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**Lethal Effects of the Non-native
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Aquatic Organism (*Hyalella azteca*)**



Honors Thesis

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Advisor: Ryan W. McEwan, Ph.D.

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Abstract

The invasive plant *Lonicera maackii* (Amur honeysuckle) has had a variety of ecological effects as it continues to spread through the eastern United States including the loss of plant biodiversity and alterations in ecosystem function in forests. Streams meander through many forests where Amur honeysuckle is present and recent evidence suggests that this terrestrial invasion has consequences for stream biology. Leaves of Amur honeysuckle have been shown to have strong negative effects on terrestrial insects and we hypothesized that these negative effects may also occur in aquatic macroinvertebrates. In this set of experiments we used a sequence of microcosm assays to assess the influence of Amur honeysuckle leachate on the macroinvertebrate *Hyaella azteca*, which is a standard “model” aquatic organism to toxicity assessment. In the lab, *H. azteca* were exposed to riparian honeysuckle leaf leachate (made by soaking 10 g leaves in 100 mL dechlorinated water for 24 h) in 48 h acute, static toxicity tests. This was repeated throughout the growing season. When exposed to an Amur honeysuckle leachate dilution series (6.25%, 12.5%, 25%, 50%, 100%), *H. azteca* survival was significantly decreased in all dilutions in the spring and fall trials. However, the summer trials showed no significant decrease in survival in nearly all the dilutions. These results suggest (a) strong toxic effects of Amur honeysuckle foliage on a model aquatic organism that (b) varies throughout the year, potentially in relationship to biochemical changes associated with phenology. Future experiments regarding the chemical composition and toxicity of these leaves should be mindful of the season in which the leaves are gathered. This study supports the importance of management of Amur honeysuckle in headwater stream riparian zones which are particularly vulnerable to perturbations and are increasingly a focus of conservation.



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Introduction

The biology of headwater streams is strongly linked to subsidies from the vegetation in the riparian zone. The biology of the riparian zone is has been linked to stream metabolism and overall stream health (Wallace *et al.* 2015, Tank *et al.* 2010, Young *et al.* 2008) and traits of riparian vegetation such as leaf chemistry and canopy density can influence many factors in the aquatic system including light availability (Baxter *et al.* 2005), nutrient input rates (Harner *et al.* 2009), and water temperature (Clinton *et al.* 2010). Allochthonous inputs from the riparian zone include organic materials such as leaves and woody material, and are closely linked to aquatic food webs (Wallace *et al.* 2015; Bailey *et al.* 2001, Baxter *et al.* 2005, Vannote *et al.* 1980). Aquatic macroinvertebrates utilize these inputs as habitat, refuge, and food, and these subsidies can be strongly affected by changes in the composition of riparian vegetation (Bailey *et al.* 2001). Aquatic macroinvertebrate species are divided into functional feeding groups based on their roles in the food web (Cummins and Klug 1979). Invertebrates that “shred” leaf materials are an important component of headwater stream food webs because they “process” leaf material that can also be utilized other organisms (Wallace and Webster 1996). Organisms in this “shredder” functional feeding group are particularly likely to respond to alterations in allochthonous materials from the riparian zone.

Changes in the composition of the riparian forest community can drive strong shifts in allochthonous subsidies to streams. Riparian habitats are especially vulnerable to plant invasion because of the availability of resources and the frequency of disturbances (Stohlgren *et al.* 1998, Nakamura *et al.* 2000) and exotic species can have unique morphological and chemical characteristics that may strongly alter the quality of allochthonous subsidies. One such invader is *Lonicera maackii* (Amur honeysuckle), an exotic, invasive shrub that has proliferated across much of the eastern United States (Luken and Thieret 1996, Hutchinson and Vankat 1997). Negative effects for native vegetation have been observed following *L. maackii* invasion including reduced biodiversity, reduced fecundity of native herbs, and allelopathic suppression of germination in native plants (Collier *et al.* 2002, Miller and Gorchoy 2004, McEwan *et al.* 2010). In terrestrial experiments using *L. maackii*, toxicity effects have been observed

with the generalist herbivore *Spodoptera exigua* (Cipollini *et al.* 2008) and the highly polyphagous gypsy moth caterpillars (*Lymantria dispar*) avoided consumption of the leaves (McEwan *et al.* 2009). When *L. maackii* invades the riparian zone of headwater streams it has strong potential to alter the nature of allochthonous subsidies entering the stream (McNeish *et al.* 2012; McNeish and McEwan 2016). Alterations of riparian subsidies may have significant ramifications for the macroinvertebrate communities of invaded streams, and may be particularly significant for shredders given that they interact directly with leaf materials (McNeish *et al.* 2012).

