1   **Ecological consequences of urbanization on a legume-rhizobia mutualism**

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3   David Murray-Stoker[1,2,3,*] and Marc T. J. Johnson[2,3]

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5   [1]Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario

6   M5S 3B2, Canada

7   [2]Department of Biology, University of Toronto Mississauga, Mississauga, Ontario, Canada L5L

8   1C6

9   [3]Centre for Urban Environments, University of Toronto Mississauga, Mississauga, Ontario,

10   Canada L5L 1C6

11   *Corresponding author: David Murray-Stoker dstoker92@gmail.com

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13   David Murray-Stoker, ORCID https://orcid.org/0000-0002-4774-6948

14   Marc T. J. Johnson, ORCID http://orcid.org/0000-0001-9719-0522

15   **Abstract**

16   Mutualisms are key determinants of community assembly and composition, but urbanization can

17   alter the dynamics of these interactions and associated effects on ecosystem functions. Legume-

18   rhizobia mutualisms are a model interaction to evaluate the ecological and ecosystem-level

19   effects of urbanization, particularly urban-driven eutrophication and nitrogen (N) deposition.

20   Here, we evaluated how urbanization affected the ecology of the mutualism between white

21   clover (*Trifolium repens*) and its rhizobial symbiont (*Rhizobium leguminosarum* symbiovar

22   *trifolii*) along an urbanization gradient. We found that the abundance of rhizobium nodules on

23   white clover decreased with urbanization. White clover acquired N from mixed sources of N

24   fixation and uptake from the soil for the majority of the urbanization gradient, but white clover

25   primarily acquired N from the soil rather than N fixation by rhizobia at the urban and rural limits

26   of the gradient. Importantly, we identified soil N as a critical nexus for urban-driven changes in

27   the white clover-rhizobium mutualism. Taken together, our results demonstrate that urbanization

28   alters the ecological consequences of a legume-rhizobium mutualism, with direct and indirect

29   effects of the urban landscape on an ecologically-important mutualistic interaction.

30   Keywords: mutualism, nitrogen, stable isotopes, urban ecology

31 **Introduction**

32   Urbanization is a major driver of ecosystem change at local and global scales, consistently

33   altering the ecological setting in terms of both biotic and abiotic factors (Grimm et al. 2008, Seto

34   et al. 2010). Biotic changes frequently include fragmented habitats, homogenization of species

35   composition, reduced abundance and diversity of native species, reduced vegetation cover, and

36   increased abundance of non-native species (McKinney 2002, Grimm et al. 2008, Groffman et al.

37   2014, Aronson et al. 2016). Common changes to the abiotic environment include increased

38   impervious surface cover, elevated temperatures, higher pollution levels (e.g., air, water, light,

39   noise), and increased nutrient deposition (Grimm et al. 2008, Groffman et al. 2014, Stevens et al.

40   2018). However, there is limited research regarding the direct and indirect impacts of biotic and

41   abiotic changes on species interactions in urban environments (Youngsteadt et al. 2015, Miles et

42   al. 2019). Elucidating how urbanization affects the ecological consequences of species

43   interactions is important for understanding the drivers of biodiversity and ecosystem change in

44   urban environments.

45   Species interactions are key determinants of community composition (Wisz et al. 2013,

46   Leibold and Chase 2017), but urbanization can disrupt these interactions (Raupp et al. 2010,

47   Aronson et al. 2016, Miles et al. 2019) and their associated ecosystem functions (Ziter 2016). In

48   urban landscapes, natural habitats are frequently fragmented and degraded, which can result in

49   reduced species diversity and altered community composition (Williams et al. 2009, Aronson et

50   al. 2016). Such ecological changes can alter antagonistic interactions (e.g., predator-prey, host-

51   parasite; Rocha and Fellowes 2018, Parsons et al. 2019), competition (De León et al. 2019,

52   Thomson and Page 2020), and mutualisms (Irwin et al. 2014, Rocha and Fellowes 2018, Rivkin

53   et al. 2020). Mutualisms can be important for community and ecosystem stability, and

54 disruptions to these interactions caused by urbanization may be particularly problematic for

55 maintaining ecosystem functions. For example, pollinator abundance, diversity, and composition

56 often change with urbanization (Harrison et al. 2018, Santangelo et al. 2020), which can shift the

57 balance of the benefits conveyed between interacting plants and pollinators (Irwin et al. 2014,

58 Rivkin et al. 2020, Santangelo et al. 2020). As another example, plant-microbe interactions are

59 important for community assembly and nutrient cycling (van der Heijden et al. 2008), and

60 urbanization can affect these interactions through altered soil chemistry mediated by pollution

61 and nutrient deposition (Grimm et al. 2008, Stevens et al. 2018). As plant-microbe interactions

62 are frequently nutrient-provisioning mutualisms, pollution and nutrient deposition that cause

63 changes in the diversity and composition of microbial communities can have cascading effects

64 on nutrient cycles in urban ecosystems (Galloway et al. 2003, Kaye et al. 2006). More broadly, if

65 urbanization frequently alters mutualistic interactions, this may have cascading effects on

66 communities and ecosystems.

67   Mutualisms between legumes and rhizobia are an ideal system for evaluating the ecological

68 impacts of urbanization on species interactions. In these mutualisms, rhizobia fix atmospheric

69 nitrogen in exchange for photosynthate and housing in nodules by their host plant (Hirsch 1992,

70 Poole et al. 2018). Urbanization can disrupt these interactions, specifically through nitrogen (N)

71 deposition and enrichment (Grimm et al. 2008, Zhang et al. 2012). Nitrogen deposition can

72 inhibit the formation of nodules (Streeter and Wong 1988, Omrane and Chiurazzi 2009),

73 precluding the development of the mutualism or reducing plant reliance on rhizobia for

74 providing N (Vergeer et al. 2008, Weese et al. 2015, Regus et al. 2017). Additionally, N

75 deposition can reduce N fixation rates by rhizobia (Cleland and Harpole 2010, Zheng et al.

76 2019). Although N deposition and short-term application of nutrient-rich fertilizers can benefit

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77 both legumes and rhizobia (Simonsen et al. 2015, Forrester and Ashman 2018), chronic and

78 long-term exposure to increased N can reduce the ecological benefits of N fixation and cause the

79 evolution of less beneficial rhizobia (Weese et al. 2015, Regus et al. 2017). Using the legume-

80 rhizobia mutualism as a model system, it is possible to study how urbanization and nutrient

81 deposition alter interactions in an ecologically-important mutualism.

