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1 **Base cations and micronutrients in soil aggregates as affected by enhanced**
2 **nitrogen and water inputs in a semi-arid steppe grassland**

3

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17 **Abstract**

18 The intensification of grassland management by nitrogen (N) fertilization and
19 irrigation may threaten the future integrity of fragile semi-arid steppe ecosystems by
20 affecting the concentrations of base cation and micronutrient in soils. We extracted
21 base cations (~~of~~ exchangeable calcium (Ca), magnesium (Mg), potassium (K), and
22 sodium (Na) and extractable micronutrients of iron (Fe), manganese (Mn), copper
23 (Cu), and zinc (Zn) from three soil aggregate sizes classes (microaggregates, <0.25
24 mm; small macroaggregates, 0.25-2 mm; large macroaggregates, >2 mm) from a
25 9-year N and water field manipulation study. There were significantly more base
26 cations (but not micronutrients) in microaggregates compared to macroaggregates
27 which was related to greater soil organic matter and clay contents. Nitrogen addition
28 significantly decreased exchangeable Ca by up to 33% in large and small
29 macroaggregates and exchangeable Mg by up to 27% in three aggregates but
30 significantly increased extractable Fe, Mn and Cu concentrations (by up to 262%,
31 150%, and 55 %, respectively) in all aggregate size classes. However, water addition
32 only increased exchangeable Na, while available Fe and Mn were decreased by water
33 addition when averaging across all N treatments and aggregate classes. The loss of
34 exchangeable Ca and Mg under N addition and extractable Fe and Mn in soil
35 aggregates under water addition might potentially constrain the productivity of this
36 semi-arid grassland ecosystem.

37

38 **Key words** Nitrogen deposition • Irrigation • Calcium • Magnesium • Manganese •

39 Copper

40 **Introduction**

41 Micronutrient availability constrains net primary productivity (NPP) (Li et al., 2010,
42 2012), and deficiencies in soil micronutrients, including Fe, Mn, Cu, and Zn, are a
43 problem threatening food production worldwide (Jones et al., 2013). Base cations (i.e.,
44 exchangeable Ca, Mg, K, Na) are the predominant exchangeable cations in the
45 calcareous soils (Zhang et al., 2013). They are essential for soil buffering capacity
46 particularly in soils affected by atmospheric acid deposition (Lieb et al., 2011), serve
47 as good indicators of soil fertility (Zhang et al., 2013), and are critical nutrients for
48 both plant and microbial metabolism (Cheng et al., 2010). For instance, Ca regulates
49 plant cell signaling, cell division, and carbohydrate metabolism (McLaughlin and
50 Wimmer, 1999), and Mg is important for photosynthesis and energy storage (Lucas et
51 al., 2011). Biogeochemical processes may be driven by base cation and micronutrient
52 supply; for instance, root-surface phosphatase activity is correlated with available Ca
53 and Mg (Gabbrielli et al., 1989) and Mg and Zn availability are important for litter
54 decomposition (Powers and Salute, 2011).

55 The availability of base cations and micronutrients is influenced by
56 environmental changes, such as altered N and water ~~availabilities~~ availability
57 (Treseder, 2008). The availability of base cations varies with edaphic properties, such
58 as soil pH (Katou, 2002), organic matter fractions (Oorts et al., 2003) and soil particle
59 sizes (Beldin et al., 2007). Prolonged N inputs generally causes soil acidification and
60 subsequent losses of soil cations (McLaughlin and Wimmer, 1999; Cheng et al., 2010),

61 and micronutrient availability may increase under soil acidification (Malhi et al., 1998)
62 causing toxicity to both plants and soil microorganisms in extreme cases (Bowman et
63 al., 2008; Horswill et al., 2008). Changes in precipitation regime and soil moisture
64 levels may interact with inorganic N affecting soil microbial activities (Wang et al.,
65 ~~2014~~, 2015a) including the decomposition of soil organic matter (SOM) and nutrients
66 release and their subsequent transport in the soil (Dungait et al., 2012; Nielsen and
67 Ball, 2014). In sandy soils, increased precipitation might promote leaching of nitrate
68 and counter-ions (such as base cations) (Ochoa-Hueso et al., 2014).

