1   Title page

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3   **Soil enzymes associated with carbon and nitrogen cycling in invaded and native secondary forests of**

4   **northwestern Argentina**

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18

19   **Abstract**
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21   *Background and Aims* Alien success has frequently been associated with changes in the concentrations of soil

22   nutrients. We aim to investigate the effects of plant invasion on soil nutrients, potential enzyme activity and

23   litter elemental composition and stoichiometry.

24   *Methods* We compared stands of secondary forest invaded by *Ligustrum lucidum* and those dominated by

25   natives, and performed litter and soil chemical analyses on 3 native and 2 exotic tree species.

26   *Results* Soils of invaded sites had 20% and 30% increase in β-glucosidase and alkaline phosphatase activity,

27   higher Olsen-phosphorus (P) and potassium (K) concentrations and lower nitrogen (N) concentration and N:P,

28   N:K and ammonium:Olsen-P ratios. Invaded and non-invaded sites differed in their overall nutrient

29   composition and enzyme activity. Natives and exotics differed in nine of the 16 litter elemental composition

30   and stoichiometry variables analyzed.

31   *Conclusions* The low N:P ratio in litter, the decrease in soil N in invaded stands and the low N concentration

32   of exotics suggest that N is the limiting nutrient and that exotic success is related to higher N uptake and use

33   efficiency. The higher investment in the acquisition of soil resources, higher nutrient uptake and use

34   efficiency of limiting nutrients contribute to the success of exotics in this subtropical forest.

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37   Keywords: soil enzyme, nitrogen, phosphorus, litter*,* stoichiometry*, Ligustrum lucidum*

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Introduction

Litter decomposition in terrestrial ecosystems is largely the result of the activity of soil enzymes from communities of bacteria and fungi. This activity is in turn conditioned by physical factors (e.g. temperature, soil humidity and soil pH) and litter characteristics (Sinsabaugh et al. 1993; Kourtev et al. 2002, Tharayil et al. 2013). Vegetational cover modifies both environmental and litter characteristics and consequently the abundance, diversity and activity of microbial communities (van der Putten et al. 2007). Importantly, invasions by exotic species represent a rapid change in community composition and are thus likely to affect the control of litter decomposition and, in turn, the concentrations of soil nutrients and stoichiometric relationships (Ehrenfeld et al. 2001; Ehrenfeld 2003; Allison and Vitousek 2004; Joanisse et al. 2007; Flory and Clay 2010).

β-glucosidases, proteases, ureases and phosphatases are the most important soil enzymes involved in the carbon (C), nitrogen (N) and phosphorus (P) cycles in the soil (Sardans and Peñuelas 2005; Sardans et al. 2008). β-glucosidases break down labile cellulose and related carbohydrates with 1-4 glucosidic bonds, degrading plant cell walls and thus contributing to the firsts phases of plant cell tissues decomposition, which then facilitate the activities of other enzymes such as proteases and phosphatases (Debosz et al. 1999; Sardans et al. 2008; Stege et al. 2010). Proteases are involved in the first phase of N mineralization by hydrolyzing the peptide bonds of amino acids. Ureases regulate the release of $N-NH_4$ by urea hydrolysis, which is essential in the chain of hydrolysis of amino compounds. Phosphatases regulate the hydrolysis of O-P bonds, releasing orthophosphate from organic matter (Sardans and Peñuelas 2005).

Water and resource limitation partly constrain the production and activities of soil enzymes (Criquet et al. 2004; Allison and Vitousek 2005; Sardans and Peñuelas 2010). Plant invasions generate changes in a forest's capacity to take up and use soil resources (water and nutrients) by both introducing a new species and by modifying the capacities of native species, hence invasions can modify soil nutrient and water contents, that in turn may affect the activities of soil enzymes (Kolb et al. 2002; Ehrenfeld 2003; Allison and Vitousek 2004; Joanisse et al. 2007). Several studies have described the effects of plant invasion on the concentrations of soil nutrients (Evans et al. 2001; Allison and Vitousek 2004) but little is known about its effects on soil stoichiometry and the mechanisms underlying these changes (Sardans and Peñuelas 2012). The objective of the present study was to discern the impacts of plant invasion on the activities of soil enzymes and on soil and litter nutrient concentrations and stoichiometry by studying forest stands in sites heavily invaded by *Ligustrum lucidum* (Oleaceae) and in sites dominated by native species in a montane forest of northwestern Argentina.

L. lucidum is an evergreen, shade-tolerant tree with high resprouting capacity, survival and growth rates (Easdale et al. 2007). Invasion by *Ligustrum* modifies soil moisture, light availability, litter depth and plant diversity (Lichstein et al. 2004; Aragón et al. 2014; Ayup et al. 2014). The litter of this invasive species has a significantly higher decomposition rate than those of three of the most common native species in the area (Aragón et al. 2014). Here we analyzed, for the first time to the best of our knowledge, the effects of plant invasion on the elemental composition and stoichiometry of both soil and litter and on the activities of soil enzymes. Given *Ligustrum* high growth rate, we hypothesize a potentially high demand of resources. In

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77　　addition to *Ligustrum* demands, the environmental conditions (i.e., lower soil moisture and light availability)

78　　present in invaded stands, could lead to increases in soil-enzyme concentration in order to compensate for the

79　　unfavorable conditions. All these in turn, could result in increases in soil and litter nutrient concentrations

80　　and in changes in soil and litter stoichiometries.

