recovery of growth rates occurred within 96-h of transferring cells to untreated media. Overall, development of *A. maculatum* embryos was not affected by exposure to atrazine at concentrations and durations exceeding those found in the environment.

1. Introduction

The yellow-spotted salamander (*Ambystoma maculatum*) produces gelatanous egg masses that are colonized by a green alga commonly referred to as *Oophila amblystomatis*. Several closely-related strains of this chlamydomonad have been identified in *A. maculatum* egg capsules (Kerney et al., 2011; Graham et al., 2013; Kim et al., 2014; Rodriguez-Gil et al., 2014), and as well as from eggs of *A. gracile* (Northwestern salamander), *Lithobates sylvatica* (wood frog) and *L. aurora* (red-legged frog) (Kim et al., 2014). To date, most observations on the occurrence and life stages of this alga, and most experimental research into the embryo-alga relationship have focussed on *A. maculatum*. A number of potential benefits of symbiosis have been identified, such as provision of oxygen (Pinder and Friet, 1994) or fixed carbon (Graham et al., 2013), and uptake of ammonia (Goff and Stein, 1978) by the algae. While the relationship may not be mutualistic under all environmental conditions (Bianchini et al., 2012), a number of studies have reported a significant positive correlation between embryonic development and density of algae (Gilbert, 1942, 1944; Marco and Blaustein, 2000; Graham et al., 2013), resulting in questions over the potential for indirect impacts to development of salamanders if algal growth is inhibited.

Notable declines in populations of amphibians have been reported over the past 15 years as a result of habitat loss and alterations, invasive species, disease, and chemical contaminants (Mann et al., 2009; Lesbarrieres et al., 2014). While the spotted salamander has not been assessed under COSEWIC (Committee on the Status of Endangered Wildlife in Canada) or the U.S. Fish and Wildlife Service, it is listed as a "specially protected amphibian" under Ontario's Fish and Wildlife Conservation Act (1997) in light of these types of threats. Since its wide distribution includes major agricultural regions of North America, and given the apparent benefits derived

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from the algal association, exposures to herbicides that impair algal performance represents a plausible adverse outcome pathway (AOP) for this species (Van Der Kraak et al., 2014).

There has been at least one previous study on the role of herbicides on this algal-salamander relationship. Olivier and Moon (2010) exposed clutches of *A. maculatum* eggs to the photosystem II inhibitor atrazine (as the formulation Aatrex®) throughout embryonic development (beginning at Harrison stage 10–17). They reported significant mortality and impairment of hatching success, as well as visible differences in algal growth within the eggs at nominal concentrations as small as 50 μg/L. As noted by the authors, confirmation of this effect using the active ingredient (AI) was determined to be a necessary next step. Considering the low likelihood of atrazine and its formulated adjuvants co-occurring in aquatic systems due to markedly different individual physiochemical properties (e.g., solubility, partitioning coefficients, stability, volatility etc.) (Andrus et al., 2013, 2015; van der Kraak et al., 2014), results of toxicity testing with the AI alone are an important component in understanding risk in the environment, and particularly the aquatic environment. As a result, we aimed to confirm this range of sensitivity with whole egg masses using technical-grade atrazine and a robust study design (see Harris et al., 2014). This study aimed to assess the impacts of atrazine on development of embryos of *A. maculatum* by measuring hatching success, size and stage of *A. maculatum* larvae at hatching following exposure throughout embryonic development under near-field conditions. In addition, we have addressed questions raised in a weight of evidence review on atrazine and amphibians (see Van Der Kraak et al., 2014).

2. Methods

2.1. Egg mass exposures

Egg masses of *A. maculatum* were collected from a shallow lake (Lost Ray Lake; 17T 692041 5051565) in Algonquin Provincial Park, Ontario, Canada on May 13th and 14th, 2014 under permit from Ontario Parks. Eggs were transported to the Hagan Aqualab at the University of Guelph where initial stage (Harrison and Wilens, 1969) and approximate number of embryos per egg mass were recorded (SI-Table 1). Masses with initial counts in excess of 65 embryos were divided in two using a sterile scalpel to obtain a set of egg masses with approximately 22–55 embryos at stages 22–29. One test unit per concentration received a portion of a split mass, with remaining units receiving an intact egg mass. SI-Table 1 identifies all treatments, initial stage, number of embryos (initial and final counts) and whether splitting occurred for each egg mass. Based on final counts (hatched embryos plus unhatched embryos and undeveloped eggs), two egg masses contained significantly more embryos than initially counted (~30% more; 10–3 and 300–2), so actual range of number of embryos per egg mass was 22–86.

