Testing for effects of pollution on a snail-borne parasite in Michigan lakes

Submitted by

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Abstract

My thesis project focused on testing for potential effects of herbicides, specifically 2,4-D and glyphosate, on avian schistosome parasites in Michigan lakes. Avian schistosomes are a diverse group of snail-borne flatworm parasites that cause a rash called "swimmer's itch" in humans. My project was part of a larger effort by Dr. Raffel's lab team to measure the abundance of these parasites at 38 inland lake shorelines across Michigan. My own project focused on how pollutants affect the parasite's intermediate hosts, snails. Chemical pollutants might affect snail populations by killing the invertebrate predators of snails or by influencing the growth rates of the snail's food source (algae). We collected water samples from each site at two time points during the summer and brought them back to the lab for chemical analysis. We also measured population densities of snails and potential invertebrate predators of snails (i.e., crayfish), and measure algal growth rates using standard periphyton samplers and a chlorophyll assay. Ultimately, I hope my work will help to inform management efforts by local lake associations by determining whether and how pollution influences these parasites and their snail hosts.

Introduction

Herbicide and nutrient pollution in lakes and other water bodies can have profound effects on aquatic ecosystems, with impacts sometimes lasting beyond the point when the chemicals are no longer detectable in water (Hasenbein, et. al. 2016). One important effect of these contaminants is to increase snail population densities by increasing growth rates of algae attached to surfaces (periphyton), and by decreasing populations of invertebrate predators that feed on snails (Preston, et. al. 2013). Snails are important intermediate hosts for a wide variety of trematode parasites, including those that cause the damaging human disease schistosomiasis (Brant & Loker, 2013). Although schistosomiasis is not currently present in the U.S., schistosome parasites of birds (avian schistosomes) are common causes of dermatitis for people who swim in Michigan lakes (Horak, et. al. 2015)

All trematode parasites have complex life cycles with multiple hosts (Preston, et. al. 2013). Preston goes on to say that schistosome parasites have two-host life cycles including a snail intermediate host and a vertebrate definitive host. Snails release an infective stage called cercariae into the water, and these seek and penetrate the skin of the vertebrate definitive host (Jothikumar, et. al. 2015). In avian schistosomes, the cercariae move through the water looking for a bird to infect as their definitive host. However, cercariae occasionally mistake humans for birds and penetrate the skin. This often results in a severe itchy rash known as “swimmer’s itch”, more technically referred to as cercarial dermatitis, which can take several days to subside (Kolarova, et. al. 2013). The rash is caused by a robust immune response, which is generally assumed to kill the cercariae and keeps them from completing their life cycle in a mammalian host (Kolarova, et. al. 2013). Swimmer’s itch is considered an emerging disease by some

authors, because it is becoming a problem in areas that previously had few or no cases of dermatitis (Horak et al 2015).

The abundance of snail intermediate hosts is perhaps the most important driver of swimmer's itch risk, and past studies have found certain types of pollution to be important drivers of snail abundance. Agricultural contaminants in particular have been shown to increase snail population densities via multiple mechanisms. Fertilizers can increase nutrient concentrations in lakes and wetlands, leading to increased growth of the surface algae that many snails eat (Rasmussen, et. al. 2015). Paradoxically, herbicides can also increase periphyton growth because they kill the algae growing up in the water column (phytoplankton), leading to clearer water (i.e., lower turbidity), more sunlight penetration, and more periphyton growth (Rohr et al. 2008; Rohr & Crumrine 2005). Insecticides can also increase snail abundance by killing arthropod predators of snails such as crayfish or prawns (Nagai, et. al. 2016). These pollution effects have been shown to be important drivers of abundance for the snail hosts of other trematode parasites, including parasites of amphibians and humans. However, to my knowledge no one has investigated whether pollution has similar effects on the snail intermediate hosts for avian schistosomes.

The primary goal of this study was to examine whether among-site differences in avian schistosome abundance could be explained by effects of chemical contaminants. My study focused on possible effects of two commonly applied herbicides (2,4-D and Glyphosate) on the abundance of snails and avian schistosomes in Michigan lakes. Based on our hypothesized effects of herbicides on phytoplankton, water clarity, attached algae, and snails (discussed above), I predicted that sites with higher herbicide concentrations would have lower turbidity, higher light penetration, higher growth rates of attached algae, and higher snail densities. I tested

these predictions by measuring among-site variation in these variables at 38 sites on 16 lakes in northern Michigan, in collaboration with other members of the Raffel lab.