81

82　　**Methods**

83　　**Study Site**

84　　The study was conducted in the lower montane forest of Sierra de San Javier, Tucumán, Argentina (26º 70' S,

85　　65º 35' W) at approximately 800 m a.s.l. The area represents the southern-most limit of the subtropical

86　　Andean montane forest (also known as *Yungas*), which extends from Bolivia to the province of Catamarca in

87　　Argentina (Cabrera and Willink 1980; Grau and Brown 1995). Average annual precipitation ranges from 1300

88　　to 1500 mm distributed in a monsoonal regime with dry winters and wet summers (Bianchi and Yañez, 1992).

89　　The mean annual temperature is 18 ℃, with frosts occurring from June to August. Most of the Sierra de San

90　　Javier piedmont was cleared for crop production and grazing during the early twentieth century (Grau and

91　　Brown 1995; Brown et al. 2001), but many cleared areas were abandoned in the last two decades and

92　　currently have forests at different stages of regeneration (Grau and Aide 2007; Grau et al. 2008). Many of

93　　these secondary forest stands are colonized by several exotic species (Grau and Aragón 2000), but *L. lucidum*

94　　is by far the most abundant exotic species and the only one that forms monodominant forest stands in this

95　　area.

96

97　　**Studied species**

98　　　　We included five tree species in this study: two exotics (*L. lucidum* and *Morus* sp.) and three natives

99　　(*Cinnamomum porphyrium, Cupania vernalis and Myrsine laetevirens*). *L. lucidum* is an Asian tree that

100　　colonizes areas of varying land-use histories and ages. It is more abundant in secondary forest stands but also

101　　grows in openings in old-growth forests (Aragón and Morales 2003). *L. lucidum* is evergreen, shade tolerant

102　　and has a high growth rate (Easdale et al. 2007). Importantly, its distribution is expected to expand in the near

103　　future (Grau et al. 2008). *Morus* sp. is also of Asian origin, but unlike *Ligustrum*, it is deciduous and shade

104　　intolerant (Grau et al. 1997; Easdale et al. 2007). *Morus* sp. is a fast-growing species as well and is most

105　　abundant on the edges of young secondary forest stands that were reclaimed from citrus orchards.

106　　　　The native species in this study are all late successional and bird dispersed, with relatively high growth

107　　rates compared to the other native species in this area (these species are among the 10 fastest-growing species

108　　among a set of 29 species studied by Easdale et al. (2007)). They are also among the most abundant in the

109　　canopy or subcanopy strata (Grau et al. 1997; Easdale et al. 2007). *C. porphyrium* is a semi-deciduous, tall,

110　　shade-tolerant tree abundant in the canopies of secondary and old-growth forests (Grau et al. 1997; Easdale et

111　　al. 2007). *C. vernalis* has similar life-history characteristics, but it integrates into the subcanopy stratum. Its

112　　saplings account for approximately 70% of the saplings in native and invaded forest understories (Grau et al.

113　　1997; Lichstein et al. 2004). *M. laetevirens* is an evergreen tree, of intermediate height (it sometimes

114　　integrates into the canopy) and has the highest growth rate among the three native species studied (Easdale et

115   al. 2007). Unlike the other species, *Myrsine* bears fruit during the winter, partially coinciding with *L. lucidum*

116   fructification. The morphological and demographic characteristics of *Myrsine* are also more similar to those

117   of *Ligustrum* (e.g. maximum growth rate, growth in well-lit conditions and density in secondary forests)

118   (Easdale et al. 2007).

119

120   **Experimental design and sampling**

121   To evaluate the activities of soil enzymes in native and invaded forests, we used a paired design with five

122   invaded-native stand pairs .We considered as invaded those stands which had *L. lucidum* as the dominant

123   species in the canopy and occurring at densities higher than 500 ind./ha. Native stands were dominated by *C.*

124   *porphyrium*, *Blepharocalix salicifolius* and *C. vernalis* among others, and even though some individuals of *L.*

125   *lucidum* were present, especially as saplings, they could be considered rare. Even though *Morus* sp is

126   abundant in many secondary patches, it does not form mono-dominant stands. For this reason, we only

127   considered two forest types: stands invaded by *Ligustrum,* and stands dominated by native species.

128   Importantly, native and exotic species co-occur in the different stands but at very dis-similar abundance. For

129   more details about species composition and stands characteristics see Grau et al. 1997; Aragón and Morales

130   2003 and Easdale et al. 2007. Within each pair, the stands were similar in age (between 30 and 50 years of

131   succession), altitude (between 550 and 700 m), slope and soil type (typically hapludoll with loam sandy

132   texture with 50-30-20 % of loam, sand and clay respectively) and were larger than 2 ha. Pairs were selected

133   based on the greatest similarity in age and the smallest geographic separation (from 200 and 500 m between

134   the members of each pair). For more details about the location of the stands in the field see Aragón et al.