82   In this study, we evaluated the hypothesis that urbanization alters the ecological and

83 ecosystem-level consequences of a nutrient-provisioning mutualism. We used the mutualism

84 between the legume white clover (*Trifolium repens*) and its rhizobial symbiont (*Rhizobium*

85 *leguminosarum* symbiovar *trifolii*) as a tractable model interaction. We conducted our study

86 along an urbanization gradient in Toronto, Canada (Fig. 1). Our study focused on three primary

87 questions: (Q1) does rhizobia nodulation vary along an urbanization gradient? (Q2) How does

88 the source of plant nitrogen (i.e., from soil or gaseous N_2 fixed by rhizobia) change along the

89 urbanization gradient? And (Q3) how do urban landscape features influence the interactions

90 between soil N, plant N, and rhizobia nodulation? We predicted that: (1) urbanization would alter

91 investment in rhizobia by *T. repens*, causing increased nodulation with decreased urbanization;

92 (2) increased N availability in the soil due to urbanization would reduce N fixation by rhizobia

93 and alter the source of N for *T. repens*; and (3) the changes in landscape features associated with

94 urbanization cause direct and indirect effects on ecosystem structure and the white clover-

95 rhizobium mutualism (Fig. 2).

96 **Materials and Methods**

97 *Land acknowledgement*

98 We conducted our sampling at 49 sites along an urbanization gradient in the Greater Toronto

99 Area of Ontario, Canada in August 2018 (Fig. 1). Our sampling was conducted on the traditional

100 land of the Huron-Wendat, the Seneca, and most recently, the Mississaugas of the Credit First

101 Nation.

102 *Study organisms*

103 White clover (*Trifolium repens* L., Fabaceae) is a perennial, herbaceous legume native to Eurasia

104 that is now globally distributed (Baker and Williams 1987). White clover typically reproduces

105 clonally through stolons (Kemball and Marshall 1995) as well as through seed via obligately-

106 outcrossed flowers (Barrett and Silander 1992). *Rhizobium leguminosarum* symbiovar *trifolii* is

107 the primary rhizobial symbiont of *T. repens* (Martínez-Romero and Caballero-Mellado 1996,

108 Andrews and Andrews 2017). As a facultative symbiont, *R. l.* bv. *trifolii* fixes atmospheric N_2

109 into accessible NH_3 for white clover in exchange for photosynthate and hosting within root

110 nodules.

111   Nodulation is initiated when rhizobia attach to and penetrate the root. After infection of

112 the root they continue to divide within the plant host cells to form nodules (Poole et al. 2018). At

113 this stage, rhizobia differentiate into bacteroids and begin the N fixation process, whereby

114 atmospheric N_2 is converted into NH_3 by bacteroids with the aid of nitrogenase enzymes (Hirsch

115 1992, Poole et al. 2018). Urbanization and specifically N enrichment can inhibit nodulation at

116 multiple steps of the process. For example, NO_3 and NH_3 can inhibit rhizobia infection of roots,

117 the formation of nodules, or the activity of nitrogenase (Streeter and Wong 1988, Omrane and

118 Chiurazzi 2009). Another common mechanism for reduced nodulation with increased N

119 availability is plant hosts switching N sources from N fixed by rhizobia to N taken up directly

120 from the soil, resulting in lower nodulation and less investment in the symbiosis (Heath et al.

121 2010, Regus et al. 2017).

122 *Field sampling*

123   Field collections of white clover, associated rhizobia, and soil samples were collected at all 49

124   sites along the urbanization gradient (Fig. 2). At each site, 9-10 clover individuals and associated

125   roots and nodules were collected (mean $\pm$ SE $= 9.94 + 0.13$ individuals). Twenty soil cores were

126   also taken at each sampling location, with 10 cores taken immediately adjacent to collected white

127   clover and 10 taken at least 5 m from the nearest white clover plant (hereafter "bulk" cores), with

128   the requirement that bulk cores did not contain other nitrogen-fixing legumes (e.g., *Medicago*

129   *lupulina*). Each soil core was taken to a depth of 5 cm, and the top 2 cm plus organic material

130   were removed because they could confound estimates of soil nutrients. Each sample type (bulk

131   and adjacent) was combined for a composite sample of each soil core type per site, which was

132   stored at $-80°C$ until subsequent processing.

133   *Lab processing*

134   *Nodule quantification:* White clover samples were separated into leaf and root tissue. Leaf tissue

135   was collected for stable isotope analyses by cutting 6-10 fully expanded, green, non-senescing

136   leaves from each individual, which were placed into a 2 mL tube and stored in a freezer at -20°C

137   until later processing. Root tissue was collected by cutting the first five roots below the plant

138   base and directly attached to the stolon. Roots were measured to the nearest 1 mm until at least

139   10 cm of root length was measured or all collected roots were measured (root length: mean $\pm$ SE

140    $= 10.3 \pm 0.13$, range $= 1.8$-17.7 cm). Nodules were counted visually for each measured root, and

141   counts were standardized by the total length of root measured to generate an estimate of nodule

142   density per cm of root per plant.

143   *Soil sample processing:* Soil samples were prepared for N analyses by filtering, grinding,

144   homogenizing, and drying samples. Samples were taken out of storage in the freezer at -80°C

145   and thawed overnight in the lab (21.5°C). Each sample was then filtered over stacked sieves

146   (mesh diameters: 4.75 mm, 2 mm, 1 mm, and 0.5 mm) to remove large rocks and gravel. Soil

147   retained on the 0.5 mm sieve and catch pan was collected, and then samples were dried and

148   stored at 60°C for 48 hrs. Samples were homogenized into a fine powder using mortar and pestle

149   and stored in a drying oven at 60°C for approximately 4 weeks until later processing. Adjacent

150   soil N provided an estimate of N available to white clover individuals and potentially affected by

151   N-fixing rhizobia, and bulk soil N was a measure of background N availability at the site.

152   *Leaf processing:* Leaf samples were prepared for N analyses by freeze-drying, grinding, and

153   drying the tissue. A composite sample for each population was prepared by taking one leaf from

154   each of the white clover individuals per site and the composite samples were freeze-dried and

155   then homogenized using a tissue grinder (FastPrep 96, MP Biomedicals, Irvine, CA, USA).

156   Samples were dried and stored in a drying oven to achieve a constant mass at 60°C for 24-48 hrs

157   until later processing.