Methodology

I sampled 38 sites across 16 lakes spread throughout northern Michigan (Table 1; Figure 1), in collaboration with other members of the Raffel lab. Briefly, sampling occurred during the month of July and included measurements of several predictor and response variables. We collected two water samples from each site for analysis of 2,4-D and glyphosate concentrations. We also installed periphyton samplers and collected them at the end of the sampling period to measure algal growth using a Chlorophyll A assay. We measured the densities of various snail species three times at each sampling location using quadrat samplers and a bucket viewer. Finally, we assessed crayfish abundance by setting traps overnight at each site. After the sample processing, we conducted correlational analyses and used multiple regression to test for relationships between key variables.

Temperature was measured using a temperature loggers and is thought to increase the rate at which chemicals (i.e., herbicides) breakdown. Shore slope would affect the run off rates of herbicides applied in the riparian zone while shoreline composition would affect the amount of herbicides applied in the riparian zone. Substrate refers to the microbes present in the sediment. As submergent, emergent, and floating vegetation levels increase it would be expected that more herbicides are applied in an attempt to limit further growth.

Protocols

Collecting Water Samples

All water samples were collected with acid-washed HDPE sample bottles. We waded to a depth of 60 cm, rinsed the bottle with lake water at least three times, and submerged the bottle to a depth of about 0.5 m before allowing it to fill with water. We then capped the bottle, placed the water on ice, and transferred it to a freezer within six hours.

ELISA Assays

We assayed 2,4-D using a commercial ELISA test kit (PN 500086, Abraxis). We started by adding 50 microliters of each sample into the wells. Analysis was done in singlicate but two samples were analyzed per site. We then added 50 microliters of enzyme conjugate solution to each well followed by 50 microliters of antibody solution to the individual wells successively. The contents were then mixed for 30 seconds and the strips were incubated for 60 minutes at room temperature. After the incubation period, we washed the strips three times using the washing buffer using at least 250 microliters of washing buffer in each well in each washing step. The remaining buffer in the wells was removed by patting the inverted plate dry on a stack of paper towels. We then added 150 microliters of substrate (color) solution to the wells and incubated the strips for 20-30 minutes at room temperature in the dark. Finally, we added 100 microliters of stop solution to each of the wells and read the absorbance at 450nm using a microplate ELISA photometer. The photometer we used was the Synergy H1 Bioplate Reader made by Biotek (Winooski, VT, USA).

We assayed glyphosate using a commercial ELISA test kit (PN 54003A, Abraxis).The standards were derivatized according to the directions. We then added 50 microliters of the derivatized standard solutions, control, or samples into the wells of the test strips according to the working scheme given. Analysis was done in singlicate but two samples were analyzed per

site. We then added 50 microliters of the antibody solution to the individual wells successively using a repeating pipettor. The contents were mixed for 60 seconds and the strips were incubated for 30 minutes at room temperature. Next, we added 50 microliters of the enzyme conjugate solution to the individual wells and incubated the strips for 60 minutes at room temperature. We then washed the strips three times using the wash buffer solution using at least 250 microliters of wash buffer in each well for each washing step. We then added 150 microliters of substrate (color) solution to the individual wells, mixed the contents for 30 seconds, and incubated the strips for 20-30 minutes at room temperature in the dark. Finally, we added 100 microliters of stop solution to each well and read the absorbance at 450nm using a microtiter plate ELISA photometer. The photometer we used was the Synergy H1 Bioplate Reader made by Biotek (Winooski, VT, USA).

Habitat Assessment

There are two primary zones we used to define the site: the Riparian zone which consisted of an area 15 meters parallel to shoreline and 15 meters running inland from the shoreline, and the Littoral zone which consisted of 15 meters of shoreline that extended 15 meters into the lake (Figure 2).

We started by observing the riparian and littoral zones. We checked if each characteristic was present and then recorded the level of relative abundance (Table 2). We also noted the dominant types of land use, ground cover, and aquatic habitats at the site.

Chlorophyll A Assay

We obtained 3 clean plexiglass (10 cm x 10 cm) tiles and secured them to a float using poly rope. Each periphyton sampler was tied to a cinder block or sufficient anchor, and each tile

was tied so that there was 30 cm of poly rope between the tile/float and the anchor. We walked out to 60 cm depth and placed the samplers in a triangle formation leaving at least 2 meters between each anchor and made sure that they are all 30 cm from the water surface. We then adjusted the sampler so that the tile was oriented upward towards the water surface and the float was underneath the tile.