This set of experiments is designed to examine the possibility that allelopathic effects from *L. maackii* contribute to changes in stream macroinvertebrate communities using a reductionist approach. Due to the negative effects of *L. maackii* leaves on terrestrial invertebrate herbivores, we hypothesized that the generalist shredder *H. azteca* would experience reduced survival when exposed to *L. maackii* leaves and leaf leachate. To test the hypothesis, a biological assessment (bioassay) method with the organism *Hyalella azteca* was used. A bioassay is a technique that evaluates toxicity of a substance by its effects on a living organism, which can provide results at a biologically relevant level of complexity.

Experimental Methods

Hyalella azteca has been identified by the USEPA as a model organism for aquatic contaminants (USEPA 2000, Keddy *et al.* 1994) and was the subject species in this set of experiments. The method consisted of a series of 48 h exposures to different dilutions of leaf and flower leachate. The leachate was made using 100 g of leaves per 1000 mL dechlorinated tap water which was left at room temperature for 24 h. The leachate was diluted to create the following serial dilution gradient: 100%, 50%, 25%, 12.5%, and 6.25%. Each test included five treatments plus a control, and four replicates per treatment. Each replicate received 50mL of leachate and five *H. azteca* between the ages of (7 to 14 d old). The response variable for all tests was survival. Trials using *L. maackii* leaves were conducted in the months of April, July, August, September, October, and December to assess possible phenological effects. Dissolved oxygen, temperature, specific conductivity, and pH were measured at the start and termination of each test

using a YSI Sonde probe. The probe was calibrated daily. Additional parameters were measured for dilutions at the start of each test: orthophosphate, hardness, and alkalinity.

Statistical Methods

All data were screened for normality and homogeneity of variance using the Shapiro-Wilks test of normality, and Levene's test for equal variance. Normality assumptions were violated, so a Kruskal-Wallis rank sum nonparametric test was utilized with a Wilcoxon Pairwise post hoc test to screen for treatment differences ($P \leq 0.05$).

For the correlation analysis, the data were screened for normality using the Shapiro-Wilks test. If normal, the data were analyzed using Pearson's product-moment correlation; if non-normal the data were analyzed using Spearman's rank correlation. All analysis was carried out using R.

Results

Bioassays using foliage collected in the fall and spring had very strong toxic effects (Figure 1). *Hyalella azteca* survival was significantly decreased by all treatments in trials using foliage collected in from autumn 2015 and spring 2016 (Figure 1; $P < 0.001$ for all trials). For instance, in the assay using foliage collected in Oct 2015, survival in the control was 100% while every animal exposed to any level of *L. maackii* leachate died (Figure 1; top left). An identical result was found in the Dec-1 assay, with 100% mortality for every organism exposed to *L. maackii* materials. In the 13-Dec assay there was a stair-step shaped response patterns with 100% survival in the control treatment, and increasingly lower survival in higher concentrations and no survival in the highest levels of leachate (Figure 1).