158   *Soil and plant N analyses:* Soil and plant samples were weighed (soil ≈ 30 mg, plant ≈ 2 mg) on

159   a micro-balance (XP2U Mettler Toledo, Mississauga, ON, Canada) and packed into aluminum

160   capsules (Costech Analytical Technologies Inc., Valencia, CA, USA). Soil samples were

161   analyzed for % N using a Carlo Erba NA 1500C/H/N Analyzer (Carlo Erba Strumentazione,

162   Milan, Italy). Plant tissue was simultaneously analyzed for % N and N isotopes (i.e., $^{15/14}$N)

163   using a Carlo Erba NA 1500C/H/N Analyzer coupled to a Thermo Delta V IRMS system

164   (Thermo Fisher Scientific, Waltham, MA, USA). Nitrogen stable isotopes were expressed

165   relative to a standard in δ notation:

166                             $\delta = [(R_{sample} - R_{standard}) - 1] \times 1000‰$

167   where R represents the ratio of ^{15}N to ^{14}N. Plants depleted in δ^{15}N (i.e., lower δ^{15}N) suggests N

168   acquisition primarily from N fixation, while plants enriched in δ^{15}N (i.e., higher δ^{15}N) suggests N

169   acquisition primarily from the soil (Högberg 1997, Craine et al. 2015). All elemental and stable

170   isotope analyses were conducted at the Stable Isotope Ecology Laboratory at the University of

171   Georgia, USA (http://siel.uga.edu/).

172   *Landscape metrics*

173   Land use and land cover metrics were calculated for each site to quantify urbanization. We

174   calculated percent impervious surface cover (ISC) manually using Google Earth Pro 7.3.2.5776

175   (Google Inc., Mountain View, CA, USA) by drawing a 100-m radius around each site and using

176   the polygon tool to draw and measure ISC. We also calculated the normalized difference

177   vegetation index (NDVI), a measure of vegetation cover or site 'greenness', for each site using

178   landsat imagery. We calculated NDVI from 1 June to 31 August at 16-day intervals for each year

179   from 2014-2018 to generate a mean NDVI for each site; NDVI was measured at a spatial

180   resolution of 250 m. Each site received 6 measurements per year, for a total of 30 measurements.

181   NDVI was calculated using the "MODISTools" package (version 1.1.1; Tuck et al. 2014).

182   Finally, for each site, we calculated the distance from the urban center as the distance on an

183   ellipsoid (i.e., geodesic distance) using the "geosphere" package (version 1.5-10; Hijmans 2019);

184   coordinates for the urban center were selected as Toronto City Hall (43.651536°N, -

185   79.383276°W). Distance from the urban center has been shown to be correlated with multiple

186   measures of urbanization in Toronto, and it has been identified as an important predictor of the

187   ecological and evolutionary effects of urbanization in other studies (Thompson et al. 2016,

188   Johnson et al. 2018, Rivkin et al. 2020).

189   *Statistical analyses*

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190   *Nodule density mixed-effects models:* We used linear mixed-effects models to analyze the

191   response of nodule density against distance from the urban center, percent ISC, and NDVI. We

192   fitted the linear mixed-effects models as:

193                           $\text{nodule density} = \text{intercept} + x_i + (1 \mid \text{site}) + e_i$

194   where nodule density was the response, x_i was the fixed effect (distance from the urban center,

195   percent ISC, or NDVI), site was a random effect, and e_i was the residual error associated with the

196   fixed effect. A full model with all predictor variables was not fitted because high

197   multicollinearity between variables (Pearson r > |0.5|) made it difficult to disentangle the effects

198   of individual variables (Supplementary material Appendix 1 Fig. A1). Instead, we used structural

199   equation modelling (see below) to integrate the variables into a single analysis.

200   All linear mixed-effects models were fitted using the "lme4" (version 1.1-25, Bates et al.

201   2015) and "lmerTest" (version 3.1-2; Kuznetsova et al. 2017) packages, with models estimated

202   using restricted maximum likelihood. We calculated partial *F*-tests of fixed effects using Type II

203   sums of squares, and denominator degrees of freedom were approximated using the Satterthwaite

204   correction for finite sample sizes (Satterthwaite 1946). Response and predictor variables were

205   standardized to a mean of 0 and standard deviation of 1 prior to analyses, and model assumptions

206   were inspected using the DHARMa package (version 0.3.3.0; Hartig 2020). Conditional R^2, a

207   measure of the variance explained by the fixed and random effects (Nakagawa et al. 2017), was

208   calculated for the models using the "r.squaredGLMM()" function in the "MuMIn" package

209   (version 1.43.17; Barton 2020).

210   *White clover $\delta^{15}N$ and soil N generalized additive models:* Changes in white clover $\delta^{15}N$ and soil

211   N (bulk and adjacent) in response to urbanization were analyzed using generalized additive

212   models (GAMs) using the "mgcv" package (version 1.8-33; Wood 2011, 2017). Generalized

213   additive models are a flexible approach for analyzing data, as non-linear predictors can be fitted

214   with non-parametric smoothing functions to identify effects of the predictors (Hastie and

215   Tibshirani 1986, Wood 2017). We fitted the GAMs as:

$$y_i = \text{intercept} + s(x_i) + e_i$$

217   where y_i was the response (white clover δ^{15}N, bulk soil N, or adjacent soil N), x_i was the

218   predictor (distance from the urban center, percent ISC, or NDVI), s was the non-linear

219   smoothing function for the associated predictor, and e_i was the residual error. Thin-plate

220   regression splines with shrinkage terms (method call: bs = "ts") were used to smooth all

221   predictors (Wood 2003), and each smooth estimate was constrained to a mean of zero. Residual

222   error was assumed independent with a mean of zero and constant variance. White clover δ^{15}N

223   GAMs were fitted to a Gaussian distribution and soil N GAMs were fitted with a logit link to the

224   "betar" distribution, part of the "mgcv" family, to model non-binomial, proportional data.

225   Similar to the nodule density mixed-effects models, high concurvity between variables (Pearson

226     $r > |0.5|$) precluded the use of a single GAM with all predictor variables (Supplementary material

227   Appendix 1 Fig. A1). Instead, all variables were integrated into the structural equation model

228   (detailed below). All GAMs were estimated using maximum likelihood.