We used a meter stick to measure and record the final depth of each tile (write the depth on the field sheet). We then removed the tile from the noodle float and placed it into its corresponding Ziploc bag. We made sure to avoid touching the top of the tile and stored the bagged tile in cooler on ice. The periphyton tiles were processed the same day as they were collected.

We started by setting up a vacuum filtration apparatus. The periphyton tiles were kept on ice until they were processed. We used forceps and a toothbrush to hold the tile still in the pan and brushed the surface of the tile, removing periphyton and allowed it to collect in the pan. A squirt bottle with tap water was used to rinse the tile and the toothbrush and all liquid was kept in the pan. We then used a vacuum pump and filter tower set up to concentrate the sample onto a Whatman GF/F glass microfiber filter (0.7 µm; Whatman Inc., Kent, U.K.). We made sure to thoroughly rinse the pan onto the top of the filter paper. If filtering was done in the field, foil squares were kept in a waterproof bag on ice and were placed in a freezer within 6 hours.

The filters were folded, labeled in tin foil, and placed in the freezer at -20 °C until fluorometric analysis. A 90% Methanol solution (10% water) was made, and each filter was placed in its labeled tube and exactly 5 mL of methanol was added to each tube. We then capped

all of the tubes and placed them in the fridge for a 24 hour period to promote algal cell lysis following a modified version of the EPA method 445.0.

The samples were centrifuged for 5 minutes at 1500 rpm to remove any suspended particles from the sample liquid. We pipetted 200 µL of each sample, and fluorometric analysis (Synergy H1 microplate reader, Biotek, Winooski, VT, USA) was used to determine chlorophyll A in relative fluorescence units; fluorescence (emission) was recorded at the 680 nm detection wavelength using an excitation wavelength of 440 nm. We calculated the average fluorescence for the three tiles to obtain an index of periphyton growth potential at each site.

Turbidity Measurement

Turbidity measurements were taken once a week for five weeks during the survey. To take a turbidity measurement, we collected a sample in a clean container. We then filled the sample cell (glass vials) to the line, approximately 15 mL, then capped the cell. The cell was handled by the top to avoid getting fingerprints on the sides of the cell. The outside of the cell was thenwiped with a Kim wipe to remove any fingerprints or water spots. A thin film of silicone oil was applied around the outside of the cell, and the cell was wiped with a soft cloth to obtain an even film over the entire surface. The Hach Portable Turbidimeter Model 2100P was used to take turbidity measurements.

Statistical Analyses:

To determine if we had detected among-site variation in glyphosate or 2,4-D concentrations, we first conducted a 1-way ANOVA to test for effects of Site, using each individual water sample as a unit of replication. Prior to among-site analyses, we averaged the measurements of each predictor or response variable for each site. Count data and turbidity

measurements have highly skewed distributions, so we log-transformed these variables ($\log_{10}$[N+1]) prior to calculating their averages. We next did an exploratory analysis testing for Pearson correlations between all pairwise combinations of variables measured. For each response variable of interest (snail abundance, periphyton growth, turbidity, 2,4-D concentration) we followed up on potentially explanatory ($r > 0.2$) predictor variables using multiple linear regression (function "lm" in program R). Contributions of predictors to each model were assessed using F-tests with a Type II sums of squares procedure (function "Anova" in the "car" package). Non-significant predictors ($P > 0.05$) predictors were removed sequentially from each model via backwards selection.

Note that all Log transformations were done using $\log_{10}$.

Results

Although our ELISAs only detected very low levels (below 2 parts per billion) of 2,4-D, we detected highly significant site-to-site variation in concentrations of this herbicide. This suggests that the assay successfully detected variation in 2,4-D, despite the concentrations being below the stated detection limit of the ELISA kit (< 2 ppb). Figure 3A shows that we found most of the lakes had similar concentrations of 2,4-D, with a few exceptions. Elk Lake and Deer Lake had very low levels of 2,4-D, with readings below our zero standard (Fig. 3A). Intermediate Lake, Portage Lake, and Lime lake had much higher levels of 2,4-D than other lakes.

In contrast, we were unable to detect significant among-site variation in glyphosate, which also had concentrations below the stated detection limit of the assay. These data are represented in Figure 3B. Indeed, many measurements indicated levels of glyphosate that were lower than the zero standard. These can be interpreted as zero concentrations.