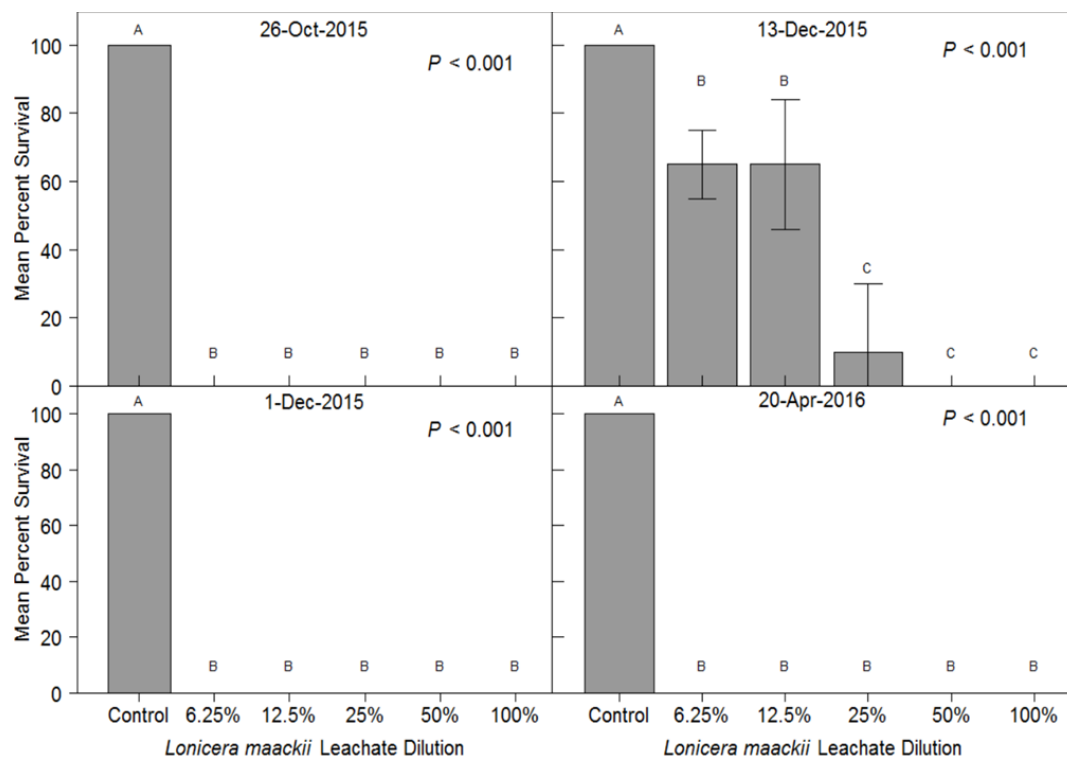


Figure 1: Mean percent survival of *H. azteca* in *L. maackii* leaf leachate during autumn of 2015 and spring of 2016. Capital letters indicate statistical differences.

In contrast to the fall and winter results, assays using foliage collected in the summer indicated little toxicity of *L. maackii* materials. There were no statistically discernable effects on survivorship in assays using foliar materials collected in July and early September (top and bottom panels, Figure 2) and survivorship was > 80% in all assays. Analysis of *H. azteca* survival exposed to foliar material collected in August suggest a significant overall treatment effect ($P = 0.005$) and post-hoc tests revealed that the 25% dilution had significantly lower survivorship than lower percentage treatments and the control but was not different than the higher concentrations (Figure 2; middle).

Across all trials, the leachate dilution treatment was significantly correlated to the survival responses throughout the year ($r_s = -0.457$,

$P=0.002$ Figure 3). The physicochemical parameters measured during the experiments showed differences in correlations with survival effects and leachate dilution treatments (Table 1). Dissolved oxygen ($r_s = 0.437$), initial pH ($R^2 = 0.238$), orthophosphate ($r_s = -0.546$) and conductivity ($r_s = -0.603$) were moderately correlated to survival, but were much more strongly correlated to the leachate dilution strength ($r_s = -0.920$, $R^2=0.723$, $r_s = 0.807$, and $r_s = 0.841$, respectively) throughout all trials (Figures 3, 4, 5, and 6). In all of