229   *Path analysis:* A structural equation model (SEM) was constructed to evaluate the direct and

230   indirect causal pathways through which distance from the urban center, percent ISC, NDVI, soil

231   N (bulk and adjacent), nodule density, and white clover δ^{15}N interact. Basic univariate and

232   multivariate equations are considered structural if there is sufficient evidence that the predictor

233   or set of predictors has a causal effect on the response (Grace 2006), and SEM is the modelling

234   of a multivariate relationship with two or more structural equations. The robustness of a SEM is

235   based on the overall model fit to the data rather than individual causal pathways within the model

236   (Mitchell 1992, Grace 2006, Grace et al. 2010). Model fit is assessed by comparing expected and

237   observed covariance between predictor and response variables in the SEM using χ^2 tests

238   (Mitchell 1992, Grace 2006, Grace et al. 2010), and the SEM is considered consistent with the

239   data when expected and observed covariance of the SEM are not different. We fit the

240   hypothesized SEM (Fig. 2), including all causal and correlational pathways, and no removal or

241   addition of causal pathways occurred. Distance from the urban center was fitted as an exogenous

242   variable (i.e., independent variable that affects other variables but is not affected by other

243   variables), while all remaining variables were fitted as endogenous variables (i.e., variables

244   affected by the exogenous variable and that can affect other endogenous variables). Distance

245   from the urban center was fitted to account for extraneous sources of urbanization and

246   environmental variation not explained by the other predictors. All data were standardized by

247   scaling individual variables to a mean of 0 and standard deviation of 1 prior to analysis, and the

248   SEM was estimated using maximum likelihood with robust Satorra-Bentler scaled test statistics

249   (Satorra and Bentler 2001, Rosseel 2012).

250   Results of the SEM were reported as standardized path coefficients, which show the

251   direction and magnitude of causal pathways between variables and allow for comparison of the

252   strength of relationships within the SEM (Wright 1934, Mitchell 1992, Grace 2006). Direct

253   effects within the SEM are the standardized path coefficient associated with the causal pathway,

254   while indirect effects are quantified by multiplying each path coefficient linking one variable to

255   another within the SEM. For example, in the hypothesized SEM (Fig. 2), an indirect effect of

256   distance from the urban center on bulk soil N is quantified as the path coefficient of distance on

257   percent ISC (percent ISC ~ distance) times the path coefficient of percent ISC on bulk soil N

258   (bulk soil N ~ percent ISC). The sign (+ or −) of an indirect effect is the sign of the last causal

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259   pathway in the link. Indirect or compounding pathways cannot be calculated across correlational

260   pathways as the direction of the effect is not unidirectional (Wright 1934, Grace 2006).

261   The SEM was fitted and analyzed using the "lavaan" package (version 0.6-7; Rosseel

262   2012). All above analyses were conducted using R (version 4.0.2; R Core Team 2020) in the

263   RStudio environment (version 1.4.869; RStudio Team 2020), with data management and figure

264   creation facilitated using the "tidyverse" (version 1.3.0; Wickham et al. 2019). All data and R

265   code are deposited on Zenodo (https://doi.org/10.5281/zenodo.4459723).

266   **Results**

267   *Nodule density, white clover $\delta^{15}N$, and soil N*

268   Urbanization had effects on nodule density, white clover $\delta^{15}N$, and soil N, although the measure

269   and effect of urbanization varied among response variables. Nodule density increased with

270   increasing distance from the urban center ($\beta = 0.146$, SE = 0.066, P = 0.031, $R^2 = 0.147$, Table 1,

271   Fig. 3) and NDVI ($\beta = 0.152$, SE = 0.064, P = 0.021, $R^2 = 0.146$, Table 1, Fig. 3), and nodule

272   density decreased with increasing percent ISC ($\beta = -0.199$, SE = 0.063, P = 0.003, $R^2 = 0.151$,

273   Table 1, Fig. 3). These effects translated to an increase of (estimate $\pm$ SE) 0.006 ± 0.003 in

274   nodules per cm of root (i.e., nodule density) for each 1 km from the city centre, -0.004 ± 0.001

275   lower nodule density for each 1 percent increase in impervious surface cover, and 0.007 ± 0.003

276   greater nodule density for each 100 unit increase in NDVI (Supplementary material Appendix 1

277   Table A1). White clover $\delta^{15}N$ was strongly and non-linearly predicted by distance from the urban

278   center ($F = 10.893$, P < 0.001, $R^2 = 0.671$, deviance explained = 71.0%) but not percent ISC or

279   NDVI (Table 2, Fig. 4). White clover $\delta^{15}N$ was higher and positive at the urban (0-10 km) and

280   rural (40-47 km) extremes of the gradient, while white clover $\delta^{15}N$ was lower and negative for

281   the majority of the gradient (10-40 km, Fig. 4). Bulk soil N was predicted by distance from the

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282   urban center ($\chi^2 = 3.097$, P = 0.043, $R^2 = 0.079$, deviance explained = 11%; Supplementary

283   material Appendix 1 Table A2, Fig. A2), where bulk soil N decreased with increasing distance

284   from the urban center. Additionally, bulk soil N was weakly predicted by percent ISC ($\chi^2 =$

285   1.722, P = 0.098, $R^2 = 0.020$, deviance explained = 6%; Supplementary material Appendix 1

286   Table A2, Fig. A2), where bulk soil N increased with increasing percent ISC; bulk soil N was not

287   predicted by NDVI (Supplementary material Appendix 1 Table A2, Fig. A2); adjacent soil N

288   (i.e., immediately surrounding the plant) was only weakly predicted by percent ISC ($\chi^2 = 2.277$,

289   P = 0.070, $R^2 = 0.069$, deviance explained = 9%; Supplementary material Appendix 1 Table A2,

290   Fig. A2).

291   *Path analysis*

292   The SEM demonstrated that urbanization had direct and indirect effects on ecosystem structure

293   (i.e., soil N) and the white clover-rhizobium mutualism (Fig. 5). Our hypothesized SEM had

294   good fit between the predicted and observed covariance ($\chi^2 = 0.009$, df = 1, P = 0.926, Fig. 5).

295   Distance from the urban center had a direct negative relationship with white clover δ^{15}N: as the

296   distance from the urban center increased, white clover δ^{15}N decreased (path coefficient = −0.60,

297   SE = 0.16, P < 0.001). Bulk soil N decreased with increasing distance from the urban center

298   (path coefficient = −0.29, SE = 0.18, P = 0.099). Further effects of distance from the urban center

299   were mediated through percent ISC on nodule density (percent ISC ~ distance path coefficient =

300   −0.55, SE = 0.11, P < 0.001; nodule density ~ percent ISC path coefficient = −0.39, SE = 0.23, P

301   = 0.082; compound path coefficient = |−0.55| x −0.39 = −0.21). Although distance from the

302   urban center was positively related to NDVI (path coefficient = 0.63, SE = 0.08, P < 0.0001),

303   there were no further effects of NDVI in the SEM. Percent ISC and NDVI were negatively

304   correlated (path coefficient = −0.45, SE = 0.13, P = 0.001). Bulk soil N had a negative effect on

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305   white clover δ^{15}N (path coefficient = −0.23, SE = 0.11, P = 0.040), in which increased bulk soil

306   N decreased white clover δ^{15}N. Nodule density and adjacent soil N were negatively correlated

307   (path coefficient = −0.29, SE = 0.11, P = 0.010). Bulk soil N was directly linked to distance from

308   the urban center and then embedded in correlational pathways between adjacent soil N and

309   nodule density (Fig. 5). All path coefficients and associated measures of variation are provided in

310   the supplement (Supplementary material Appendix 1 Table A3).