Since 2,4-D had significant among-site variation, we looked for any correlations with other variables we tested for. Using only variables with an r-value of greater than 0.3 or less than -0.3, we found four variables that correlated with 2,4-D concentrations. Those variables were buildings ($F_{1,36}$ =3.5794, P = 0.067), submergent vegetation ($F_{1,36}$ = 6.3466, P = 0.016), emergent vegetation ($F_{1,36}$ = 5.5392, P = 0.024), and total vegetation ($F_{1,36}$ = 6.7004, P = 0.014). The strongest correlation was between 2,4-D and total vegetation (Figure 4a). The amount of total vegetation was determined by the habitat assessment conducted at each site prior to sampling that site.

We found no correlation between 2,4-D and snails ($F_{1,36}$ = 0.0522, P = 0.821), 2,4-D and turbidity ($F_{1,36}$ = 0.301, P = 0.587; Figure 4d), or 2,4-D and chlorophyll ($F_{1,36}$ = 0.5459, P = 0.465; Figure 4c). The best predictors of snail abundance were turbidity ($F_{1,35}$ = 12.1, P = 0.001) and cobble substrate ($F_{1,35}$ = 5.8, P = 0.021). As turbidity increased, the lognumber of snails decreased. There was a weak positive correlation between periphyton growth and snail abundance, but it was not significant (P > 0.1).

Discussion

The strong negative relationship between water turbidity and snail density supported our prediction that snails would be more abundant in locations with greater water clarity. The correlation we found between 2,4-D concentration and total vegetation is most likely due to people applying this herbicide in an attempt to limit, or even reverse, the growth of aquatic weeds. However, other predictions of our core hypothesis were not supported by our results. We did not find any relationship between herbicides and its proposed response variables, turbidity, light penetration, chlorophyll, and snail abundance. We did find a positive trend between snail

abundance and periphyton growth; however, it was a far weaker pattern than the correlation between snails and turbidity and not statistically significant.

Our failure to detect the predicted effects of herbicides on water clarity could be due to a multiple reasons. First, we might not have measured the most important pollutants in the system. The two herbicides we tested for are some of the most widely applied pesticides in the country, but there are many other chemical pollutants that we were unable to measure and which might impact these lake ecosystems (Grube, et. al. 2011). For example, the herbicide atrazine, which has a much longer half-life than glyphosate, significantly reduced phytoplankton abundance in a previous study, resulting in significantly increased nutrient availability, water clarity and sunlight penetration to attached algae (Rohr, 2008). We also have not completed our analysis of water nutrient levels, which might also be important predictors of algae growth. Elevated nutrient availability can also lead to increased periphyton in lakes and wetlands where algae growth is limited by nitrogen or phosphorus availability (Bondar-Kunze, et. al. 2016).

Second, it is possible that glyphosate pollution is in fact an important factor in these lake ecosystems, but we failed to detect it due to its very short half-life (Larras, et. al. 2013). Certainly it seems likely that riparian owners use glyphosate-containing products as part of their regular lawn maintenance (e.g., "weed and feed" prodcuts). It is possible that glyphosate-containing products are only used at certain times of the year – for example, in spring when people start mowing lawns – such that any effects would occur over a short time period. If this was the case, the chemical would likely have completely degraded prior to our sampling period in July and August. If this is the case, our measurements were simply too infrequent and too insensitive to detect this potentially important variation.

Our failure to detect a relationship between snail abundance and periphyton growth is a bit surprising, considering that this was the hypothesized mechanism for the snail-turbidity relationship that we did detect. One possibility is that our measurements of periphyton growth in shallow water are not representative of periphyton growth out in deeper water, where water clarity and light penetration might have more important, limiting effects on attached algae. Deep-water algal growth could be important for snail populations, which regularly move between deep and shallow parts of clear-water lakes such as those in northern Michigan (Jarcho & Van Burkalow, 1952; Howard & Walden, 1965). Snail abundance might be more affected by factors other than algal periphyton. Snail abundance can be influenced by the abundance of proper substrates and invertebrate predators. Invertebrate predator levels can be reduced by insecticides as mentioned by Nagai, et. al. (2016). Snails also eat a variety of food sources, including plant matter, detritus, and bacterial and fungal periphyton. It is possible that these other food sources are very important to snail abundance, or they may even relate to turbidity in some way.

Future research testing for the effects of water pollutants in this system could be done by assaying water samples for nutrient levels and other important pesticides, such as organophosphate insecticides and other common herbicides like atrazine. The insecticides might play a role in limiting arthropod predators of snails which could lead to an increase in snail abundance. It might also be interesting to collect water samples more frequently from some select lakes with heavy urbanization or agricultural activity, to determine when are the best times to sample for particular pollutants.