135   (2014). We established a 3 x 3 m plot in each stand, avoiding edges and gaps in the canopy.

136   Three soil cores (diameter 6 cm) from the top 10 cm of the soil profile were collected from each plot of

137   the five invaded/native pairs in April 2012. Each soil sample was kept at approximately 5 ºC until analyzed.

138   In the laboratory, we first sieved the soil through a 2-mm mesh and then analyzed the two fractions for soil-

139   enzyme activity and concentrations of C and N and the main nutrients Ca, Fe, Mg, Mn, and Na. For the soil

140   analyses (i.e., enzymes and nutrients) five pairs of stands (invaded and non-invaded forests in each pair) in 5

141   different sites were considered to account for the potential site to site variability. Each pair was taken as a

142   block (5 repetitions), and forest types (two levels) as treatment (fixed factor).

143   The litterfall of 3-8 individuals of each of the five studied species was collected in invaded and native sites

144   between May and September 2011 into plastic bags suspended underneath each plant. Leaves were air-dried

145   for 3-5 days and stored in open paper bags until further analysis.

146

147   **Chemical analyses**

148   **Activities of soil enzymes**

149   To determine *β*-glucosidase activity, we incubated 5 g of soil for 3 h at 37 ºC with acetate buffer (2 M, pH

150   6.2 diluted in 1000 ml of distilled water) and with salicin (*β*-glucosido-saligenin) as a substrate (Tabatabai

151   1994). The solutions were filtered (Millipore 0.45-μm HA nitrocellulose filter) and the saligenin released

152   from the substrate was determined colorimetrically after coloring with 2,6-dibromomchinon-4-chloroimide in

153   a borate buffer (0.2 M, pH 10). At pHs above 9, saligenin forms a blue indophenol dye with 2,6-

154   dibromchinon-4-chloroimide, which was then measured at 578 nm with a Helios α spectrophotometer

155   (Thermo Scientific, Waltham, MA, USA) against the reagent blank. We calculated the saligenin content by

156   referring to a calibration curve obtained with standards containing 0, 10, 20, 50 and 100 μg of saligenin per

157   ml. *β*-glucosidase activity was expressed as μg of saligenin released per gram of soil per hour. For the

158   analyses of all enzymes we first dried the soil samples by freezing in order to prevent protein damage by heat.

159   To determine protease activity, we used the method of Ladd et al. (1976) using casein as substrate.

160   Briefly, 5 mL of substrate solution (casein 2%, w/w) was added to 1 g of soil sample. We added 5 mL of Tris

161   (Tris-hydroxymethyl-aminomethane) buffer (0.05 M, pH 8.1) and then incubated for 2 h at 50 ℃. After

162   incubation, the remaining substrate was precipitated with trichloroacetic acid. Thereafter, samples and

163   controls were filtered immediately. For photometric analysis, 5 mL of the filtrate was added to 7.5 mL of

164   alkali reagent in a test tube, mixed well, 5 mL of Folin-Ciocalteu's phenol reagent were added, and mixed

165   again. Alkali reagent is a mix of three solutions: a) 50 g of sodium carbonate, 60 mL of 0.1 M NaOH in 600

166   mL of distilled water (1000 mL); b) 5g of copper sulfate pentahydrate in 1000 mL of distilled water (20 mL);

167   and c) 10 g of sodium potassium tartate in 1000 mL of distilled water (20 mL). Before colorimetric

168   measurement, the samples, controls and standards were filtered (Millipore 0.45-μm HA nitrocellulose filter)

169   to prevent interference from the precipitates formed by the casein reaction products. The solutions were then

170   allowed to stand at room temperature for exactly 90 min for color development. We measured the extinction

171   at 700 nm with the spectrophotometer against the reagent blank and calculated the tyrosine content by

172   referring to a calibration curve obtained with standards containing 0, 100, 250, 1000 and 1500 μg of tyrosine

173   per ml. Protease activity was expressed as μg tyrosine per gram of soil per hour.

174   We used the Kandeler and Gerber (1988) method for determining urease activity. An aqueous (controls) or

175   a buffered urea solution (samples) was added to 5 g of soil sample and incubated for 2 h at 37 °C. Released

176   ammonium (NH_4^+) was extracted with 2 mol L^{-1} KCl and quantified by a modified Berthelot reaction

177   (Schinner et al. 1996). The solutions were shaken for 30 min and filtered (Millipore 0.45-μm HA

178   nitrocellulose filter) to prevent interference from possible precipitates. The determination was based on the

179   reaction of sodium salicylate with NH_3 in the presence of sodium dichloroisocyanurate, which forms a green

180   complex under alkaline pH conditions. The extinction was measured at 690 nm against the reagent blank.