69 Soil aggregate structure predominantly controls SOM dynamics (Six et al., 2004)
70 and microbial activities (Dorodnikov et al., 2009), and soil aggregate stability can
71 serve as an indicator for grassland ecosystem health (Reinhart et al., 2015). In
72 comparison with macroaggregates, microaggregates provide preferential sites for soil
73 C stabilization (Wang et al., 2015b) and the SOM herein is more microbial-processed
74 as evidenced by natural abundance stable ^{13}C values (Gunina and Kuzyakov, 2014;
75 Wang et al., 2015b). More microbial-processed SOM (i.e. more functional groups)
76 and potentially higher mineral contents within microaggregates (Creamer et al., 2011)
77 would purportedly provide more binding sites for base cations and micronutrients.
78 Therefore, dynamics of soil base cations and micronutrients in aggregate scale would
79 be a good indicator for soil health and for the potential of metal nutrients
80 sustainability. However, studies concerning aggregate-scale distribution of base
81 cations and micronutrients under enhanced N input and precipitation are still rarely
82 seen.

83 Semi-arid steppe grasslands support diverse animal and plant species (Kang et al.,
84 2007) and are experiencing or will experience enhanced atmospheric nitrogen (N)
85 deposition and precipitation (Niu et al., 2009; Bai et al., 2010; ~~Wang et al., 2014,~~
86 ~~2015a;~~ Zhang et al., 2014). Previously, we demonstrated that water addition promoted
87 the incorporation of microaggregates into macroaggregates and enhanced
88 decomposition rates within microaggregates compared to macroaggregates, and that
89 the addition of N depressed extracellular enzyme activities within soil aggregates as a
90 result of soil acidification, after 9-years in a field experiment in the semi-arid steppe
91 grasslands of Inner Mongolia (Wang et al., 2015a,b). In this study, we investigated the
92 changes of base cations and micronutrients within the soil aggregates. We
93 hypothesized that (1) both concentrations of base cation_s and micronutrient_s would
94 increase in microaggregates because of the increased abundance of adsorption sites
95 provided by greater SOM and mineral contents therein, and (2) that increased N and
96 water inputs would decrease base cations and increase the availability of
97 micronutrients within soil aggregates due to soil acidification and leaching in the
98 free-draining soil.

99

100 **Materials and methods**

101 *Study sites and experiment design*

102 The experiment was conducted in the Inner Mongolia Restoration Ecological
103 Research Station (IMRERS) in the south of Duolun County, Inner Mongolia, China
104 (42°02'27"N, 116°17'59"E, elevation 1,324 m a.s.l). The topography of the

105 experimental area is flat. The mean annual temperature is 2.1 °C, ranging from -17.8 °C
106 in January to 18.8 °C in July, and mean annual precipitation is 379.4 mm (Xu et al.,
107 2012). The soil is a Haplic Calcisols according to the FAO classification (IUSS
108 Working Group WRB, 2015) with a texture of sandy loam (0-10 cm): 63% sand, 20%
109 silt, and 17% clay (Wang et al., 2014). The chemical characteristics of the 0 – 10 cm
110 depth of whole soil are given in Wang et al. (2014).