Acknowledgements

The completion of this thesis project would not have been possible without the guidance of my mentor, Dr. Thomas Raffel, the other members of the Raffel lab, the various lake associations, and everyone that opened their homes to us this summer. First, and foremost, a huge thank you to Dr. Raffel as his guidance has brought me a long way from the guy that could not even properly tie knots. I have learned many things about lab techniques, proper protocol, experimental design, and statistical analyses that will be invaluable in my future academic career. I would then like to thank Madelyn Messner and Jason Sckrabulis for all the guidance they provided Aleena and I in the field. I must also thank the 16 lake associations from across Northern Michigan for making this entire project possible by providing us with funding as well as sampling sites on their lakes. I would like to thank all of the people that opened up their homes to us, gave us a place to stay, fed us, and shared stories with us. Their role was as crucial as any in the project as without them, this project would not have been possible. Thus, thank you to all of the following: Ken and Teresa Dennings, Russ and Kathy Kittleson, Steve and Kathy Young, Sandra and Ken Michalik, Anne Covy, Holly and Rich Gerdert, John and Gayle Popa, Denny and Luci Lavis, Scott and Kelly Zimmerman, Bob and Dotty Blank, and Bob and Peggy Haverberg. Thank you to all of the volunteers that collected daily water samples at all of the sites. Finally, I want to extend a big thank you to my good friend Aleena Hajek. Even though you made me put out all of your crayfish traps, refused to actually touch the crayfish, and forced me to watch the Bachelorette, thank you for making this project feel less like work and more like fun. Sorry I had to beat you in Monopoly and Ticket to Ride.

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Table 1. List of all lakes, site ID, and latitude and longitude coordinates.

Lake	Site ID	GPS Coordinates
Crystal	CA	44.66502, -86.24453
Crystal	CC	44.68975, -86.20743
Crystal	OH	44.64629, -86.09312
Crystal	ON	44.63804, -86.16975
Deer	DS	45.17076, -84.97124
Douglas	BS	45.56009, -84.67489
Douglas	BW	45.58848, -84.72621
Elk	BK	44.88647, -85.36201
Glen	DO	44.85531, -86.01307
Glen	KA	44.89071, -85.95938
Glen	ME	44.86815, -85.92953
Hamlin	JD	44.06929, -86.41986
Hamlin	MB	44.02759, -86.45231
Hamlin	PP	44.05144, -86.45583
Hamlin	PR	44.01614, -86.47054
Higgins	DH	44.43672, -84.70445
Higgins	GT	44.49592, -84.69906
Higgins	KB	44.46567, -84.68034
Higgins	SS	4.464110, -84.74430
Intermediate	JG	45.02279, -85.23637
Intermediate	TP	45.06957, -85.26011
Leelanau	NF	45.04364, -85.72026
Leelanau	PS	45.00279, -85.77054
Lime	MA	44.89570, -85.84868
Little Traverse	RC	44.92495, -85.85420
Margarethe	DL	44.65653, -84.78121
Margarethe	LB	44.63559, -84.79324
Margarethe	SD	44.62734, -84.78608
Margarethe	SF	44.66086, -84.81658
Platte	BB	44.69307, -86.07571
Platte	IN	44.67515, -86.07893
Platte	RA	44.69484, -86.11979
Portage	NP	44.36551, -86.23814
Portage	VP	44.36241, -86.20688
Skegemog	KG	44.80942, -85.34577
Walloon	RK	45.32866, -85.04487
Walloon	W2	45.26464, -85.00223
Walloon	W3	45.30802, -84.98653