181   Sodium nitroprusside was used as a catalyst to increase the sensitivity of the method approximately 10-fold.

182   We calculated the NH_4^+ content by referring to a calibration curve obtained with standards containing 0, 1,

183   1.5, 2, and 2.5 mg NH_4^+ L^{-1}. Urease activity was expressed as μg NH_4^+ released per gram of soil per hour.

184   Phosphatase activity was determined by adding 4 mL of THAM solution (Tris-hydroxymethyl-

185   aminomethane with citric, maleic and boric acids), buffer (tris hydroxymethil aminomethane, maleic acid,

186   citric acid monohydrate and boric acid in 500 mL of 1 M NaOH, at pH 6.5 for acid phosphatase assays or pH

187   11 for alkaline phosphatase assays) and 1 mL of p-nitrophenyl phosphate solution (as a substrate) prepared in

188   the same buffer to 1 g of soil in a flask. We then swirled the flask for a few seconds to mix the contents. The

189   stoppered flask was incubated at 37 ℃ for 1 h, and then1 mL of 0.5 M $CaCl_2$ and 4 mL of 0.5 M NaOH were

190 added. The flask was again swirled for a few seconds to stop the reaction. The solution was filtered as above

191 to prevent the appearance of possible precipitates. The fading of the intensity of the yellow color in the

192 calibration standards, samples and controls was measured at 398 nm against the reagent blank. We calculated

193 the p-nitrophenol content by referring to a calibration curve obtained with standards containing 0, 10, 20, 30,

194 40 and 50 ppm of p-nitrophenol. Phosphatase activity was expressed as μg p-nitrophenol per gram of soil per

195 hour.

196

197 **Chemical analyses of litter and soil**

198 For chemical analyses of foliar tissue, leaves were dried in an oven at 60 ºC to a constant weight and then

199 ground in a CYCLOTEC 1093 (Foss Tecator, Hoganas, Sweden) and stored in desiccators until analysis. C

200 and N contents were determined from 0.7 mg of pulverized dried sample by combustion coupled to gas

201 chromatography in an Elemental Analyzer CHNS Eurovector 3011 Thermo Electron Gas Chromatograph

202 model NA 2100 (C.E. Instruments-Thermo Electron, Milan, Italy). For the other nutrients (Ca, Fe, Mg, Mn, S,

203 P, K and Na), 0.25 g of pulverized dried sample was diluted with the acid mixture HNO_3 (60%) and H_2O_2

204 (30% w/w) and digested in a MARSXpress microwave system (CEM, Matthews, NC, USA). The digested

205 solutions were brought to a final volume of 50 mL with ultra pure water and at 1% HNO_3. Blank solutions (5

206 mL of HNO_3 with 2 mL H_2O_2 without any sample biomass) were regularly analyzed. After digestion, the

207 concentrations of Ca, Fe, Mg, Mn, S, P, K and Na were analyzed with an Optima 4300DV ICP-OES (Optical

208 Emission Spectrometer for Inductively Coupled Plasma, Perkin-Elmer, Waltham, MA, USA). To assess the

209 accuracy of the biomass digestion and analytical procedures, we used certified biomass NIST 1573a (tomato

210 leaf) standards. To analyze the soil samples, we followed the same protocols used for the foliar tissues, but we

211 filtered the samples with a 0.45 μm microfilter.

212 In addition to C, N and the other nutrients, we also determined immediately available P (Olsen P) and

213 ammonium. The P available to plants in the soil was determined by Olsen's method (Olsen et al. 1954). This

214 method measures the relative availability of orthophosphate (PO_4-P) extracted in 0.5 M $NaHCO_3$ adjusted to

215 pH 8.5. Phosphorus content in 1 g of soil was determined spectrophotometrically at 882 nm in an acidic

216 medium of 0.24 M H_2SO_4 by reacting with ammonium molybdate using ascorbic acid as a reductant in the

217 presence of antimony potassium tartrate. Phosphorus concentration was determined using a calibration curve

218 built with seven solutions containing 0.0, 0.25, 0.50, 0.75, 1, 2 and 3 mg L^{-1} PO_4-P.

219 To determine the ammonium concentration in the soil, we used a procedure similar to that described for

220 urea. Released ammonium (NH_4^+) was extracted with a 2 mol L^{-1} KCl solution added to 1 g of dried soil. The

221 determination was based on the reaction of sodium salicylate with NH_3 in the presence of sodium

222 dichloroisocyanurate and using sodium nitroprusside as a catalyst. The measurement was performed at 690

223 nm using a calibration curve similar to that described for urea.

224 **Statistical Analyses**

225 We analyzed soil-enzyme activities, ammonium and Olsen-P concentrations and ammonium:Olsen-P

226 ratios in the soil with two-way ANOVAs, with forest types as treatments (invaded and native) and the five

227 pairs of sites as blocks (repetitions). In the case of leaf litter, we first considered two sources of variation:

228 forest types (litter collected from individuals occurring in invaded or in native stands: two levels) and species

229 (five levels). Secondly, we explored the differences only among species, and lastly we grouped species in

230 native (three species) and exotics (two species) (i.e., two levels).