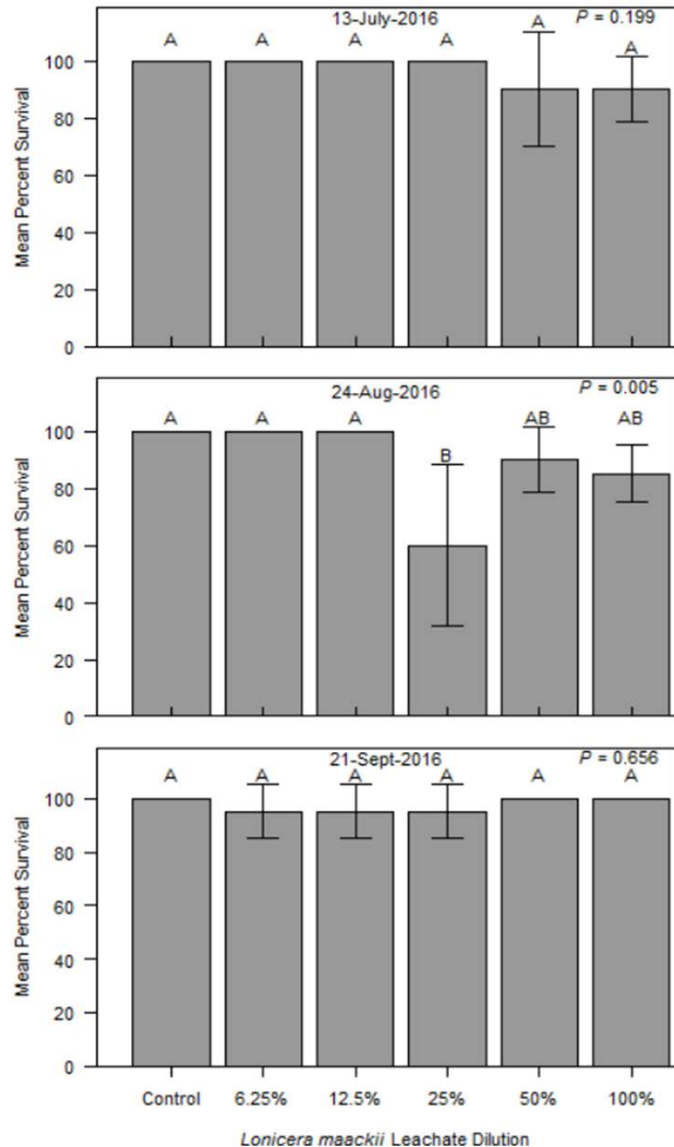


Figure 2: Mean percent survival of *H. azteca* in *L. maackii* leaf leachate during the summer of 2016. Capital letters indicate statistical differences.

these correlation models $P < 0.01$. These results indicate that these parameters were changing significantly with the leachate dilutions even in the trials when the survival was unchanged.

Table 1

Pysicochemical Parameter	Survival Correlation		Leachate Dilution Correlation	
	P-value	r_s (R^2 for pH)	P-value	r_s (R^2 for pH)
DO	0.004	0.437	<0.001	-0.920
pH	<0.001	0.238	<0.001	0.723
Orthophosphate	<0.001	-0.546	<0.001	0.807
Conductivity	<0.001	-0.603	<0.001	0.841

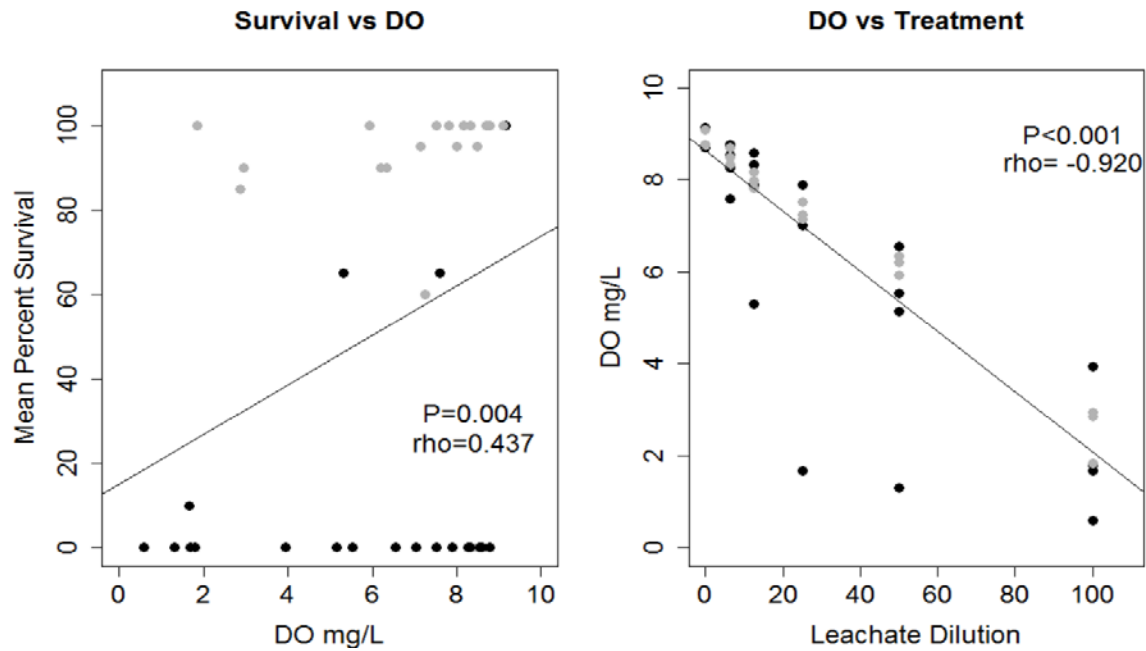


Figure 3: Correlation analysis of dissolved oxygen in relation to mean percent survival and leachate dilution treatments. Grey points represent data from summer trials and black points represent data from the spring and fall trials.