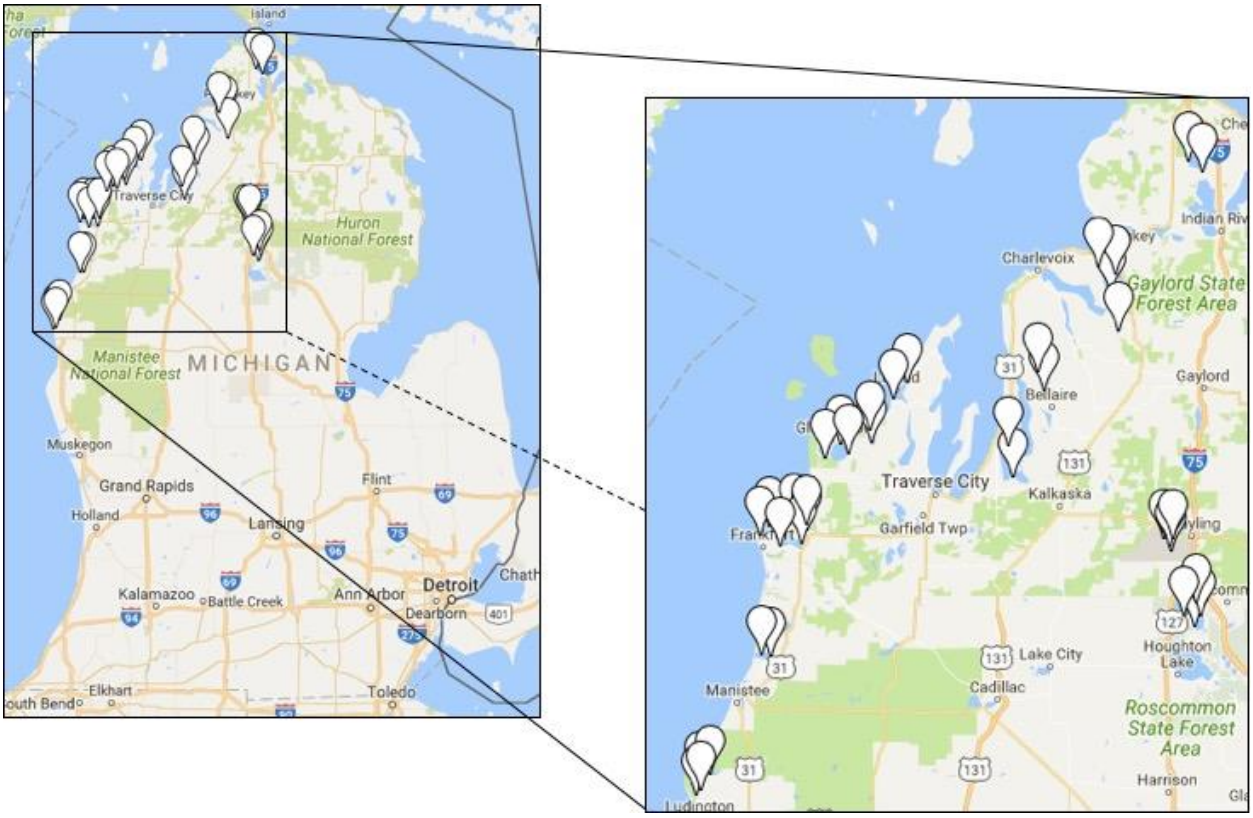

Figure 1. Map of Michigan with all 38 sampling sites marked across 16 lakes. The inset shows a close up of Northwest Michigan with all sites marked.

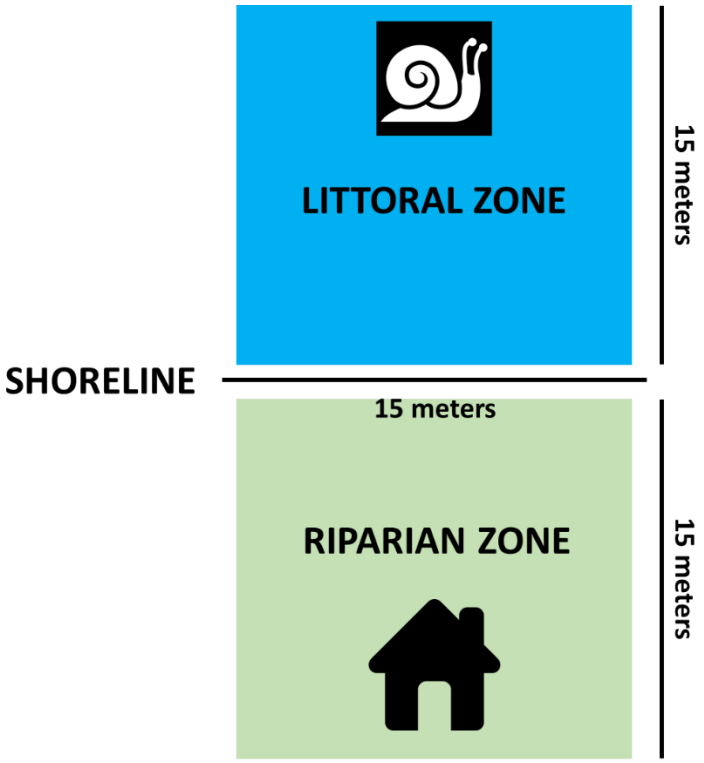

Figure 2. Shows the littoral and riparian zones as described in the methods.

Table 2. Table used for habitat assessment. Marks were given for the abundance of each category according to the following scale: 0= Absent, 1= Sparse (<10%), 2= Moderate (10-40%), 3= Heavy (40-75%), 4= Very Heavy (>75%).