231 We performed principal component analyses (PCA) using correlation matrices with 20 variables for the

232 soil (five enzymes, 12 nutrients including ammonium and Olsen P and three ratios) and 13 variables for the

233 litter (10 nutrients and three ratios). The differences among treatments (invaded and native for the soil and

234 five species for the litter) in the variable distribution in the multidimensional space defined by the PCA were

235 tested using an ANOVA for the scores of sites (n=10) or individuals (10 of each species). In addition, to

236 specifically evaluate if the invasion of *L. lucidum* changed over all studied soil variables, nutrient

237 compositions and potential enzyme activities in the soil and nutrient composition in the litter, we used a

238 multi-response permutation procedure (MRPP) based on Euclidean distance (Biondini et al. 1985). MRPP is a

239 non-parametric procedure for testing the null hypothesis of differences between groups or entities. It provides

240 a statistic, δ, that is the weighted mean of within-group distances and is associated with a *p*-value that

241 indicates the likelihood of δ being equal to or smaller than that observed by chance (McCune and Mefford

242 1999). For the soils, we used a block-MRPP considering the five pairs of invaded/native sites, and for the

243 litter, we grouped data from all species and considered forest type as the only source of variation. All the

244 multivariate analyses were performed with the PC-ORD 5 program (McCune and Mefford 1999). In addition,

245 for the litter, we performed univariate ANOVAs with 16 variables (10 nutrients and six ratios) and with

246 species status (native or exotic) as the classification factor. We also made multiple comparisons among

247 species (five levels).

248

Results

250 **Enzymes and nutrients in the soil**

251 Invaded sites had an approximately 20% increase in *β*-glucosidase activity and a 30% increase in alkaline

252 phosphatase activity. The soil of invaded sites had a lower N concentration (both as NH_4^+ and total N), higher

253 Olsen-P concentration (40%) and lower ammonium:Olsen-P ratio (67%) (Table 1). Invaded stands also had a

254 higher K concentration (13%) and lower N:P and N:K ratios (25% and 39%, respectively) (Table 2). The first

255 two axes of the PCA ordination that considered 20 variables explained 66% of the total variance (Figure 1).

256 The first axis (explaining 42.8% of the total variance) was related to the variability among blocks, and the

257 second axis (explaining 23.4% of the total variance) separated invaded from native stands (ANOVA with

258 PCA scores: Axis I: *F* for blocks = 7.1, *p* = 0.04; Axis II: *F* for forest type = 17.27, *p* = 0.01). Invaded sites

259 were associated with higher enzyme activities and Olsen-P and K concentrations, while the

260 ammonium:Olsen-P ratio and Mg and Fe concentrations were higher in the native sites (Figure 1). This

261 pattern was reinforced by the MRPP results. Overall, the distances within invaded and native stands were

262 smaller than expected by chance (chance-corrected within group agreement = 0.19, *p* = 0.03), so we can

263 conclude that the invaded and native sites significantly differed in their nutrient compositions and enzyme

264 activities.

Chemical analyses of leaf litter

266   The litter of the different species had similar elemental compositions in the highly invaded stands and the

267 native stands (Table A1, appendix). This pattern was evident from both the univariate and the MRPP analyses

268 (chance-corrected within group agreement = 0.01, p = 0.15). In contrast, the litter of the different species had

269 several differences in elemental composition and stoichiometry. The native and exotic species as groups

270 differed in nine of the 16 studied variables (Table 3). Exotics had higher concentrations of Ca and K and

271 lower concentrations of Mn, P, C and N. Differences among species were also evident through the PCA

272 ordination, especially between the natives *Cinnamomum* and *Cupania* and the other studied species, whereas

273 the two exotics and the native *Myrsine* all shared some characteristics (Figure 2a). The first three axes of the

274 PCA ordination explained 63% of the total variability. Two groups could be distinguished along the first axis

275 (29.7% of the variability) (Figure. 2a, letters on the top): exotics and *Myrsine* on the positive side and

276 *Cinnamomum* and *Cupania* on the negative side. Exotics and *Myrsine* had higher concentrations of Ca and K

277 and lower N:K and P:K ratios compared to the remaining two species (Figure 2b). The second axis (20.4% of

278 the variability) separated three groups: *Cupania* with litter concentrations of Mg, Fe, S, Na, P and Mn higher

279 than those for *Cinnamomum* and exotics and *Myrsine* with intermediate characteristics (Fig. 2a letters on the

280 right). The third axis (13.6% of the variability) separated, although to a minor degree, exotics (*Ligustrum* and

281 *Morus*) from *Myrsine* (Figure 3a letters on the right), and overall, the invasive from the native species as

282 groups (Fig 3 a, right arrows) Exotics had lower N concentrations and higher Ca concentrations (Figure 3b)

283 (Table 3).