111 The experimental design is described in detail in Wang et al. (2014). Briefly,
112 experimental plots were set up and run for 9 years. A split-plot design was applied
113 with water and N addition being the two treatments. In April 2005, twelve 8 m × 8m
114 plots were established in each of seven treatment blocks with 1 m buffer zone
115 between any two adjacent plots. Each block was divided into two main plots with
116 Based on water treatment (either ambient precipitation and/or 180 mm water addition);
117 each of seven blocks was divided into two main plots as treatments. Each main plot
118 was divided into six subplots and nitrogen treatments (urea pellets) were applied to a
119 randomly selected subplot (dispersed on the top of the soil) (Xu et al., 2012). Two
120 subplots were phosphorus addition treatments which were not considered in this study.
121 Additional water (approximately 50% of mean annual precipitation) was added to the
122 water addition plots by sprinkling 15 mm weekly during the middle of the growing
123 season from June to August as over 65% of annually total precipitation occurs during
124 this time. The chemical composition of the irrigation water was listed as Table S1.
125 Nitrogen additions were split applications with half applied in early May and the other
126 half in late June at the rates of 0 (control plots, defined as CK), 5 (N₅), 10 (N₁₀), and

127 15 (N_{15}) $g N m^{-2} yr^{-1}$ (Xu et al., 2012). The background N inputs (atmospheric
128 deposition plus fertilizer application) in this area are about $5 g N m^{-2} yr^{-1}$, so these
129 manipulations represent 100%, 200% and 300% surplus of nitrogen compared to the
130 background N inputs (Wang et al., 2015a).

131

132 *Soil sampling and aggregate-size fractionation*

133 In September 2013, top soils (0 – 10 cm) were sampled by compositing five randomly
134 selected cores within each plot from four out of seven blocks. Fresh soil samples were
135 stored at 4°C during transportation to the laboratory. Soil aggregates were isolated by
136 a dry-sieving method according to Dorodnikov et al. (2009) to minimize the
137 disruption in microbial activities and to prevent leaching of available nutrients by wet
138 sieving. Briefly, fresh bulk soil samples (gravimetric water content of 10-15%) were
139 gently passed through a 5 mm screen and transferred to a nest of sieves (2 and 0.25
140 mm) on a mechanical shaker Retsch AS200 Control (Retsch Technology, Düsseldorf,
141 Germany). The sieves were mechanically shaken (amplitude 1.5 mm) for 2 min to
142 separate the aggregates > 2 mm (large macroaggregates), 2 – 0.25 mm (small
143 macroaggregates), and < 0.25 mm (microaggregates). The mass proportions of large
144 macro-, small macro-, and microaggregates were 30.3-44.8%, 38.3-42.8%, and
145 15.3-31.1%, respectively (Wang et al., 2015b). No correction was made for sand
146 because sand grains can be completely embedded within macroaggregates. The
147 chemical characteristics of bulk soil and soil aggregates (0-10 cm) are given in Table
148 1 (Wang et al., 2014, 2015a,b).

149

150 *Soil physiochemical and biological parameters*

151 Soil aggregate pH and electrical conductivity (EC) was determined in a 1:5 (w/v)
152 soil-to-water extract with a PHS-3G digital pH meter (Precision and Scientific Corp.,
153 Shanghai, China) and a Orion 150A+ conductivity meter (Thermo Fisher Scientific
154 Inc., Beverly, USA), respectively. Soil organic carbon (SOC) within aggregate classes
155 was determined for acid-fumigated soils (to remove carbonates) using elemental
156 analyzer (EA1112, ThermoFisher Scientific, Japan). Concentration of soil aggregate
157 ammonium was determined colorimetrically from 2 M KCl soil extracts (Wang et al.,
158 2015b).

159

160 *Extraction and analysis of base cations and micronutrients*

161 Soil aggregate base cations were extracted and analysed using the method of
162 Ochoa-Hueso et al. (2014). Briefly, 2.5 g of each soil aggregate fraction was extracted
163 by 50 ml 1 M ammonium acetate ($\text{CH}_3\text{COONH}_4$) (pH 7.0). The slurry was orbitally
164 shaken for 30 min at 150 rpm and then filtered through Whatman no. 2V filter paper
165 (quantitative and ash-free). Micronutrients (Fe, Mn, Cu, Zn) within soil aggregates
166 were extracted by diethylenetriamine pentaacetic acid (DTPA) (Lindsay and Norvell,
167 1978). 10 g of soil aggregates was weighted into Erlenmeyer flask to mix with 20 mL
168 0.005 M DTPA + 0.01 M CaCl_2 + 0.1 M TEA (triethanolamine) (pH 7.0). The mixture
169 were shaken at 180 rpm for 2 h and filtered. All of the extractable metal cations were
170 analyzed by atomic absorption spectrometer (AAS, Shimadzu, Japan). The sum of

171 exchangeable Ca, Mg, K, and Na was defined as effective cation exchange capacity
172 (ECEC).