Riparian Ground Cover (<15 m from shore)	• Woody shrubs & saplings [ ] • Herbs / grasses [ ] • Barren (dirt/sand/rock) [ ]
Riparian Canopy (<15 m from shore)	• Deciduous [ ] • Broadleaf / Evergreen [ ] • Coniferous [ ] • Mixed [ ]
Riparian Human Influence (<15 m from shore)	• Buildings/houses [ ] • Park facilities / man-made beach [ ] • Docks / boats [ ] • Roads / railroad [ ] • Agriculture [ ] • Lawn [ ] • Other: [ ]
Littoral Substrate (<15m from shore into lake)	• Bedrock (car) [ ] • Boulders (basketball) [ ] • Cobble (tennis ball) [ ] • Gravel (ladybug) [ ] • Sand (gritty) [ ] • Silt, Clay, Muck (not gritty) [ ]
Littoral Aquatic Vegetation (<15 m from shore into lake)	• Submergent [ ] • Emergent [ ] • Floating [ ] • Total Cover [ ]

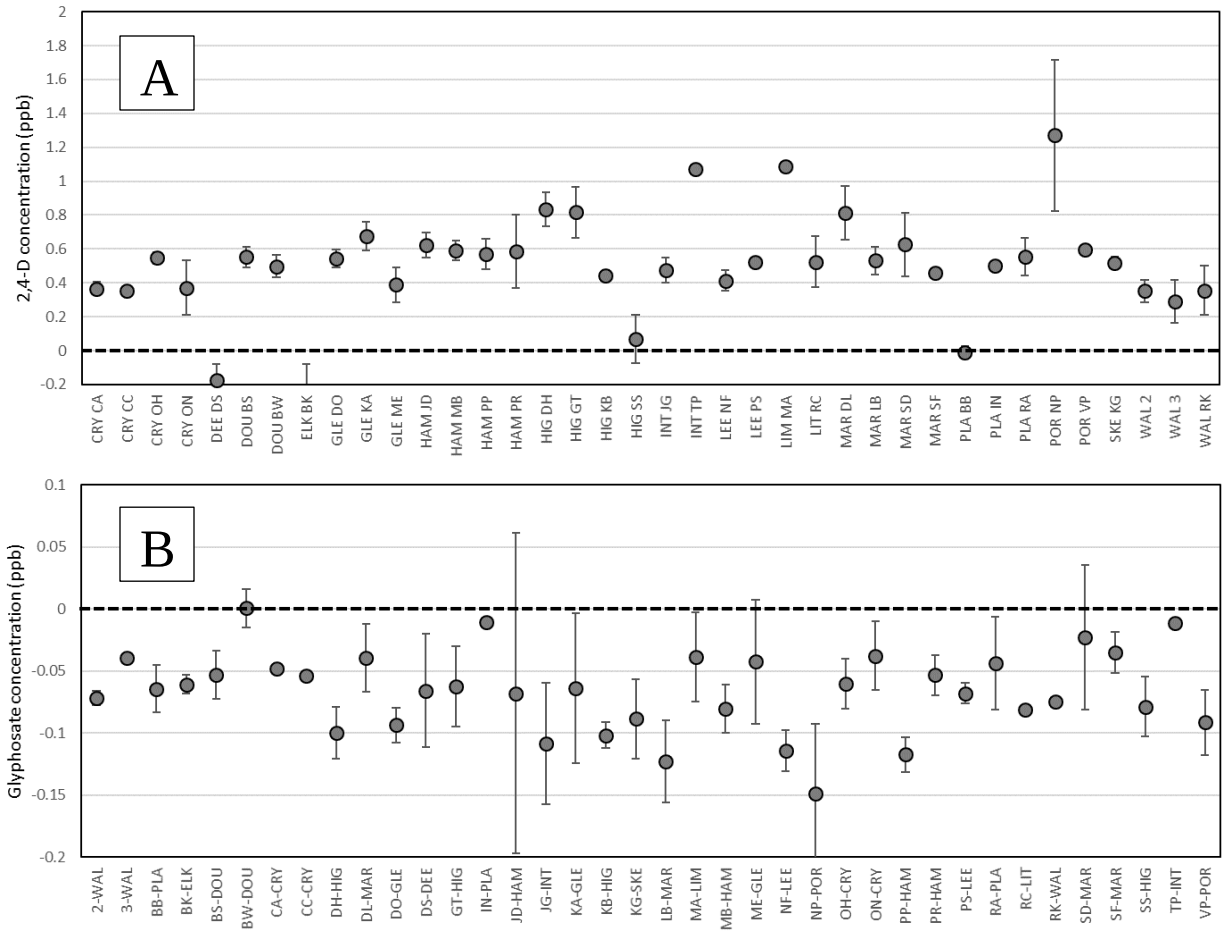

Figure 3. Plot A shows 2,4-D concentrations at each site. Plot B shows glyphosate concentrations at each site.

