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1 **Lethal effects of leaf leachate from the non-native invasive shrub Amur honeysuckle (*Lonicera***
2 ***maackii*) on a model aquatic organism (*Hyalella azteca*)**

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7

8 ABSTRACT

9 The invasive shrub *Lonicera maackii* (Amur honeysuckle) is a problematic species in the eastern
10 United States and there is growing evidence that materials from this species have toxic effects
11 on some organisms. We used a sequence of microcosm bioassays to assess the influence of *L.*
12 *maackii* leaf leachate on the macroinvertebrate *Hyalella azteca*, which is a standard aquatic
13 organism for toxicity assessment. In a laboratory setting, *H. azteca* were exposed to a leaf
14 leachate dilution series (6.25%, 12.5%, 25%, 50%, 100%) in 48 h toxicity tests. This was repeated
15 throughout the growing season to assess the potential for changes in leaf toxicity due to
16 phenology. Strong toxic effects were found when *H. azteca* was exposed to a *L. maackii*
17 leachate from autumn ($P < 0.05$) and, in fact, all organisms died when exposed to any level of
18 concentration in most trials. Mean percent survival also decreased significantly in all dilutions
19 in the spring ($P < 0.05$ for all treatments); however, little toxicity was detected in growing
20 season trials. These results suggest (a) strong toxic effects of *L. maackii* foliage on a model
21 aquatic organism that (b) varies throughout the year, potentially in relationship to biochemical
22 changes associated with phenology.

23

24 Keywords: Phenology; ecological invasion; toxicity; allochthonous subsidy; terrestrial-aquatic

25 linkages; allelopathy

26

27 **Introduction**

28

29 The transfer of energy and nutrient resources from the riparian zone into headwater streams is
30 strongly linked the diversity and composition of aquatic biota and overall stream health (Wallace
31 et al. 2015, Tank et al. 2010, Young et al. 2008). Traits of riparian vegetation such as leaf
32 chemistry and canopy density can influence many factors in the aquatic system, including light
33 availability (Baxter et al. 2005), nutrient input rates (Harner et al. 2009), and water temperature
34 (Clinton et al. 2010). Allochthonous inputs from the riparian zone include organic materials,
35 such as leaves and woody material, and are closely linked to aquatic food webs (Wallace et al.
36 2015, Bailey et al. 2001, Baxter et al. 2005, Vannote et al. 1980). Aquatic macroinvertebrates
37 utilize these inputs as habitat, refuge, and food, and these subsidies can be strongly affected by
38 changes in the composition of riparian vegetation (Bailey et al. 2001). Riparian habitats are
39 vulnerable to plant invasion because of the increased availability of resources and the frequency
40 of disturbances, such as flooding (Stohlgren et al. 1998, Nakamura et al. 2000). In these habitats,
41 the novel morphological and chemical characteristics of invasive species may strongly alter the
42 quality of allochthonous subsidies entering the stream.

43 In the eastern United States the invasive shrub *Lonicera maackii* (Amur honeysuckle) has
44 proliferated in a variety of habitats, including disturbed forests, urban areas, and riparian
45 ecosystems (Luken and Thieret 1996, Hutchinson and Vankat 1997). The impacts of *L. maackii*
46 invasion on terrestrial ecosystems include reduced biodiversity, reduced fecundity of native
47 herbs, and allelopathic suppression of germination in native plants (Collier et al. 2002, Miller
48 and Gorchoy 2004, Dorning and Cipollini 2006). Low levels of terrestrial insect herbivory have
49 been noted for *L. maackii* (Lieurance et al. 2015), and experiments indicated that the generalist

50 herbivore *Spodoptera exigua* (Cipollini et al. 2008) and the highly polyphagous gypsy moth
51 caterpillar (*Lymantria dispar*) avoided consumption of the leaves (McEwan et al. 2009b).
52 Aquatic macroinvertebrates interact directly with terrestrial organic subsidies (Cummins and
53 Klug 1979, Wallace and Webster 1996); therefore, *L. maackii* invasion into riparian areas may
54 have significant ramifications for in-stream biota. In fact, McNeish et al. (2012) provide
55 evidence that the macroinvertebrate community colonizing *L. maackii* leaf packs differs from that
56 of native species. McNeish et al. (2015) found that removal of *L. maackii* from a riparian
57 corridor resulted in significantly higher density of macroinvertebrates in riffle habitats, and
58 McNeish and McEwan (2016) provide an overall framework in which they propose that stream
59 macroinvertebrate community structure is mediated by changes to in-stream subsidies associated
60 with *L. maackii* invasion. Seeking to clarify the mechanism of this effect, Custer et al. (2017)
61 used a series of microcosm assays to investigate the influence of *L. maackii* flower and fruit
62 materials on stream macroinvertebrates. They found strong evidence that both of these materials
63 are toxic to some species in field and laboratory settings. The novel fruit and flower materials in
64 the Custer et al. (2017) experiments are an important subsidy; however, to our knowledge no
65 work has yet assessed the potential for toxic effects of leaf materials.