174 *Statistical analyses*

175 ~~A split-plot design~~ Three-way ANOVAs with a split-plot design ~~was/were~~ used to test
176 the effect of water (between subject), N addition (within subject) and soil aggregate
177 sizes on electrical conductivity (EC), the concentrations of base cations (exchangeable
178 Ca, Mg, K, and Na) and available micronutrients (Fe, Mn, Cu, and Zn). ~~The effects of~~
179 ~~N addition rates on~~ Multiple comparisons with Duncan design were performed to
180 determine differences in EC, base cations, and available micronutrients ~~were~~
181 ~~determined by one-way ANOVA among N rates~~ and run separately for ambient and
182 enhanced precipitation within each soil aggregate. Pearson correlation analysis was
183 used to examine the relationships between base cations and available micronutrients.
184 All statistical analyses were performed in SPSS 16.0 (SPSS, Inc., Chicago, IL, U.S.A.)
185 and statistical significance was accepted at $P < 0.05$.

187 **Results**

188 *Responses of electrical conductivity (EC) ~~and total base cations~~ to N and water*
189 *addition*

190 Under ambient water conditions, soil EC was significantly reduced in N addition plots
191 from 272.5 to 179.2 $\mu\text{s cm}^{-1}$ in large macroaggregates, from 249.4 to 188.6 $\mu\text{s cm}^{-1}$ in
192 small macroaggregates, and from 295.3 to 173.9 $\mu\text{s cm}^{-1}$ in microaggregates in soil

193 ~~aggregate size classes~~, except for N₅ in macro- and microaggregates as
194 compared to the control (CK) (Fig. 1a, Table 2). Under elevated water conditions, EC
195 values were significantly smaller in N₁₅ plots across soil aggregate size classes (Fig.
196 1a). The N₁₅ treatment significantly decreased soil EC from 237.8 to 194.5 $\mu\text{s cm}^{-1}$ in
197 large macroaggregates, from 247.5 to 192.9 $\mu\text{s cm}^{-1}$ in small macroaggregates, and
198 from 293.5 to 192.7 $\mu\text{s cm}^{-1}$ in microaggregates (Fig. 1a). Soil EC values were
199 significantly larger in water addition plots at N application rates of N₁₀ in large
200 macroaggregates, N₅ and N₁₀ in small macroaggregates, and N₁₀ in microaggregates
201 comparing to respective N plots of ambient precipitation (Fig. 1a).

202 ~~Total base cations (sum of exchangeable Ca, Mg, K, and Na) in soil aggregates~~
203 ~~increased in the order: small macroaggregates < large macroaggregates <~~
204 ~~microaggregates averaging across all N and water treatments (Table 2, Fig. 1b). Soil~~
205 ~~base cations decreased with N addition in both large and small macroaggregates under~~
206 ~~both ambient and elevated water conditions (Fig. 1b).~~

207
208 Response of ECEC, Exchangeable-exchangeable Ca, Mg, K, and Na and
209 DTPA-extractable micronutrients to N and water addition

210 Total base cations ~~he ECEC~~ (sum of exchangeable Ca, Mg, K, and Na) in soil
211 aggregates increased in the order: small macroaggregates < large macroaggregates <
212 microaggregates averaging across all N and water treatments (Table 2, Fig. 1b). Soil
213 base cations decreased with N addition in both large and small macroaggregates under
214 both ambient and elevated water conditions (Fig. 1b).