Beginning May 16th, egg masses at stage 23–29 (n = 5 for controls; n = 3 for treatments) were exposed to technical-grade atrazine (96.2% pure) in non-chlorinated Guelph well water (as used by Bianchini et al. (2012)) at nominal concentrations of 0, 3, 10, 30, 100, and 300 μg/L until hatching was complete (29–66 d). Concentrations were chosen to reflect environmentally relevant exposures, as well as concentrations at which responses in isolated alga had previously been observed. Given the lack of field data for concentrations of atrazine and degradation rates in shallow forest pools, these exposure concentrations and durations also conservatively include continuous exposure to values 4–10-x greater than the 99th centile of annual maximums estimated for farm ponds in areas of intensive atrazine use (Giddings et al., 2005). Exposures occurred in 1.5-L glass beakers containing 1 L exposure solution (atrazine in non-chlorinated well water), made without the use of organic solvents. Exposure solutions were renewed weekly and concentrations of atrazine were tested pre- and post-renewal in duplicate sub-samples by enzyme-linked immunosorbant assay (Abraxis Atrazine Magnetic Particle Kit; limit of detection 0.05 μg/L).

Exposures occurred in a controlled walk-in Constant Temperature Control environmental chamber with datalogger, set at 10 °C, as described by a number of researchers working with *A. maculatum* egg masses over multiple weeks (Bianchini et al., 2012; Olivier and Moon, 2010; Tattersall and Spiegelaar, 2008). Full-spectrum grow lights were set on a 14:10 light:dark cycle, and measured fluence of photosynthetically active radiation (PAR) was 140–254 μmol/m^2/s, beyond the typical level of saturation for microalgae (Mayer et al., 1998) but below values measured in-water during collection (~600 μmol/m^2s). Test units were randomly positioned on four shelves with individual lighting and were gently aerated by air stones to produce near-field conditions at the time of collection (10–11 mg oxygen/L).

Along with the above-described concentration-response test for atrazine, embryo responses were measured simultaneously in untreated egg masses (stage 22–29) incubated in the dark (measured PAR fluence = 0 μmol/m^2/s) with and without aeration, under the standard light cycle without aeration, and under the light cycle without aeration at 30 μg/L atrazine (n = 3 for all alternative condition treatments). These test units were included to compare effects of varying environmental conditions (reduced aeration) on impacts of atrazine, as well as to compare the magnitude of effects due to atrazine with effects occurring as a result of substantial elimination of algal growth due to darkness. All treatments are shown in SI-Table 1.

Measurements of standard water chemistry parameters (dissolved oxygen, pH, temperature, conductivity), plus ammonium (NH_4^+) were conducted for all units daily using a YSI ProPlus. PAR was measured approximately weekly at six locations on each shelf using a quantum fluence metre (Apogee Instruments). Hardness, alkalinity, and phosphate were measured approximately weekly in the test solution source water (non-chlorinated groundwater) by Hach test kit.

All egg masses were photographed weekly to track visible differences in algal growth. An additional egg mass was maintained under each treatment (both standard and alternative) for assessment of effective quantum yield of photosystem II ("PSII yield") by PAM fluorometry within the eggs themselves. Weekly, two to four eggs were removed from each designated mass using a sterile scalpel, and placed in a disposable 4-well polycarbonate dish. PSII yield of each egg was measured under ambient light using a Mini-PAM Portable Chlorophyll Fluorometer (Heinz Walz, Effeltrich, Germany). On days 14, 21, and 28, five saturation pulses were administered to each egg 30 s apart and the mean of the last three fluorescence values was used in subsequent calculations and statistical modelling (Vallotton et al., 2008). On day 7, each egg received two saturation pulses (as methods were still being established).

Hatched larvae were removed once a day using a wide-mouth pipette, staged according to Harrison and Wilens (1969) and photographed in a Petri dish on 2 mm graph paper. The length of each larva was later determined using ImageJ software and any obvious gross malformations (haemorrhage, incomplete gill development, spinal curvature) were noted. Time to hatch was calculated for each larva as the number of days from study initiation to hatching, and averaged within each egg mass. Hatching success for each egg mass was calculated as the total number of hatched larvae divided by initial number of embryos. Due to the difficulty in counting