311   **Discussion**

312   Our results show that urbanization alters the ecology of the white clover-rhizobium mutualism

313   and associated patterns in soil nutrients, with support for this conclusion from three key results.

314   First, we observed increased investment in nodules by white clover with decreasing impervious

315   surface cover, increasing NDVI, and greater distance from the urban center (Q1). Second, we

316   found that the source of nitrogen used by white clover varied along the urbanization gradient

317   (Q2). White clover δ^{15}N suggested acquisition of N through N-fixing rhizobia for the majority of

318   the urbanization gradient, although increased δ^{15}N at the urban and rural extremes suggested

319   direct uptake of N sources from the soil. Finally, we observed direct and indirect effects of

320   urbanization on ecosystem structure and white clover-rhizobium interactions (Q3). Urbanization

321   altered the landscape, changing the amount of green and impervious surface cover surrounding

322   each white clover population, with these changes cascading onto soil N, nodule density, and

323   white clover δ^{15}N. Given this evidence, our results suggest that urbanization alters the ecology of

324   the white clover-rhizobia mutualism through direct and indirect pathways.

325   *Effects of urbanization on the white clover-rhizobium mutualism*

326   Increasing urbanization is frequently associated with increased N deposition and enrichment

327   (Grimm et al. 2008, Zhang et al. 2012), and legumes are expected to have lower investment in

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328   rhizobia with greater N availability in the soil (Heath and Tiffin 2007, Heath et al. 2010, Regus

329   et al. 2017). Our results supported this prediction, whereby nodule density, a common measure

330   of investment in mutualistic interactions with rhizobia by host plants (Heath and Tiffin 2007,

331   Heath et al. 2010), increased with decreasing effects of urbanization; these results are supported

332   by other empirical studies in nonurban systems that found reduced investment in nodules with

333   increased N (Heath and Tiffin 2007, Heath et al. 2010, Lau et al. 2012). In our study, nodule

334   density was further influenced by the urban landscape and its effects on the local soil

335   environment. Increasing distance from the urban center was associated with negative effects on

336   bulk soil N and, while nodule density was also negatively related to impervious surface cover,

337   there were entangled dependencies between nodule density, bulk soil N, and adjacent soil N.

338   Given that soil N can regulate nodule formation and development (Streeter and Wong 1988,

339   Omrane and Chiurazzi 2009) and cause shifts in the cost-benefit balance of the white clover-

340   rhizobium mutualism (Lau et al. 2012, Weese et al. 2015, Regus et al. 2017), urban-driven

341   changes to local soil N is a likely driver linking urbanization to altered ecological dynamics in

342   the white clover-rhizobium mutualism.

343   In addition to nodule density, white clover $\delta^{15}N$ varied with urbanization. Comparing

344   relative $\delta^{15}N$ signatures allows for assessing and tracing the source of N used by plants (Högberg

345   1997, Robinson 2001, Craine et al. 2015). For the majority of the urbanization gradient, white

346   clover $\delta^{15}N$ was frequently bound between –1‰ and 0‰, suggesting that white clover primarily

347   acquired N through fixation by rhizobia but other sources of N were also used (Högberg 1997,

348   Craine et al. 2015). By contrast, populations at the urban and rural extremes of the gradient

349   exhibited high values of $\delta^{15}N$, which suggests decrease reliance on rhizobia for N and increased

350   uptake of N from the soil. A potential explanation for this pattern is the application of fertilizer

16

351   and management practices, which would increase white clover δ^{15}N (Trammell et al. 2016,

352   2020). Although we did not use experimental plant lines to determine the δ^{15}N signatures of

353   white clover relying solely on N fixation or on soil acquisition (Högberg 1997, Robinson 2001,

354   Craine et al. 2015), white clover acquiring N solely through fixation by rhizobia has a δ^{15}N of –

355   2‰ to –1‰ (Högberg 1997). Experimentally quantifying the contributions of N fixation and soil

356   uptake along the urbanization gradient would help elucidate the relative importance of these two

357   processes and pools of N. Our existing data show that urbanization is at least partially

358   responsible for variation in nodule density and white clover δ^{15}N due to shifts in the costs and

359   benefits of the rhizobia-plant host interaction.

360   *White clover-rhizobium-soil interactions*

361   Depleted δ^{15}N in legumes is associated with N fixation by rhizobia, and we expected increased

362   bulk soil N to lead to a decrease in white clover δ^{15}N, consistent with decreased fixation.

363   Contrary to our expectation, increased bulk soil N was associated with decreased white clover

364   δ^{15}N. While contrary to our predictions, increased N can stimulate nodulation, N fixation, and

365   other metabolic processes in rhizobia (Streeter 1985, Simonsen et al. 2015, Forrester and

366   Ashman 2018). Our observation of increased adjacent soil N with decreasing nodule density is

367   consistent with inhibition of nodulation due to high N (Streeter and Wong 1988, Omrane and

368   Chiurazzi 2009). Conversely, decreased adjacent soil N with increased nodule densities could

369   also suggest less beneficial rhizobia that fix less N are colonizing roots, which could reduce the

370   enrichment of soil N resulting from the white clover-rhizobium mutualism. Manipulative

371   experiments are needed to disentangle these two possibilities to understand the causal

372   explanation for our observed patterns.

17

373   Our expectation that lower white clover δ^{15}N would increase adjacent soil N was not

374   supported. While both distance from the urban center and bulk soil N negatively affected white

375   clover δ^{15}N, we did not observe a feedback from white clover δ^{15}N to adjacent soil N. In

376   contrast, nodule density was negatively correlated to adjacent soil N, despite predicting a

377   positive correlation. A potential explanation for this response is that white clover produced fewer

378   and larger nodules: larger nodules can convey greater benefits to white clover, increasing N

379   fixation and associated effects on soil N (Porter and Simms 2014, Gano-Cohen et al. 2020). We

380   only measured nodule count and density, so we were unable to test this hypothesis.