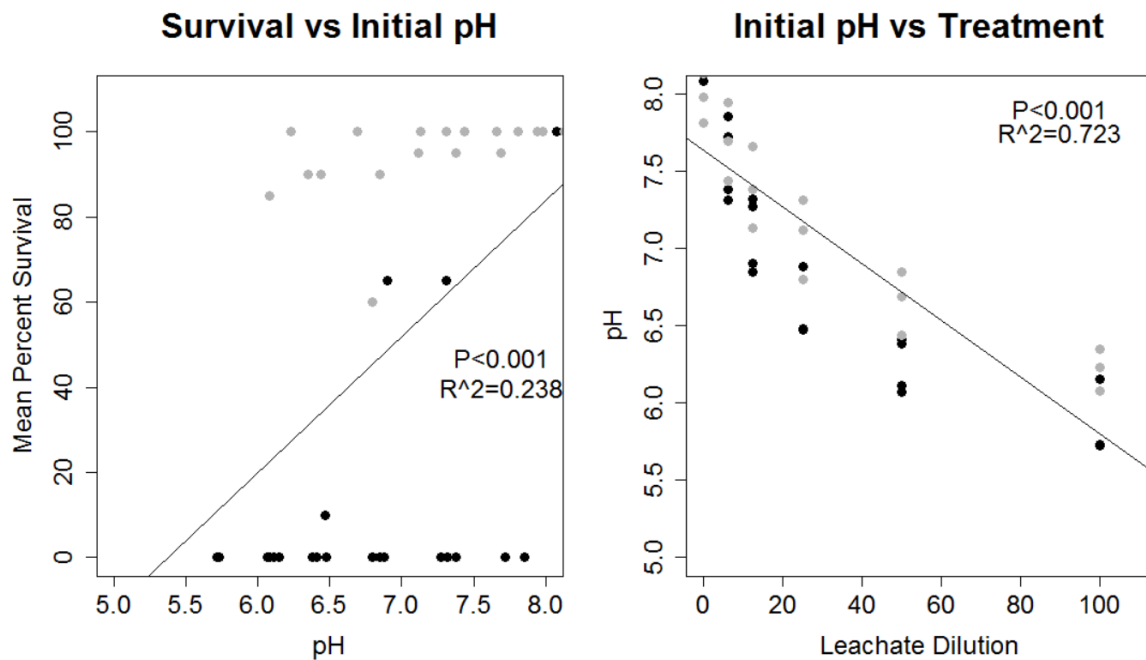


Figure 4: Linear regression analysis of pH in relation to mean percent survival and leachate dilution treatments. Grey points represent data from summer trials and black points represent data from the spring and fall trials.