66 In this project a series of microcosm assays was used to assess the possibility for toxic
67 effects of *L. maackii* foliage on stream macroinvertebrates. In particular, we used a dilution
68 sequence of *L. maackii* leachate to assess toxic effects on the generalist shredder *Hyaella azteca*.
69 Through these experiments, we explicitly tested the hypothesis that *H. azteca* would experience
70 reduced survival when exposed to *L. maackii* leaf leachate. In deciduous forests invaded by *L.*
71 *maackii*, leaf drop is autumnal and therefore our methods focused on leaves during this season;
72 however, to provide an assessment of possible seasonal variation we included assays in spring

73 and summer. Though a static microcosm assay may not reflect conditions in a flowing stream in
74 all conditions throughout the year, we have observed that local headwater streams hold
75 significant quantities of *L. maackii* foliage in the fall and often have such low flow that they are
76 effectively a series of pools. Therefore, functionally, this design mimics field conditions that are
77 relevant at least in some portions of the year and provides a clear, though reductionist,
78 assessment of toxicity for a model macroinvertebrate species.

79

80 **Materials and Methods**

81

82 *Bioassay methods*

83

84 Our bioassay testing method consisted of a series of 48 h exposures to different dilutions
85 of leaf leachate. The leachate was made using 1000 mL dechlorinated tap water in which 100 g
86 of leaves were soaked at room temperature for 24 h. The mixture was poured through a 250
87 micrometer sieve and diluted with dechlorinated tap water to create the following serial dilution
88 gradient: 100%, 50%, 25%, 12.5%, 6.25%. The 10:1 ratio of our 100% dilution has been used in
89 allelopathy studies of *L. maackii* making results from that concentration comparable to other
90 projects (e.g., Custer et al. 2017, McEwan et al. 2010) while the dilution sequence adds realism
91 to the experiment given that in some natural situations the aquatic system will be flowing which
92 could potentially dilute leachate concentrations depending on upstream vegetation. Each trial
93 included these five treatments plus a control (dechlorinated tap water), and four replicates per
94 treatment. Each replicate received 50 mL of leachate and five *H. azteca*. After 48 h, the
95 organisms were counted and the mean percent survival was recorded for each replicate. Trials

96 were conducted in October and December to represent the senescence and leaf drop of *L.*
97 *maackii* (McEwan et al. 2009a, Arthur et al. 2012). Further trials were conducted in April to
98 assess potential toxicity in just unfurled leaves, and in July, August and September prior to any
99 signs of senescence to assess summer conditions. In a natural system, leaf drop would be
100 insignificant in the growing season; however, we tested leaves experimentally during this period
101 to assess potential shifts associated with phenology and also because there may be potential for
102 toxicity associated with throughfall (McEwan et al. 2012a). All leaves were gathered directly
103 from shrubs within the riparian zones of headwater streams in northern Montgomery County,
104 Ohio.

105 Dissolved oxygen, temperature, specific conductivity, and pH were measured at the start
106 and termination of each test using a YSI ProSeries probe (Yellow Springs, Ohio, USA).
107 Additional water chemistry parameters measured for each dilution at the start of each test were
108 orthophosphate, hardness, and alkalinity. The leachate dilutions were stored at 4°C until the
109 analyses were carried out. Orthophosphate was measured using Hach method PhosVer 3[®], within
110 48 hours of dilution. Hardness and alkalinity were measured using titrations.

111 *Hyalella azteca* has been identified by the USEPA and Environment Canada as a model
112 organism for aquatic contaminants (USEPA 2000, Constable et al. 2003, Keddy et al 1994). This
113 species is widely distributed in freshwater habitats, found in regional streams, and is a primary
114 consumer making it a good test organism for our experiments. *Hyalella azteca* were cultured in
115 the lab from starter cultures provided by the USEPA using dechlorinated City of Dayton tap
116 water, and fed a slurry of Tetramin/wheat grass. Organisms were selected for testing when they
117 reached age range between 7 – 14 d.