215 When averaging all the N and water treatments, the greatest concentrations of
216 exchangeable Ca, Mg, and K were all detected in microaggregates, followed by large
217 macroaggregates (Fig. 2a, b, c). Microaggregates contained significantly more
218 exchangeable Na than the large macroaggregates averaging across all the treatments
219 (Fig. 2d). Under ambient precipitation, exchangeable Ca was significantly less in
220 N-addition plots with N₅, N₁₀, and N₁₅ in large macroaggregates, and in N₁₀ and N₁₅
221 plots in small macroaggregates as compared to control plots by as much as 33 %
222 (between CK and N₁₅ treatments) (Fig. 2a). Under elevated water conditions, N
223 addition significantly decreased exchangeable Ca in both large (N₅, N₁₀, and N₁₅) and
224 small macroaggregates (N₁₀ and N₁₅) by as much as 26 % (Fig. 2a). Under ambient
225 water conditions, exchangeable Mg was significantly lower in N₁₅ in large
226 macroaggregates, in N₅ and N₁₅ in small macroaggregates, and in N₁₅ in
227 microaggregates by 4 - 27 % (Fig. 2b). Under increased water inputs, N addition
228 decreased exchangeable Mg in N₁₀ and N₁₅ plots in both large and small
229 macroaggregates and in N₅, N₁₀, and N₁₅ in microaggregates by 2 - 19 % (Fig. 2b).
230 Both exchangeable K and Na showed no response to N addition across all
231 aggregate-size classes (Fig. 2c, d). Significant positive water effects were only seen
232 on the concentration of exchangeable Na when comparing the means of all N
233 treatments and aggregate classes between ambient precipitation and water addition
234 (Table 2).

235 DTPA-extractable micronutrients were distributed differently across
236 aggregate-size classes (Fig. 3, Table 2). Significantly less extractable Fe and Mn were

237 detected within small macroaggregates compared to large macro- and
238 microaggregates (Fig. 3a, b). Extractable Cu increased in the order large macro- >
239 micro- > small macroaggregates when averaging all the N and water treatments (Fig.
240 3c). The largest extractable Zn was observed in microaggregates, while no significant
241 difference was found for Zn between large and small macroaggregates (Fig. 3d).
242 Following N additions, DTPA-extractable Fe, Mn, and Cu significantly increased
243 within all soil aggregates under both ambient and elevated water conditions by as
244 much as 262 %, 150 %, and 55 %, respectively (Fig. 3a, b, c). No overall N effects
245 were detectable on extractable Zn (Table 2), but Zn decreased significantly with N₁₀
246 and N₁₅ treatments under water addition within microaggregates (Fig. 3d). Extractable
247 Fe and Mn significantly decreased with water addition under N addition rates of 5, 10,
248 and 15 g N m⁻² yr⁻¹ within large macroaggregates (Fig. 3a,b, Table 2).

249

250 *Correlations between soil physicochemical parameters, base cations and*
251 *micronutrients*

252 Significant positive correlations were observed between SOC and soil exchangeable
253 Ca, Mg, and K, and between pH and EC, exchangeable Ca, Mg, and Na (Table 3).
254 There were significant positive relationships between exchangeable Ca vs. Mg, Ca vs.
255 K, Ca vs. K, Mg vs. K, and Mg vs. Na (Table 3). Micronutrients Fe, Mn, and Cu were
256 negatively correlated with soil pH across all soil aggregate size classes (Fig. 4a, b, c).

257 Within macroaggregates, ~~total base cations~~ECEC significantly and negatively
258 correlated with extractable ammonium (~~Table Fig. S1a5a~~).