284

Discussion

Effects of invasion on soil and litter elemental composition and stoichiometry

287   The soils of invaded stands had lower N concentrations (both as NH_4^+ and total N), higher extractable K

288 concentrations, higher plant-available P concentrations and a general trend toward higher soil-enzyme

289 activities. All these data suggest higher rates of nutrient cycling, mainly of N, in the invaded stands. These

290 results are in agreement with several studies reporting significant impacts of alien plants on the availability of

291 soil nutrients, decomposition of organic matter, nutrient cycling and soil stoichiometry (Sardans and Peñuelas

292 2012, Tharayil et al. 2013). In a recent review of the effect of invasive plants on N and P availability, C:N:P

293 ratios of soils and rates of soil decomposition, mineralization and nutrient cycling from 65 studies conducted

294 in environments with unclear limitations of nutrients (except some conducted mainly in arid and semiarid

295 areas of the USA), 48 studies reported increases in the availability of soil nutrients, 14 reported decreases and

296 three were inconclusive (Sardans and Peñuelas 2012). Most of the 14 studies reporting decreases in soil

297 nutrients were studies with *Bromus tectorum*, an invasive grass of semiarid areas of the USA (but see Castro-

298 Díez et al. 2013), indicating that most invasions in nutrient-rich ecosystems tend to increase the availability of

299 soil nutrients and hence to increase nutrient cycling. In forests invaded by *Ligustrum*, however, we found a

300 decrease in nutrients, particularly N. Importantly, less is known of the effect of plant invasions on nutrient

301 imbalances. By investigating litter and soil stoichiometry and soil-enzyme activity in Argentine subtropical

302 forests, we have shown that the success of invasive plants is associated with an overall change in soil nutrient

303 composition and function, mainly by a decrease in the most limiting nutrient, here N, increasing the

304 imbalances with other nutrients such as P, which tends to increase its availability in the soil (i.e. decreasing

305 N:P ratio). This result sheds light on the role of N:P ratios in plant invasions, which has remained

306 inconclusive (Sardans and Peñuelas 2012).

307 Even though we cannot effectively discriminate plant available N with our data, there are several

308 indications that N appears to be the most limiting element in this area. The global litter N:P ratio is 46:1

309 ($\pm$3:1) (McGroddy et al. 2004) but was 7.4:1 ($\pm$ 1:1) in our study. Moreover, the lower soil water content in

310 invaded stands was associated with lower total N concentrations, suggesting that invasive success is related to

311 higher N uptake. In contrast, P in these soils did not appear to be limiting for plant growth. Invaded stands had

312 higher Olsen-P concentrations related to higher soil-enzyme activity and generally faster cycling of water and

313 elements. This area receives an annual precipitation above 1000 mm, so water is not likely an important

314 limiting factor. All these data thus strongly suggest that N is the limiting nutrient and that invasive plant

315 success depends on a large capacity of N uptake, reduced soil N availability and a higher limitation of N. The

316 current literature suggests that alien invasion in nutrient-rich environments frequently favors plant species

317 with high rates of photosynthesis and growth (Baruch and Goldstein 1999; Leishman et al. 2007; Mozdzer

318 and Zieman 2010; Feng et al. 2011), low costs of foliar construction (Nagel and Griffin 2001; Feng et al.

319 2007; González et al. 2010), large investments of N in photosynthetic production (Ehrenfeld 2003; Xu et al.

320 2007; Feng 2008; Shen et al. 2011), higher capacities of nutrient uptake (Zabinsky et al. 2002; Harrington et

321 al. 2004; Blank and Sforza 2007; Feng 2008; Blank 2010; Hewins and Hyatt 2010; Leffler et al. 2011; Peng et

322 al. 2011) and high levels of plasticity in the acquisition of resources as a function of pulses in nutrient

323 availability (Leffler et al. 2011). These factors indicate that higher efficiency in nutrient uptake and foliar

324 traits enabling rapid rates of growth (Leishman et al. 2007; Zabinsky et al. 2002) will help invading species to

325 succeed when resources are not limited (Bray et al. 2003; Funk and Vitousek 2007; Shah et al. 2009).

326 Similar to our findings, Lichstein et al. (2004) reported that the percentage of soil organic matter was

327 negatively correlated with *Ligustrum* basal area. That study hypothesized that this pattern could be associated

328 with litter quality or with the rapid growth rate in *Ligustrum* and presumably with rapid nutrient uptake. Our

329 results support this hypothesis, because *Ligustrum* has rather low concentrations of N and C in its leaf litter.

330 Higher soil concentrations of extractable K were associated with higher K concentrations in the leaf litter

331 of invasive species and of *Myrsine*. Previous studies have demonstrated that invaded stands have less soil

332 moisture (Aragón et al. 2014), suggesting a higher water uptake with the increasing abundance of invasive

333 species (Gerlach 2000; Levine et al. 2003; Holmes et al. 2005; van Wilgen et al. 2008) that in turn decreases

334 runoff (Dye and Jarmain 2004; Gorgens and van Wilgen et al. 2004; Holmes et al. 2005; van Wilgen et al.