118

119 *Statistical Methods*

120

121 All data were screened for normality and homogeneity of variance using the Shapiro–
122 Wilk test for normality and Levene’s test for equal variance. For all trials, normality and equal
123 variances were violated, so a Kruskal–Wallis rank sum nonparametric test was utilized with a
124 Wilcoxon Pairwise post hoc test to determine the significance of treatment differences. For the
125 correlation analysis, the data were screened for normality using the Shapiro-Wilk test. If normal,
126 the data were analyzed using Pearson’s product-moment correlation; if non-normal, the data
127 were analyzed using Spearman’s rank correlation. All analyses were carried out using R
128 statistical software (R Core Team 2017).

129

130 **Results**

131

132 Bioassays using foliage collected in the fall had very strong toxic effects (Figure 1). *Hyaella*
133 *azteca* survival significantly decreased in all trials using foliage collected in autumn 2015
134 (Figure 1; $P < 0.001$ for all trials). For instance, in the assay using foliage collected on 26-Oct-
135 2015, survival in the control was 100% while every *L. maackii* leachate treatment exhibited
136 100% mortality (Figure 1, top panel). An identical result was found in the assay using foliage
137 collected on 1-Dec-2015. In the 13-Dec-2015 assay, survival decreased with increasing dilution
138 concentration, resulting in 0% survival in the highest dilution concentration. (Figure 1, bottom
139 panel).

140 Our assays using materials collected during the growing season exhibited contrasting
141 results with strong mortality in spring and only a slight indication of toxicity in during the

142 summer. A bioassay using materials collected 20-April-2016 had an identical pattern to that
143 observed in the autumn with 100% survival in the control and complete mortality in all other
144 concentration levels (Figure 2). Every organism exposed to any concentration of *L. maackii* leaf
145 leachate died in this assay (Figure 2). In contrast to the fall and spring results, assays using
146 foliage collected in the summer indicated little toxicity of *L. maackii* materials. There were no
147 statistically discernable effects on survivorship in assays using foliar materials collected in July
148 and early September (top and bottom panels, Figure 3) and survivorship was > 80% in all assays.
149 Analysis of *H. azteca* survival exposed to foliar material collected in August suggested a
150 significant overall treatment effect ($P = 0.005$) and post-hoc tests revealed that the 25% dilution
151 had significantly lower survivorship than lower percentage treatments (12.5% and 6.25%) and
152 the control, but was not different than the higher concentrations (Figure 3).

153 Physiochemical parameters of the water within the microcosms were seemingly unrelated
154 to organism survival but were strongly and significantly influenced by changes in the leachate
155 concentration. For instance, organismal response was typically 100% survival or 100% mortality
156 across a range of dissolved oxygen (DO) concentrations (Figure 4, left panel), yet leachate
157 dilution had a strong effect on aquatic DO in the microcosms where increasing leachate
158 concentration resulted in a reduction in DO ($P < 0.001$; Figure 4, right panel). A similar pattern
159 was evident in the relationships with pH where survival was nearly always either 0 or 100%
160 (Figure 5, left panel) and a significant negative relationship ($P < 0.001$) existed wherein
161 increasing leachate concentration was correlated with decreasing pH (more acidic, Figure 5, right
162 panel). For conductivity, the basic pattern was the same (Figure 6) except that increasing
163 dilution resulted in a significant increase in conductivity of water within the microcosms (Figure
164 six, right panel).

165

166 **Discussion**

167

168 The critical role of terrestrial subsidies as a structuring factor in aquatic communities is
169 well established in a wide variety of systems (Vannote et al. 1980, Tank et al. 2010, Young et al.
170 2008). Alterations of subsidies from the terrestrial environment associated with riparian zone
171 invasion by exotic plants have been shown to have cascading impacts on in-stream foodwebs.
172 For example, riparian invasion of Japanese knotweed (*Fallopia japonica*) in the Pyrenees in
173 France resulted in altered macroinvertebrate communities and leaf litter breakdown rates (Lecerf
174 et al. 2011). Lab studies have shown changes in growth of aquatic invertebrates in response to
175 feeding on leaf litter from the invasive giant reed (*Arundo donax*) (Going and Dudley 2008). In
176 forests of the Midwestern United States, *Lonicera maackii* is a significant forest invader and is
177 well-known to create a dense stands in forested riparian zones (Luken and Thieret 1996,
178 Hutchinson and Vankat 1997, Wilson et al. 2013, McNeish and McEwan 2016). Forest invasion
179 by this species has been shown to have wide ranging implications for biogeochemical cycles,
180 wildlife, local plant communities, and stream structure and function (reviewed in McNeish and
181 McEwan, 2016). The results of this study provide further evidence that invasion by *L. maackii*
182 has the potential to re-structure aquatic macroinvertebrate communities and supports a more
183 general “novel subsidies” hypothesis of plant invasion (*sensu* Custer et al. 2017).