259

260 **Discussion**

261 *Distribution of base cations and micronutrients within soil aggregates*

262 Relatively increased clay mineral and SOM contents are commonly recognized to be
263 associated with the smallest aggregate size fractions (< 0.25 mm), both of which play
264 an essential role in retaining soil base cations especially in sandy, free-draining soils
265 similar to our experimental field plot (Oorts et al., 2003; Beldin et al., 2007). In line
266 with our initial hypothesis, a significant positive correlation was detected between
267 SOC (the largest component of SOM) and exchangeable Ca, Mg, K, and ~~total base~~
268 ~~cations~~ ECEC (Table 3), and their distribution was similar to the distribution pattern of
269 SOC and total nitrogen with the highest concentrations in microaggregates (Wang et
270 al., 2015b). Soil organic matter was more decomposed within microaggregates (Wang
271 et al., 2015b) and thus may have provided more binding sites (e.g. more carbonyl
272 functions; Golchin et al., 1994) for exchangeable base cations. It is widely reported
273 that available micronutrients correspond to both SOM and clay content which are both
274 usually more abundant in microaggregates (e.g. Sharma et al., 2004; Six et al., 2004).
275 Although we observed greater available Zn in microaggregates compared to
276 macroaggregates (~~as reported by Głab and Gondek, 2014~~), there was no significant
277 relationship of SOC with Fe, Mn, and Cu, which had quite different distribution
278 patterns from SOC within soil aggregate-size classes and was similar to the findings
279 by Głab and Gondek (2014) in bulk soil. Due to the fact that large macroaggregates
280 are responsible for providing nutrients for plants and the early stages of SOM

281 formation (Six et al., 2004), plant cycling (uptake and biomass decomposition) might
282 contribute to the higher concentrations of Fe, Mn and Cu in large macroaggregates
283 (Jobbágy and Jackson, 2001).

284

285 | *Response of EC and base cations to nitrogen and water addition*

286 Protons released when soils acidify may exchange with base cations making them
287 available for take-up by plants or transport (and loss) by water in soils (Lucas et al.,
288 2011; Malhi et al., 1998; Sharma et al., 2004). As EC represents salt concentration in
289 soil solution (Zhou et al., 2011), significantly lower EC values might suggest the loss
290 of ions, in line with the decline of ECEC under higher N rates (Fig. 1a vs. Fig. 1b).

291 Deficiencies in soil base cations caused by N deposition could pose a threat to
292 ecosystems through their effect on soil microbial activity (Vitousek et al., 1997;
293 Treseder, 2008). Many studies on the effects of chronic N addition to soil contribute
294 shifts in soil microbial community structure and activity to the reduction of soil pH
295 (Ramirez et al., 2010; Wang et al., 2014), and we previously reported soil enzyme
296 depression under N addition attributed to soil acidification (Chung et al., 2007; Wang
297 et al., 2014). Consistent with our hypothesis, we observed a decrease in Ca and Mg,
298 which are the major base cations in macroaggregates (Fig. 2). This decrease
299 corresponded to a negative correlation between NH_4^+ and ~~total base cation~~
300 ~~concentrations~~ ECEC across soil macroaggregates (Fig. S15a). Nitrogen addition
301 increased NH_4^+ concentrations across all three soil aggregate size classes (Wang et al.,
302 2015b) which may displace soil aggregate base cations and bind strongly to soil

303 surfaces (Matschonat and Matzner, 1996). We did not measure leaching rates in the
304 field experiment, but these are likely to be substantial given the sand content of the
305 soil (> 60%). Increased leaching under water addition that caused NO₃⁻ loss was
306 previously reported (Wang et al., 2015b) and may indicate a route for the loss of Ca
307 and Mg within soil aggregates, as observed by Currie et al. (1999) and Lucas et al.
308 (2011). Our results contradict those of Ochoa-Hueso et al. (2014) who reported an
309 increase of extractable base cations under N deposition in a highly buffered, limestone
310 soil in the semi-arid Mediterranean region. However, where N addition increases NPP
311 in an N-limited soil, such as those of semi-arid steppe grasslands (Xu et al., 2014) a
312 coincident uptake of base cations by vigorous plant growth may occur (Tomlinson,
313 2003). But we did not investigate changes in the cation content of plant tissues in this
314 experiment to confirm this flux. Furthermore, no effect of N addition was observed on
315 soil aggregate K and Na concentrations (although Ca and Mg decreased; Fig. 32,
316 Table 2) indicating the selective depletion of base cations (Lu et al., 2014). The
317 depletion of base cations within soil aggregate can accelerate a reduction in the
318 buffering capacity of soils and accelerate soil acidification (with increased N at
319 ambient water soil pH 6.97 decreased to 6.10, and with added water, 7.17 to 6.42;
320 Wang et al., 2015a); the dynamics of the base cations were affected substantially.