335 2008). Invasive plants can thus prevent the loss of K by increasing water uptake, decreasing runoff and taking

336 up more K. Higher K uptake should be correlated with higher concentrations of K in the litter and, in general,

337 with faster plant-soil-plant K cycling. We have observed a marginally significant higher K concentration in
338 the litter of invaded stands, especially in *L. lucidum* litter, coinciding with the lower soil moisture observed in
339 these stands (Aragón et al. 2014). Importantly, ecosystem-level impacts of an invasive species depend on the
340 combination of traits that determine its per capita effect, together with its abundance (Drenovsky et al. 2012).
341 *Ligustrum* dominance must be taken into account when assessing its potential impacts at community scale
342 (Aragon et al. 2014).
343
344

345 **Effect of invasion on potential soil enzyme activity**
346   The observed increases in phosphatase and β-glucosidase activities reinforce the general results of soil and
347 litter composition, suggesting that plant invasion accelerates nutrient uptake and nutrient-cycling rates. The
348 success of invasive plants in this semi-wet, sub-tropical ecosystem is thus associated with faster water and
349 nutrient cycles, higher soil-enzyme activities, lower concentrations of some nutrients in the soil and with
350 higher levels of nutrients in stand biomasses and faster growth. These results also suggest cascade effects,
351 because higher soil-enzyme activity and higher N uptake can be related to low ammonium:Olsen-P ratios in
352 soils. Lower soil N:P ratios impact soil trophic webs, increasing the abundance of rapidly growing microbial
353 groups (Elser et al. 2003; Fierer et al. 2007), and may be associated with the observed increase in the potential
354 activity of some soil enzymes. Microbes adjust their extracellular release of soil enzymes to maximize the
355 mobilization of substrates rich in their limiting element (Wallenstein and Weintraub 2008: Burns et al. 2013).
356 The higher C:N ratio of the soil in invaded stands is related to the higher levels of potential soil β-glucosidase
357 activity. This enzyme catalyzes the first steps of the hydrolysis of large C-chains and is critical for the further
358 action of enzymes linked to N and P mineralization (Debosz et al. 1999; Stege et al. 2010).Moreover, since β-
359 glucosidase is involved in cellulose catabolism, more abundant and extended litter production in invaded
360 stands (*Ligustrum* is a perennial species which has an extended period of litter fall) (Aragón 2000; Easdale
361 2006) may also explain the higher activity of this enzyme in this type of forest.
362   The maximum potential enzyme activity depends of the density of active enzymes present in soil. Our
363 results thus show a higher density of soil enzymes, consistent with the idea that invaded stands have a higher
364 investment in the production of soil enzymes. Our findings are thus an indication of enzyme concentration
365 and capacity in the soil but not necessarily of their actual activity in the field. As stated above, soil moisture in
366 stands dominated by *Ligustrum* is lower than in native stands (Lichstein et al. 2004; Aragón et al. 2014).
367 Humidity directly affects enzyme activity by affecting hydrolysis and hence subsequently determines nutrient
368 mineralization. Litter decomposition in four species (two natives and two exotics) tended to be lower in stands
369 dominated by *Ligustrum* (Aragón et al. 2014), and the same was true for a standard substrate (leaves of
370 *Populus* sp.) (Fernandez 2012). Consequently, invaded stands appear to invest more in the production of
371 enzymes (that accumulate in the soil) to compensate for the unfavorable environmental conditions. This is
372 particularly important given the higher growth rate of *Ligustrum* and hence its potentially higher demand for
373 resources.
374

11

Species-specific effects

We have observed clear differences between the native and invasive species and also some differences among the natives. Whereas the litter traits of *Cinnamomum* and *Cupania* clearly differed from those of the invasive species, *Myrsine* shared several characteristics with them. This observation supports previous studies that found some morphological and demographic similarities among *Myrsine*, *Ligustrum* and *Morus* (Easdale et al. 2007; Easdale and Healey 2009). Easdale et al. (2007) measured 19 demographic variables in 29 montane tree species of northwestern Argentina, and *Myrsine* was closer in multidimensional space to *Ligustrum* and *Morus* than to *Cinnamomum* and *Cupania*. *Myrsine* and the two exotic species also shared ecomorphological features (structural, biochemical and morphological) such as seed mass, maximum growth rate and foliar P concentration (Easdale 2006). The similarities were especially evident with *Ligustrum*, which also has an overlapping fructification phenology. All these similarities indicate that the morphological and life-history characteristics of *Myrsine* resemble those of the exotics in this study. The invasion of *Ligustrum* in the study area has caused changes in species cover, dominance, diversity and sapling recruitment (Lichstein et al. 2004; Aragón and Morales 2003). Importantly, native species may be affected in different ways. Exotics may specially affect species that share attributes with them and hence potentially have similar requirements (Drenovsky et al. 2012), as perhaps happens with *Myrsine*, whose recruitment and growth is particularly reduced in stands dominated by *Ligustrum* (Bartolucci 2012). In a comparison of native and invaded stands of similar age, *Myrsine* showed a reduction in sapling recruitment of approximately 85% in *Ligustrum* stands, while *Cinnamomum* and *Cupania* were less affected (between 3.5 and 50%). The same trend was found for changes in basal area of adults (an average reduction of 80% in *Myrsine*) (Bartolucci 2012). *Myrsine* has more similar elemental composition and stoichiometry with invasive species than *Cinnamonium* and *Cupania*, which seem to explain the more negative impact over *Myrsine*. The native *Myrsine* would tend to use the resources in the same way than the invasive species and should compete stronger with it.