184 A growing body of evidence suggests that riparian invasion by *L. maackii* along
185 headwater streams influences aquatic community structure and that these changes are linked to
186 alterations in the quality and quantity of terrestrial subsidies. McNeish et al. (2012)
187 demonstrated rapid breakdown of *L. maackii* foliage and a unique colonizing macroinvertebrate

188 community when compared with native leaf packs. In a riparian restoration experiment McNeish
189 et al. (2015) reported a significant increase in macroinvertebrate density following removal of *L.*
190 *maackii* and proposed a mechanistic framework that linked the invasion of *L. maackii* to aquatic
191 biota via alterations in the terrestrial subsidies. Subsequent work over a longer time frame
192 confirmed that macroinvertebrate density increased in the absence of *L. maackii* and also pointed
193 to an alteration in the functional composition of those aquatic communities (McNeish et al.
194 2017). Previous work has suggested strong toxic effects of *L. maackii* flowers and fruit, leading
195 Custer et al. (2017) to propose that these represent “novel subsidies” that may drive shifts in the
196 aquatic communities. Our series of bioassay trials support this hypothesis. Leaf leachate from *L.*
197 *maackii* leaves collected during the normal autumnal leaf drop was toxic to *H. azteca* resulting in
198 100% mortality in higher concentrations in all assays .

199 The particular cause of leaf leachate toxicity in our study is presently unknown, but is
200 likely related to leaf chemistry and not alterations in the physicochemical characteristics of the
201 microcosm water. The physicochemical parameters (orthophosphate, dissolved oxygen, pH, and
202 conductivity) were strongly correlated with changes in leaf leachate solution across all trials;
203 however, survival in our trials was nearly always either 100% or 0. For instance, there was
204 depletion of dissolved oxygen (DO) with increasing leachate concentration, which can be
205 problematic for aquatic organisms (Nebeker et al. 1992); however, and in our trials some assays
206 with the lowest levels of DO had very high survival and some assays with high levels of DO
207 exhibited 100% mortality. Ruling out these physicochemical factors as drivers of toxicity could
208 suggest that the toxic effects are being caused by allelochemicals that were not measured in this
209 experiment. Analysis of *L. maackii* leaf composition has revealed several phytochemicals that
210 may have allelopathic anti-herbivore effects, including apigenin, luteolin, and chlorogenic acid

211 (Cipollini et al. 2008). The allelopathic aspect of *L. maackii* leaves has been examined as an
212 important factor in the “enemy release hypothesis” of invasion, which holds that the invasive can
213 be more successful in its invaded range due to the absence of coevolved herbivores (Liu and
214 Stiling 2006). This hypothesis has been supported by surveys reporting low levels of *L. maackii*
215 leaf loss from herbivory and disease in the invaded range (Lieurance and Cipollini 2012) and lab
216 feeding tests with specialist and generalist insect herbivores, which show less herbivory on *L.*
217 *maackii* than on native relatives (McEwan et al. 2009b, Lieurance and Cipollini 2013). We
218 hypothesize that plant secondary compounds from *L. maackii* are the cause of the toxic effects
219 revealed in our assays.

220 We noted strong variation throughout the growing season in toxicity, possibly due to
221 annual variation in leaf chemistry associated with phenology. Phenological variation of defensive
222 chemicals has been documented in the invasive plant Dalmatian toadflax, *Linaria dalmatica*
223 (Jamieson and Bowers 2010). One hypothesis that may explain the apparent variation through
224 the growing season in leaf toxicity may be related to plant chemistry variation in response to
225 herbivory in seasons when there are fewer other sources of food for herbivores, especially in the
226 fall and winter due to the extended growing season of *L. maackii* (Fridley 2012). Arthur et al.
227 (2012) demonstrated that *L. maackii* foliage began being deposited around the same time as the
228 native forest flora in mid-October; however, McEwan et al. (2009a) did not note leaf senescence
229 until mid-December. These findings match our fields observations that *L. maackii* often drops
230 leaves that are “green” in appearance and that the autumnal color change is a relatively subtle
231 shift in hue. We recorded nearly nearly 100% survival in a trial using leaves collected in
232 September, prior to any signs of senescence and prior to known leaf drop for *L. maackii*, and
233 100% mortality for organisms exposed to leaves collected late in October when leaves are known

234 to be going through senescence. It was beyond the scope of this study to ascertain what
235 phytochemical differences may be induced in *L. maackii* during the onset of senescence.