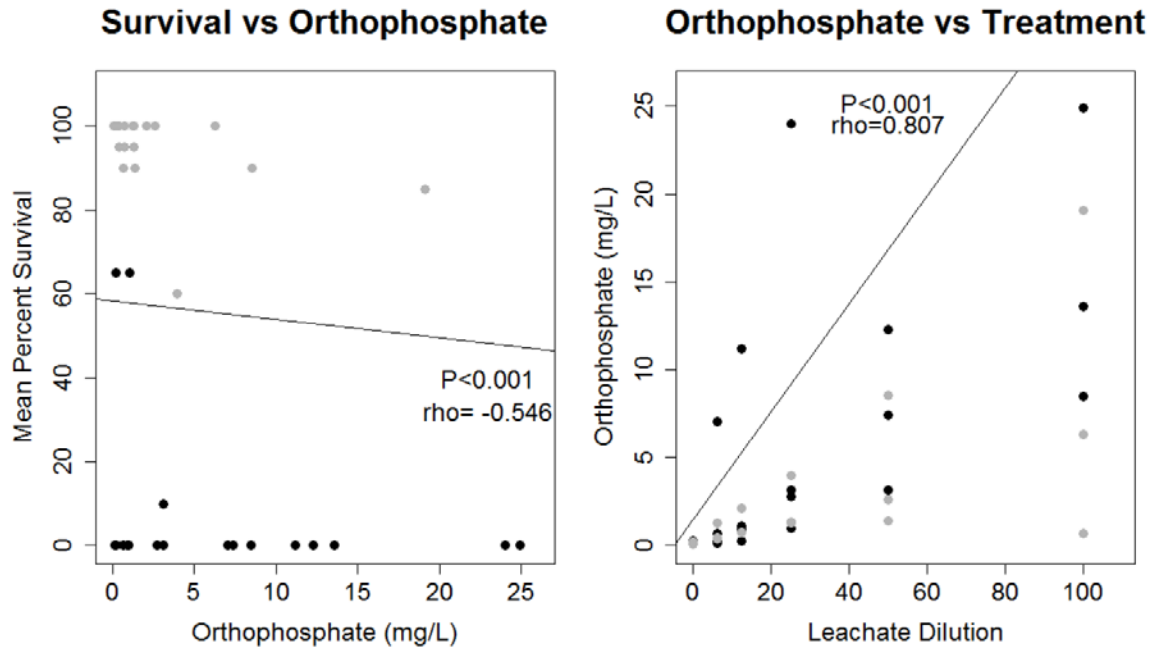


Figure 5: Correlation analysis of orthophosphate in relation to mean percent survival and leachate dilution treatments. Grey points represent data from summer trials and black points represent data from the spring and fall trials.

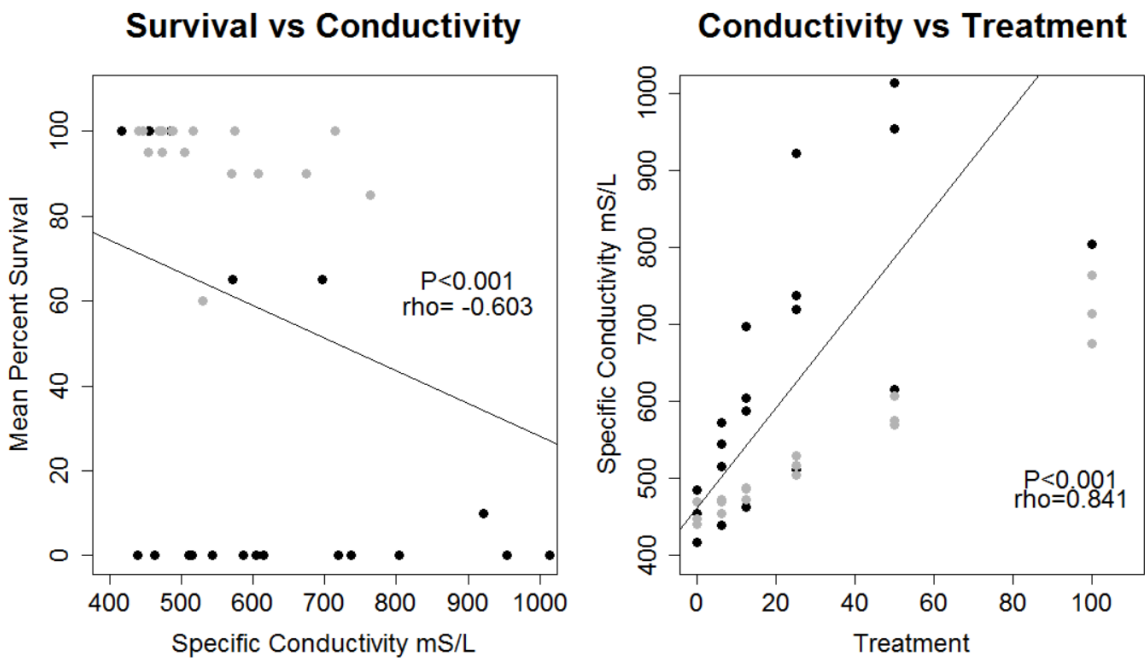


Figure 6: Correlation analysis of specific conductivity in relation to mean percent survival and leachate dilution treatments. Grey points represent data from summer trials and black points represent data from the spring and fall trials.