These results agree with those expected under the biogeochemical niche hypothesis (Peñuelas et al. 2008; 2010; Sardans and Peñuelas 2013), which claims that elemental composition (nutrient concentrations and their stoichiometric relationships) varies among plant species as a consequence of differential genotypic expression and functioning. This hypothesis predicts that different plant species growing in the same community would tend to have different elemental compositions to reduce the overlap in the use of soil resources and consequently would reduce direct competition. These results are consistent with the "competitive niche exclusion" a basic paradigm of the ecological niche theory (Bonsall et al. 2004; Phillips et al. 2004; Levine and HilleRisLammbers 2009; Alder et al. 2010). In this case the native species with more similar biogeochemical niche to the invasive species was the species first affected by the success of the invasive species. The biogeochemical niche thus appears as a useful tool to detect niche overlap intensity in the study of invasive species, frequently very difficult to be investigated in field conditions (Mooney and Cleland 2001; Davies et al. 2007). Moreover, it can provide clues of which native species would present the highest sensitivity to invasive species spread.

411   In addition, we would like to acknowledge that even though our design intended to control the potential
412   inherent variability between invaded and native stands, we cannot unequivocally assigned the differences in
413   soil enzyme or nutrients to the treatment effect. This is a common and recognized limitation in studies at
414   landscape scale such as most of the studies related to invasive species ecology (van Kleunen et al. 2010). The
415   comparative approach remains, so far, as one of the most commonly used in the field (e.g. , Leishman et al.
416   2010; Tecco et al. 2010). However, these limitations have to be taken into account when interpreting the
417   results.
418

419   **Final remarks and conclusions**
420   Even though several studies have highlighted the role of biological invasions at global scale, invasion
421   ecology still lacks general influential hypotheses (Strayer 2012). Several attempts that intended to identify
422   traits associated with invasion impacts have yield mixed results mainly because successful invasions are
423   closely linked to native community assembly and environmental filters (Drenovsky et al. 2012). In the context
424   of invasion ecology, understanding the link between invasion and ecosystem functioning is crucial to fully
425   evaluate the effect of invasive species. In this study, even though we focused on one particular exotic species,
426   we intended to provide general understanding of the potential effect of this species on the functioning of the
427   ecosystem.
428   Our results indicated that invasion by *Ligustrum* increased the maximum potential of alkaline phosphatase
429   and β-glucosidase activities, probably by compensating for the lower soil water content of the invaded soil, as
430   compared to native forest soil. These observations are supported by previously documented increases in water
431   and plant nutrient uptake in invaded stands (Ayup et al. 2014; Aragón et al. 2014). Plant invasion decreased
432   the availability of soil N, likely the limiting nutrient (litter N:P ratio of 7.4, based on mass) in these soils,
433   whereas it increased the availability of soil P. The lower soil water content and the higher growth capacity of
434   alien plants, coinciding with higher plant nutrient uptake, the large investment in soil-enzyme activity and the
435   lower N and P concentrations in litter, thus suggesting a link between the success of alien plants and a higher
436   capacity to take up nutrients and retain them in the biomass and generally in the ecosystem. Our results
437   showed that the species most sensitive to invasion, *Myrsine*, had a litter composition more similar to that of
438   the invasive plants than to other native plants. This result strongly suggests that this native species and the
439   exotics *Ligustrum* and *Morus* use resources very similarly, and thus *Myrsine* is most directly affected by
440   competitive pressure from invasive species.
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Fig. 1 Principal component analysis with three sets of variables: enzymes, nutrients and nutrient ratios (20 variables) measured in invaded (red) and native (black) stands. The symbols indicate the corresponding score group means ± SE. and the arrows represent variable eigenvectors in the space plotted by the first two PCA axes. The projection of the lines along each axis indicates their relative importance

Fig. 2 (a) Principal component analysis with 13 variables (nutrients and nutrient ratios) of the five species studied. C (*Cinnamomum*), Cu (*Cupania*) and My (*Myrsine*) are native species. L (*Ligustrum*) and M (*Morus*) are exotic species (axes 1 and 2). The symbols correspond to the score group means ± SE. Different letters indicate significant differences among species at each axis at $p < 0.05$. (b) Variable eigenvectors in the space plotted by the first two PCA axes. The projection of the lines along each axis indicates their relative importance

Fig. 3 (a) Principal component analysis with 13 variables (nutrients and nutrient ratios) of the five species studied. Codes as in Figure 2a (axes 1 and 3). The symbols correspond to the score group means ± SE. Different letters indicate significant differences among species at each axis at $p < 0.05$. When big arrows are present they indicate significant differences between invasive and exotic species. (b) Variable eigenvectors in the space plotted by the first and third PCA axes. The projection of the lines along each axis indicates their relative importance

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