236 It is virtually impossible for experimental *ex-situ* assays to reflect the full range of
237 conditions and leachate concentrations in dynamic natural streams. In our experiments, the
238 dilution series ranged from 100g/L to 6.25g/L and leaching process took place over 24 hours. In
239 a test of germination inhibition, Dorning and Cipollini (2006) used a range of concentration
240 equivalents ranging from 50g/L to 200g/L, which overlaps with some portion of our gradient,
241 and their leaching process took place over 72 hours. Watling et al. (2011b) examined the
242 influence of *L. maackii* on amphibian biology using 5g/L of leaves soaked for 48hrs. This
243 concentration is lower than our lowest concentration (6.25g/L); however, their soaking period
244 was 2× longer at 48 hours while ours was 24 hours. Presumably the shorter soaking period in
245 our assay would result in a lower concentration of leachate, though how concentration is related
246 to soaking time is presently unknown. Watling et al (2011a,b) found 3.35 mg/L of phenolics
247 resulted from their *L. maackii* treatment. In comparison, Martin and Blossey (2013) assessed
248 impacts of secondary plant compounds on larval amphibians using concentrations that ranged
249 from 0-25 mg/L. The concentration of *L. maackii* leachate in water of regional headwater
250 streams remains unknown, but likely ranges from extremely low-levels during high flow
251 conditions in the summer when leaves are on the shrubs, to potentially very high concentrations
252 during droughty conditions in the fall when leaves are being deposited into small stagnant pools
253 and rapidly decomposing (McNeish et al. 2012). Our experimental concentrations are realistic
254 representations of leachate concentrations that organisms are likely to experience during some
255 conditions in the field and provided compelling evidence of toxicity. Future work that carefully
256 tracks the transfer of secondary compounds from invasive plants into natural systems and

257 provides accurate estimations of concentrations present would provide a better framework for
258 experiments work and advance understanding of the intricate ways in which exotic plant
259 invasions may influence ecosystem processes.

260 Our results suggest there are strong negative implications for aquatic insects when the
261 leaves of *L. maackii* are deposited into stream systems. Our results were obtained via *ex-situ*
262 microcosm assays, which have a high level of experimental control and therefore less of the
263 inherent ecological variation is captured (Newman and Clements 2008). Further work that
264 continues to explore these relationships in flowing streams *in-situ* may be highly illuminating.
265 While *H. azteca* is considered a model organism, aquatic systems contain a diverse assemblage
266 of macroinvertebrates that may exhibit differential responses to the diverse array of
267 allochthonous materials and other contaminants entering a stream, including those from *L.*
268 *maackii*. Variation in toxicity across season highlights the importance of future work that isolates
269 aspects of phytochemistry that may be a source of toxicity in aquatic systems and links transfer
270 of those substances with plant phenology. In summary, this study offers experimental evidence
271 of (a) lethal effects to *H. azteca* and (b) seasonal variation in those effects. The lethal effects
272 observed in our study emphasize the importance of invasive species management in riparian
273 zones, especially when the invasive species has unique traits that may alter the quality of the
274 allochthonous subsidies that connect terrestrial and aquatic habitats.

275

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277

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290

291 **Disclosure statement**

292

293 No potential conflict of interest was reported by the authors.

294

295 **References**

296

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417

418 Figure Legends

419

420 Figure 1: Mean (\pm SD) percent survival of *Hyalabella azteca* in serially diluted *Lonicera maackii*
421 leaf leachate created with leaves collected during autumn of 2015. Capital letters indicate
422 statistically significant differences ($P < 0.05$). Absence of error bars indicates identical results in
423 all replicates.

424

425 Figure 2: Mean (\pm SD) percent survival of *Hyalabella azteca* in serially diluted *Lonicera maackii*
426 leaf leachate created with leaves collected during the spring of 2016. Capital letters indicate
427 statistically significant differences. Absence of error bars indicates identical results in all
428 replicates.

429

430 Figure 3: Mean (\pm SD) percent survival of *Hyalabella azteca* in serially diluted *Lonicera maackii*
431 leaf leachate created with leaves collected during the growing season of 2016. Capital letters
432 indicate statistically significant differences. Absence of error bars indicates identical results in all
433 replicates.

434

435

436 Figure 4: Linear regression analysis of dissolved oxygen in relation to mean percent survival and
437 leachate dilution treatments. Empty circles represent high percent survival ($>80\%$), grey circles
438 represent moderate survival ($20-80\%$), and black circles represent low survival ($<20\%$).

439

440 Figure 5: Linear regression analysis of pH in relation to mean percent survival and leachate
441 dilution treatments. Empty circles represent high percent survival (>80%), grey circles represent
442 moderate survival (20-80%), and black circles represent low survival (<20%).