Discussion

The critical role of riparian vegetation as a structuring factor in aquatic communities is well-established in a wide variety of systems (Vannotte *et al.* 1980, Tank *et al.* 2010, Young *et al.* 2008). Connections between riparian vegetation and stream biology may be changed due to the invasion of exotic terrestrial plants. This effect has been seen with the invasion of Japanese knotweed (*Fallopia japonica*) in the Pyrenees in France, which supported different macroinvertebrate communities and altered the leaf litter breakdown rate (Lecerf *et al.* 2011). Lab studies have shown changes in growth of aquatic invertebrates in response to feeding on invasive Giant Reed (*Arundo donax*) leaf litter (Going *et al.* 2008). Other experiments have shown that *L. maackii* can increase the mortality and change the behavior of several amphibian species (Watling *et al.* 2011). While some field studies have hinted at this negative effect of *L. maackii* on macroinvertebrate communities (McNeish *et al.* 2012), another study has shown that *L. maackii* leachate can actually facilitate the development of larvae from the mosquito species *Culex pipiens* (Shewhart *et al.* 2014). The results of this study support the hypothesis that *L. maackii* may be negatively affecting aquatic macroinvertebrate communities which has been observed in previous experiments (McNeish *et al.* 2012, Custer *et al.* in review).

The series of bioassay trials indicated that leaf leachate from *L. maackii* is toxic to *H. azteca*; however, the effects vary strongly through the year. These results may be associated with annual variation in leaf chemistry associated with phenology. One hypothesis is that these seasonal changes in leaf toxicity are an allelopathic response to the increased herbivory in seasons when there are fewer other sources of food for herbivores, especially in the fall due to the extended growing season of *L. maackii* (Fridley 2012). Whatever the source, the variation in toxicity indicated by our results suggests that future research that focuses on leaf biology of Amur honeysuckle, and potentially other species, carefully includes consideration of the potential for variation in leaf chemistry through the growing season.

The toxicity of leaves can be caused by a number of different factors. For example, depletion of dissolved oxygen due to cellular respiration can be just as problematic for aquatic organisms as allelochemicals that are specifically produced to

deter herbivory. We measured several physicochemical parameters in this experiment in order to determine the causes of toxicity. The physicochemical parameters (orthophosphate, dissolved oxygen, pH, and conductivity) were not highly correlated to survival across all the trials. However, the changes in these parameters were much more strongly correlated to treatment. This could suggest that the toxic effects are being caused by allelochemicals that were not measured in this experiment. Analysis of *L. maackii* leaf composition has revealed several phytochemicals that may have allelopathic anti-herbivore effects, such as apigenin, luteolin, and chlorogenic acid (Cipollini *et al.* 2008). The allelopathic aspect of *L. maackii* leaves has been examined as an important factor in the “enemy release hypothesis” of invasion, which holds that the invasive can be more successful in its invaded range due to the absence of coevolved herbivores (Liu and Stiling 2006). This hypothesis has been supported by surveys reporting low levels of *L. maackii* leaf loss from herbivory and disease in the invaded range (Lieurance and Cipollini 2012) and lab feeding tests with specialist and generalist insect herbivores, which show less herbivory on *L. maackii* than on native relatives (Lieurance and Cipollini 2013). However, the seasonal aspect of our results does not reflect the results of some of these previous studies, which show a suppression of herbivory throughout the year.

This experiment is a reductionist set of microcosm assays and as such the implications of these findings for flowing streams (in situ) are unclear. For instance, in a flowing system, such as a headwater stream, chemical composition of the water will be continuously refreshed by flow, potentially creating a much lower level of exposure than what is created in the microcosm cups. Secondly, materials from *L. maackii* will be flushed downstream periodically likely lessening exposure. Finally, macroinvertebrates exist in aquatic systems in a diverse assemblage of organisms exposed to materials from a diverse array of allochthonous materials. Even so, this study offers experimental evidence of (a) lethal effects and (b) seasonal variation in those effects of *L. maackii* foliage on a model macroinvertebrate. Further work that explores these relationships in flowing systems could be highly illuminating, and our work suggests that any future efforts to quantify allelopathy of non-native invasive species such as *L. maackii* must incorporate experimental consideration of the potential for strong seasonal variation.

Finally, the lethal effects observed in our study emphasizes the importance of invasive species management in riparian zones, especially when the invasive species has unique traits that may alter the quality of the allocthanous subsidies that connect terrestrial and aquatic habitats.

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