381   *Urbanization and soil nitrogen*

382   Urbanization had expected effects on soil N, but this was dependent on interactions between

383   white clover and rhizobium and their associated effects on soil and plant N. Bulk soil N was

384   greatest in urban areas, which was consistent with our predictions and observed patterns in other

385   studies (Pouyat et al. 2015, Regus et al. 2017, Trammell et al. 2020), and increased soil N is

386   likely a result of N deposition linked to urbanization (Grimm et al. 2008, Zhang et al. 2012,

387   Regus et al. 2017). Adjacent soil N showed a similar but weaker pattern to bulk soil N in

388   response to impervious surface cover. These results in combination with the SEM show that

389   local soil N is largely governed by exogenous N inputs (e.g., rain, synthetic fertilizer, pets) into

390   the surrounding soils and perhaps less influenced by modification of N pools by the white

391   clover-rhizobium mutualism. In contrast to soil N, white clover δ^{15}N showed a complex and non-

392   linear relationship with urbanization. Specifically, white clover δ^{15}N was lower and negative for

393   the majority of the urbanization gradient but highest and positive at the urban and rural extremes

394   of the gradient. Additional evidence from the SEM suggests that increased bulk soil N could

395   have stimulated N fixation, contrary to our expectation of lower white clover δ^{15}N with less soil

18

396   N. Taken together, these lines of evidence suggest that the effects of urbanization on the white

397   clover-rhizobium mutualism are complex, with local variation in soil N influencing dynamics

398   between white clover, rhizobium, and N.

399   We quantified total soil N to answer our focal questions for this study, but other measures

400   of soil N can be important to consider. For example, total N does not discriminate between

401   different types of N (e.g., NO_3, NO_2, and NH_3). Identifying the type and quantifying the relative

402   amounts of each species of N could provide a clearer link between urbanization and N

403   deposition, as different forms of N are important for both plant and rhizobia physiology and

404   metabolism (Wallsgrove et al. 1983). In addition to further investigation of soil N content, soil

405     $\delta^{15}N$ could integrate inputs, metabolic processes, and transformations of N in ecosystems

406   (Robinson 2001). For example, a typical fertilizer has a $\delta^{15}N$ around 0‰ and other

407   anthropogenic sources have enriched $\delta^{15}N$ signatures (Robinson 2001, Michener and Lajtha

408   2007), which can be reflected in the tissues of organisms utilizing these sources (Robinson 2001,

409   Trammell et al. 2016). Urban environments frequently have increased and less variable soil $\delta^{15}N$

410   relative to nearby nonurban or rural environments (Trammell et al. 2020); therefore, using soil

411     $\delta^{15}N$ could also help to identify a direct link from urbanization to changes in soil N and

412   ultimately legume-rhizobia mutualisms.

413   *Limitations*

414   Our study had several limitations that contextualize our conclusions. First, we focused on the

415   focal white clover-rhizobia mutualism without considering how other coexisting plants could

416   affect the focal mutualism and soil nutrient patterns. White clover was frequently collected in

417   patches of grass, especially *Poa annua*, and near other legumes, predominantly *Medicago*

418   *lupulina* and occasionally other *Trifolium* species (*T. pratense*, *T. hybridum*; DMS, personal

19

419　observation). White clover competes for space and nutrients with these other plants and this

420　competition could have affected the response of the focal mutualism to urbanization, especially if

421　plant species composition changed along the urbanization gradient as has been reported

422　elsewhere (Hope et al. 2003, Knapp et al. 2008, 2012). Other legumes might have also affected

423　the observed patterns in soil N, although we did explicitly avoid collecting soil from patches of

424　other legume species so this possibility is unlikely. Second, we did not investigate how the

425　microbial community beyond rhizobium in the soil and in the roots of white clover varies with

426　urbanization. Microbes are crucial for plant community assembly and responses to biotic and

427　abiotic stress (van der Heijden et al. 2008, Fitzpatrick et al. 2018). Additionally, the ostensibly

428　pairwise mutualistic interaction between white clover and rhizobia can be altered in the presence

429　of other bacteria or fungi (Heath and Tiffin 2007, García Parisi et al. 2015). With recent studies

430　documenting changes in microbial diversity and composition in response to urbanization (Xu et

431　al. 2014, Reese et al. 2016), it is plausible that variation in microbial communities in the mosaic

432　of urban environments could alter the ecological effects of the white clover-rhizobium

433　interaction and shift the cost-benefit balance of the mutualism (García Parisi et al. 2015,

434　Burghardt et al. 2018, Batstone et al. 2020). Notwithstanding these limitations, our study shows

435　that urbanization alters the ecological and ecosystem-level effects of the white clover-rhizobia

436　mutualism.

437　*Conclusion*

438　Our study represents an evaluation of the effects of urbanization on an ecologically-important

439　mutualism. To date, urban ecological research has principally evaluated species interactions by

440　focusing on plant-pollinator and plant-herbivore interactions (Youngsteadt et al. 2015, Aronson

441　et al. 2016, Harrison et al. 2018, Miles et al. 2019, Rivkin et al. 2020). We have extended this

442   field by investigating the mutualistic interaction between a plant (white clover) and its

443   mutualistic, microbial symbionts (rhizobia). White clover invested in more nodules and relied on

444   rhizobia for N fixation with less urbanization, and increased urbanization directly and negatively

445   affected investment in rhizobia by white clover, with soil N playing a critical role linking

446   urbanization to the mutualistic interaction between white clover and rhizobia. In conclusion, our

447   results demonstrate the direct and indirect effects of urbanization on the cost-benefit balance and

448   ecological consequences of a legume-rhizobium mutualism.

449   **Acknowledgements**

456   **Author Contributions**

457   DMS and MTJ conceived the study; DMS and MTJ conducted field sampling; DMS processed

458   samples in the lab; DMS performed statistical analyses with input from MTJ; DMS and MTJ

459   interpreted results; DMS led the writing of the manuscript with extensive contributions from

460   MTJ.

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631 **Figures (with captions)**

632

633 Figure 1: Map of the urbanization gradient in the Greater Toronto Area, ON, Canada displaying

634 all 49 sampling sites for the study. The inset displays an illustration of a typical white clover

635 individual sampled in the field, and the callout depicts a nodule attached to the root. Satellite

636 imagery was taken in 2018 and retrieved from Google. The illustration was provided by Kelly