443

444 Figure 6: Linear regression analysis of specific conductivity in relation to mean percent survival
445 and leachate dilution treatments. Empty circles represent high percent survival (>80%), grey
446 circles represent moderate survival (20-80%), and black circles represent low survival (<20%).

Figure 1

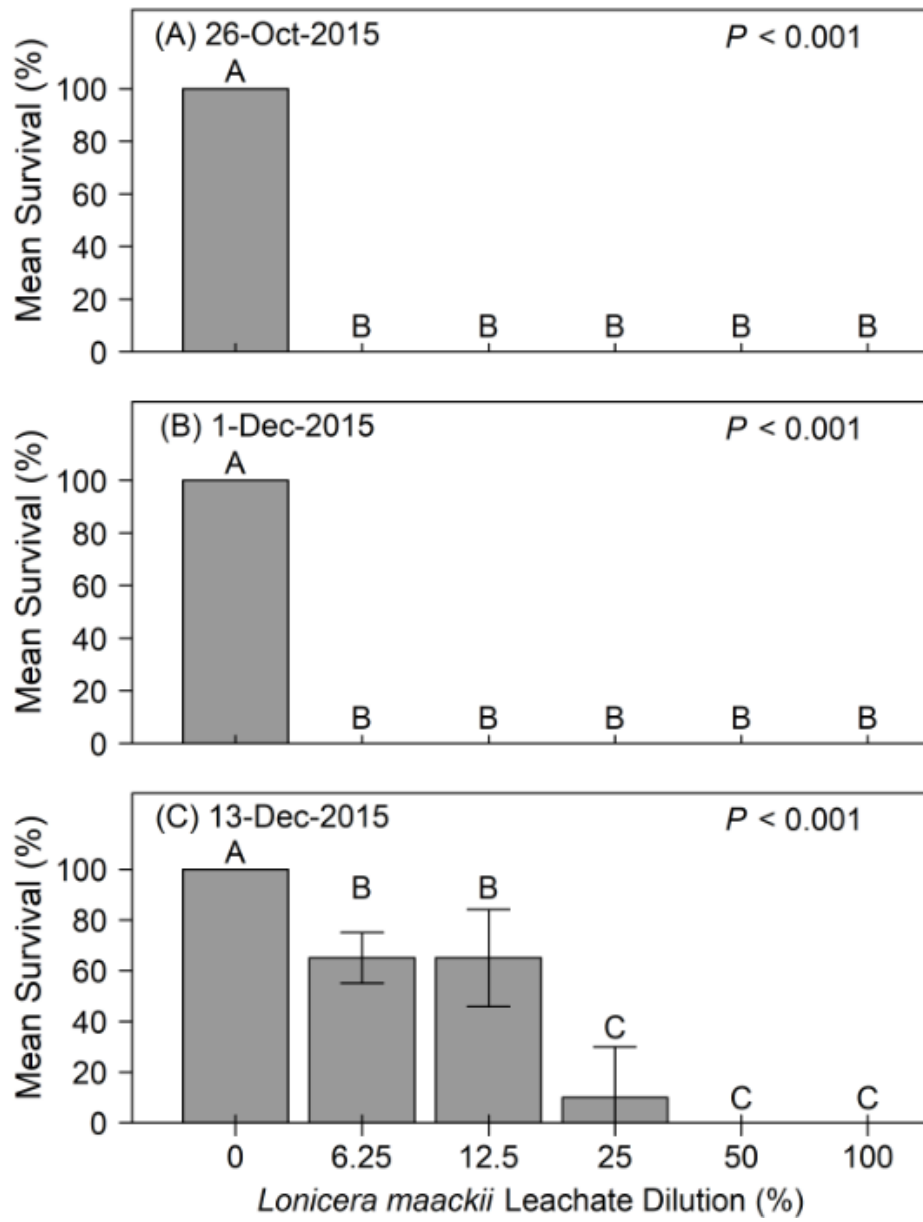


Figure 2