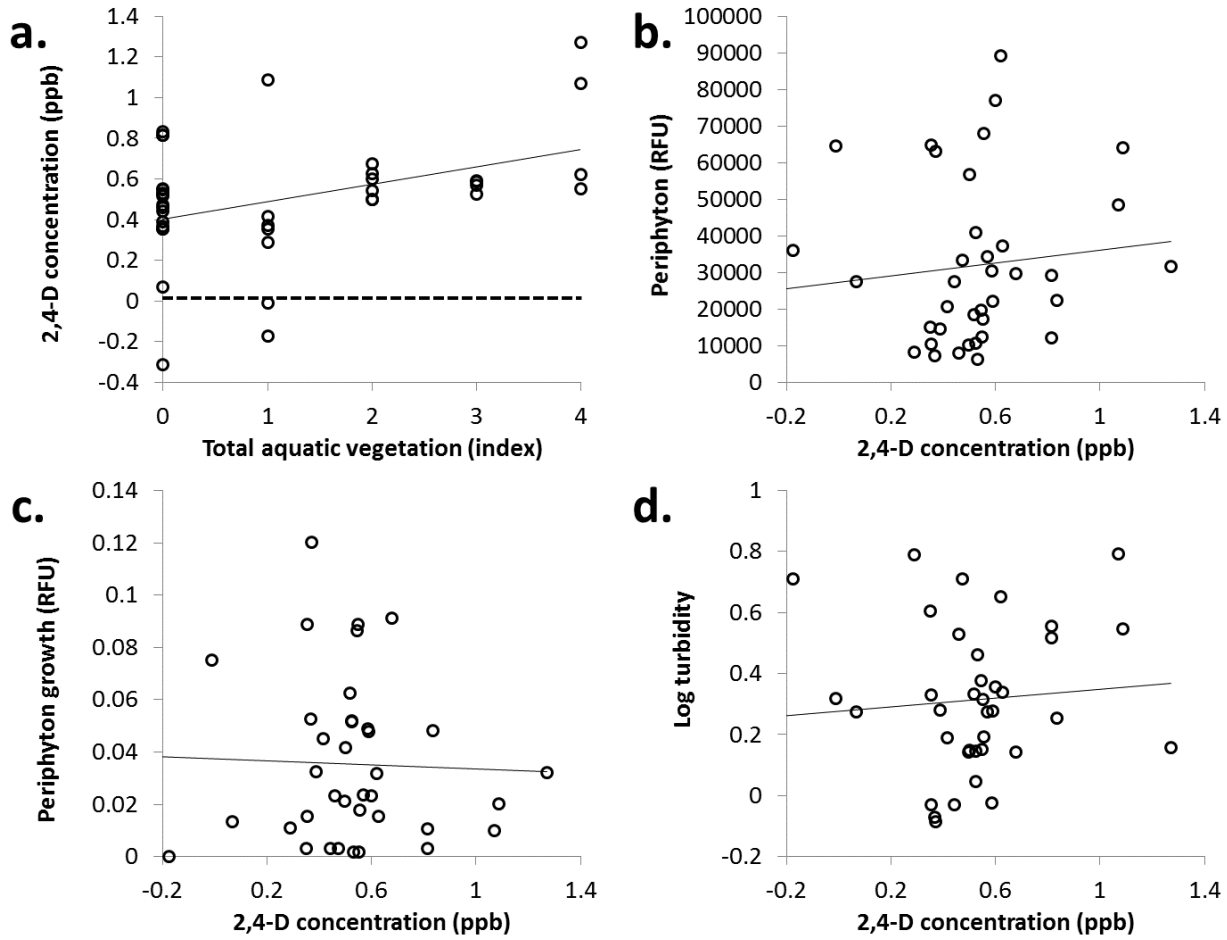

Figure 4. Graph a shows the correlation between 2,4-D concentration and Total aquatic vegetation (index). Graph b shows the correlation between Periphyton and 2,4-D concentration. Graph c shows the correlation between Periphyton growth and 2,4-D concentration. Graph d shows the correlation between the Log turbidity and 2,4-D concentration.