637 Murray-Stoker.

638
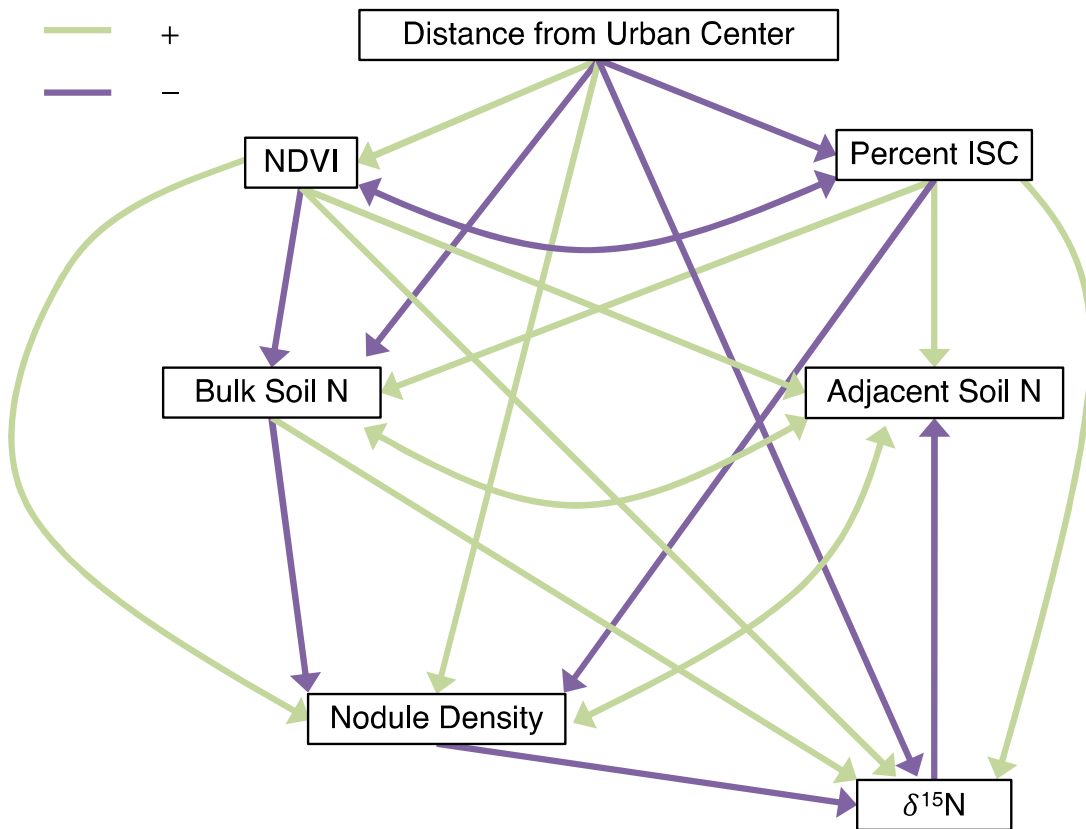
Figure 2: Hypothesized path model specifying causal and correlational pathways and the

associated direction of each relationship. The structural set of linear equations for each causal or

correlational pathway can be described by variables linking the response to the predictor (e.g.,

bulk soil N ~ percent ISC + NDVI + distance). Distance from the urban center was fitted to

account for extraneous sources of urbanization and environmental variation not explained by

other predictors. We hypothesized that changes to local environments in the urban landscape

would manifest in direct effects of urbanization (i.e., percent ISC and NDVI) on both soil N and

the legume-rhizobia mutualism (i.e., nodule density and white clover δ^{15}N), with indirect effects

of urbanization mediated through soil N.
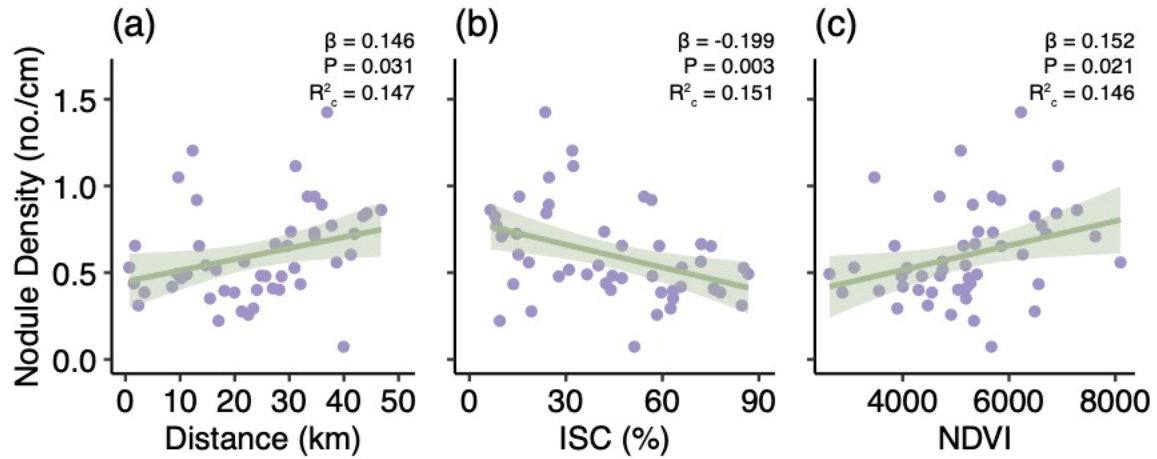
Figure 3: Plots of nodule density against (a) distance from the urban center (Distance), (b) percent impervious surface cover (ISC), and (c) normalized difference vegetation index (NDVI). Lines are lines-of-best-fit (± standard error) from a linear mixed effects model, with the parameter coefficient (β), P-value, and $R^2_{conditional}$ also provided.
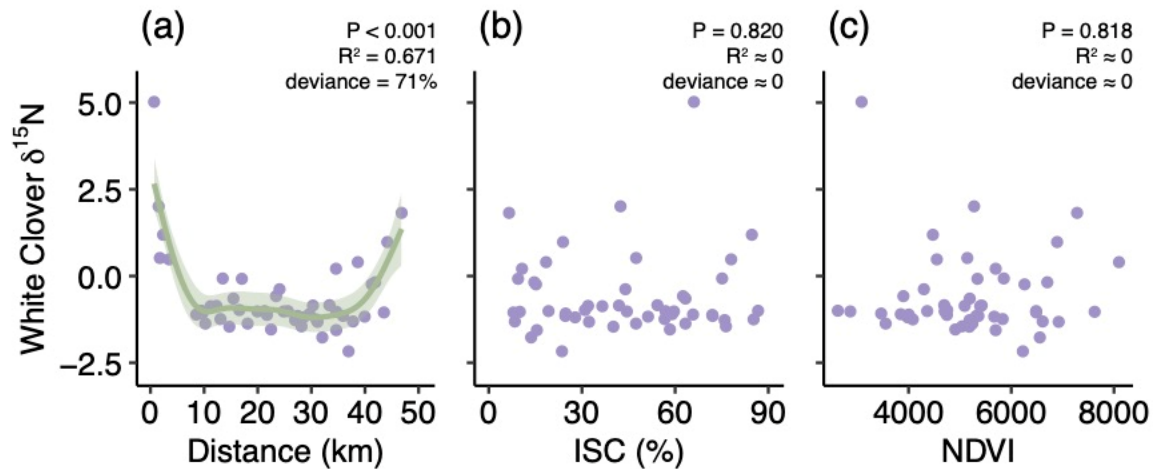
653

654   Figure 4: Plots of white clover δ^{15}N against (a) distance from the urban center (Distance), (b)

655   percent impervious surface cover (ISC), and (c) normalized difference vegetation index (NDVI).