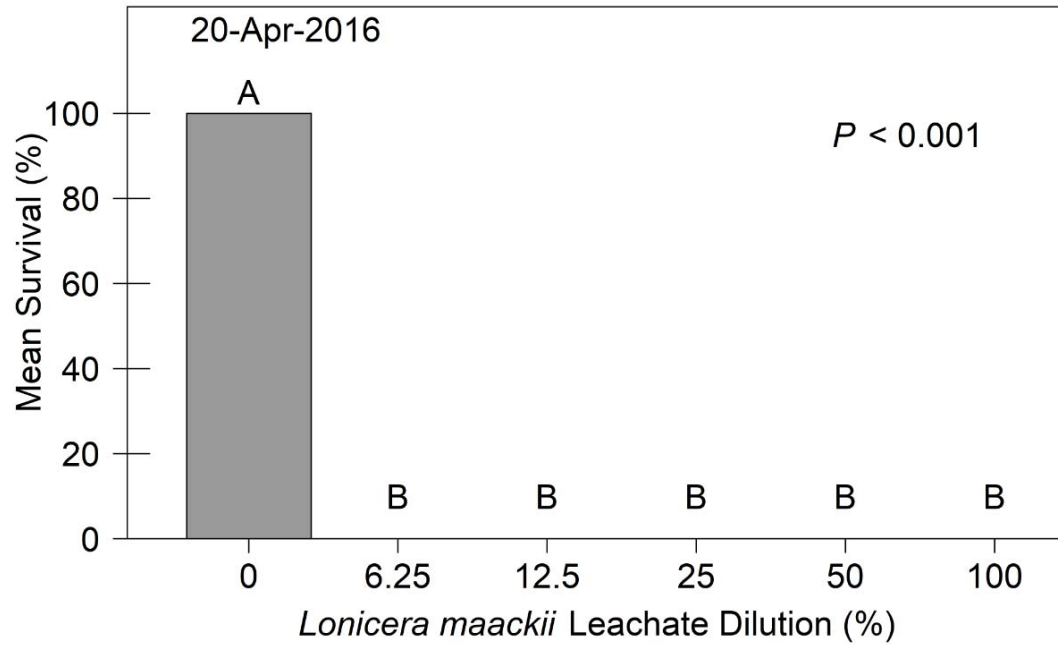


Figure 3

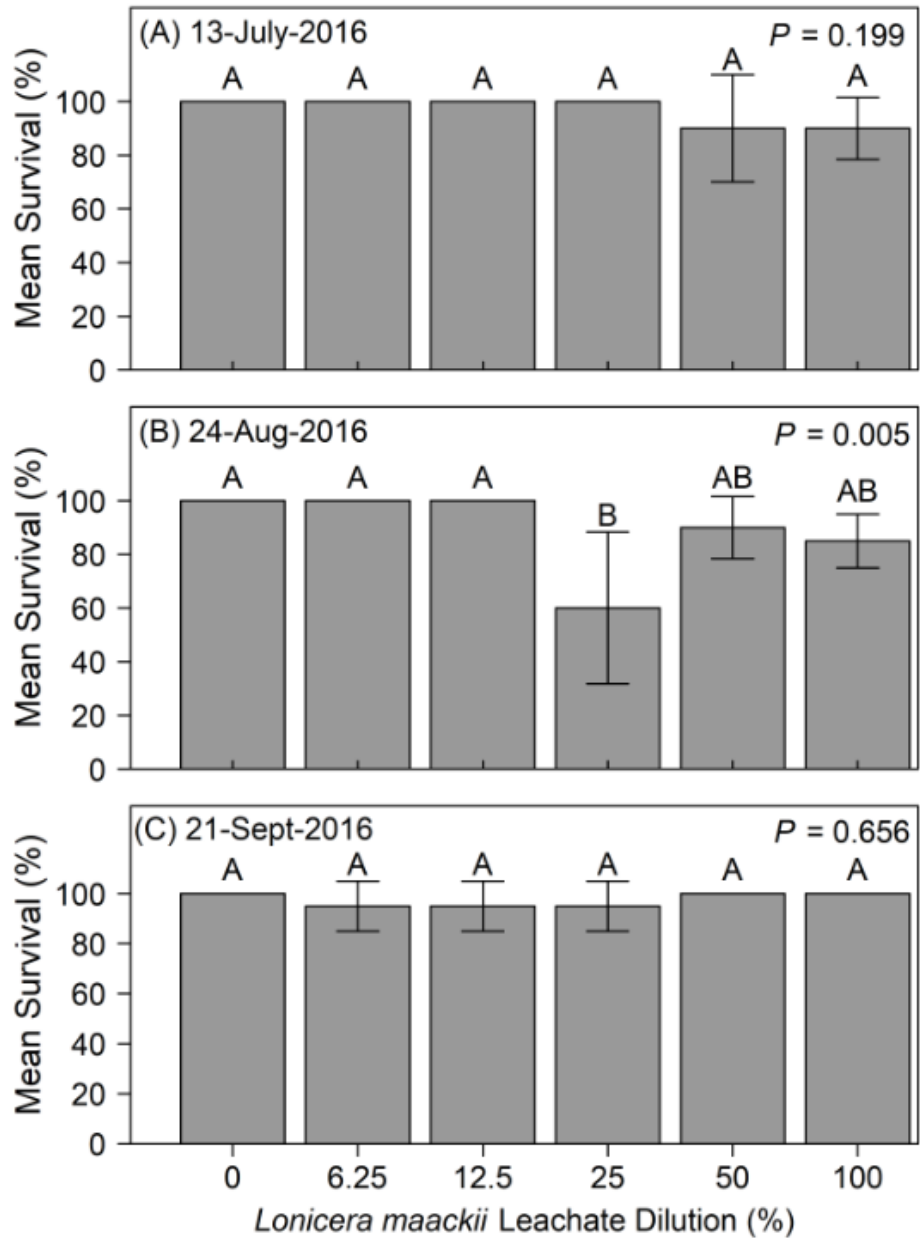


Figure 4

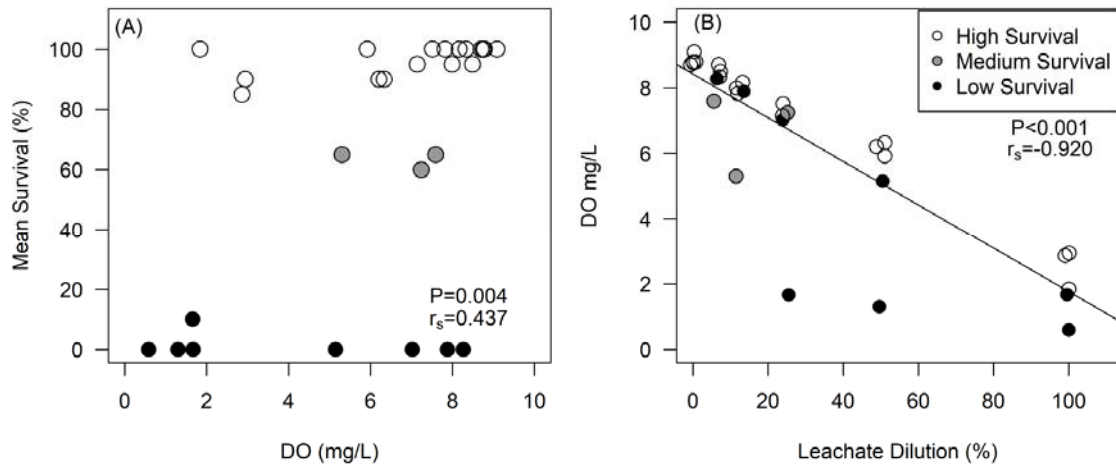


Figure 5

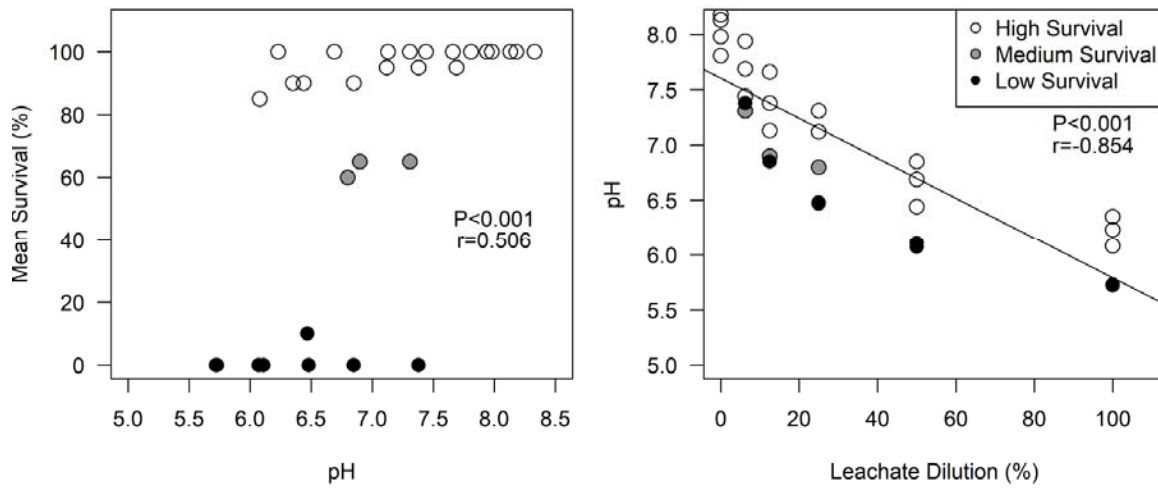


Figure 6