656   Lines are smoothed curves (± standard error) from a generalized additive model, with the P-

657   value, R^2, and deviance explained (deviance) also provided. Percent ISC and NDVI were not

658   ecologically-relevant predictors of white clover δ^{15}N so lines are not displayed.
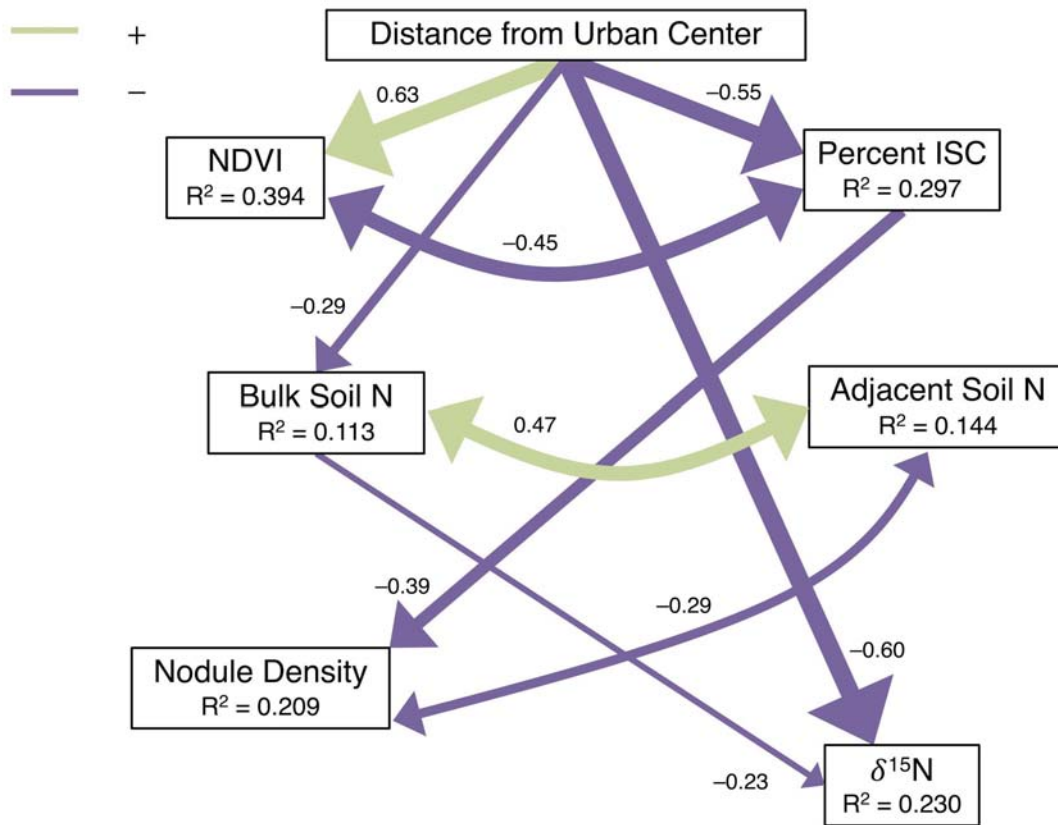
659

660   Figure 5: Path diagram showing the causal interactions between distance from the urban center,

661   percent impervious surface cover (ISC), normalized difference vegetation index (NDVI), soil N

662   (bulk and adjacent), nodule density, and white clover δ^{15}N. The model had good fit between the

663   predicted and observed covariance (χ^2 = 0.009, df = 1, P = 0.926). Lines represent influential

664   causal pathways in the model, with single-headed arrows indicating an unidirectional pathway

665   and double-headed arrows indicating a correlation between variables. Purple lines represent

666   positive pathways, while green lines represent negative pathways; non-influential pathways are

667   not displayed. Standardized path coefficients, which show the direction and magnitude of the

668   relationship between variables, are reported next to each line and line widths are scaled relative

669   to the magnitude of the path coefficients. The R^2 is reported for each endogenous variable.

670   **Tables (with captions)**

671   Table 1: Summary of the nodule density linear mixed-effects model comparing nodule density

672   against distance from the urban center (distance), percent impervious surface cover (ISC), and

673   normalized difference vegetation index (NDVI). We report coefficients (β, slope parameter),

674   standard errors of the coefficients (SE), numerator degrees-of-freedom (NDF), denominator

675   degrees-of-freedom (DDF) approximated following the Satterthwaite method, partial F-statistics

676   calculated from Type II sums-of-squares, P-values, and $R^2_{conditional}$.

Term	β	SE	NDF	DDF	F	P-value	$R^2_{condidtional}$
Distance	0.146	0.066	1	47.723	4.949	0.031	0.147
Percent ISC	−0.199	0.063	1	51.839	10.042	0.003	0.151
NDVI	0.152	0.064	1	55.268	5.640	0.021	0.146

677

678   Table 2: Summary of the white clover δ^{15}N generalized additive models (GAMs) comparing

679   white clover δ^{15}N against distance from the urban center (distance), percent impervious surface

680   cover (ISC), and NDVI. We report estimated degrees-of-freedom (EDF), which can differ from 1

681   because the values are penalized for smoothed parameters; an EDF = 1 would suggest a linear

682   relationship. Reference degrees-of-freedom (REF.df) are used for calculating F-statistics and P-

683   values for each smoothed term. We also report the R^2 and deviance explained for each model, as

684   well as the χ^2 statistic associated with the smooth term.

Term	EDF	REF.df	F	P-value	R^2	Deviance Explained
Distance	5.654	9	10.893	< 0.001	0.671	71%
Percent ISC	< 0.001	9	< 0.001	0.820	$\cong$ 0.000	$\cong$ 0%
NDVI	< 0.001	9	< 0.001	0.818	$\cong$ 0.000	$\cong$ 0%

685   Note: As the white clover δ^{15}N GAMs were fitted to a Gaussian error distribution, F-statistics
686   were calculated instead of χ^2-statistics.