321 Water addition increased EC and exchangeable Na but did not affect the other base
322 cations (Table 2; Fig. 1a, 2). ~~Although the Na concentration in the water for irrigation~~
323 ~~is uncertain, †~~The positive effect of water on soil EC and exchangeable Na ~~was~~
324 ~~unlikely~~might be caused by ~~Na~~-the relatively high EC value and Na concentration in

325 irrigative water ~~due to the undetectable water effect on Ca~~ in this calcareous grassland
326 ([Table S1](#)). We previously reported that water addition increased soil microbial
327 activity at the study site (Wang et al., 2014, 2015a): if litter decomposition rates were
328 promoted as a consequence (e.g. Liu et al., 2006) Ca, Mg and K may have been
329 released from this secondary biological source to supplement the soil pools.

330

331 *Response of micronutrients to nitrogen and water addition*

332 Micronutrient cycling relies on the balance between input (mineral weathering
333 release), recycling (plant biomass turnover), retention (sorption reactions), and
334 removal (biomass harvesting, fire, and hydrologic leaching) (Li et al., 2008).

335 Long-term N addition caused large increases in extractable Fe, Mn and Cu
336 concentrations in all soil aggregate size classes (by up to 262%, 150%, and 55 %,
337 respectively; Fig. 3a, b, c), presumably due to the decrease in soil pH which may
338 increase micronutrient release through weathering and desorption from soil minerals
339 and SOM (Malhi et al., 1998). Acidification caused by the long-term addition of N
340 negatively affected microbial activity previously observed as the depression of
341 enzyme activity in these experimental soils (Chung et al., 2007; Wang et al., 2014).

342 The mechanism underlying this biological response was not explored but is likely to
343 be complex and due in part to the effect of the changes in micronutrient supply.

344 Long-term irrigation had the opposite effect on extractable Fe and Mn within soil
345 aggregates where increases in soil aggregate pH (which usually decrease the
346 mobilization of micronutrients) were detected compared to ambient precipitation plots

347 (Wang et al., 2014). Our previous study suggested that water addition increased soil
348 pH from 7.1 to 7.3 in CK and from 5.6 to 6.1 in N₁₅ plots for large macroaggregates,
349 from 7.1 to 7.5 in CK and from 5.8 to 6.2 in N₁₅ plots for small macroaggregates, and
350 from 7.1 to 7.4 in CK and from 5.9 to 6.2 in N₁₅ plots for microaggregates (Wang et
351 al., 2015). A previous study carried out at the same plots showed that water addition
352 significantly increased NPP (Xu et al., 2010), suggesting enhanced uptake of growing
353 plants may have reduced concentrations of Fe and Mn in the soil aggregates. This is
354 consistent with the findings of Li et al. (2008) who reported that Mn and boron uptake
355 by plants outpaced their resupply by mineral weathering. However, Fe and Mn may
356 be toxic to plants (Brown and Jones, 1977), so losses by increased leaching may be
357 the most likely route for their decrease in the irrigated plots. However, neither
358 leaching nor plant performance was measured in this experiment.

359

360 **Conclusions**

361 Consistent with our first hypothesis, microaggregates had greater base cation
362 concentrations, which was ascribed to a proportion of sorption provided by increased
363 SOM and clay contents associated with the smallest aggregate size class. However,
364 extractable micronutrients (except Zn) did not follow the same concentration pattern
365 as base cations across three soil aggregate size classes. Under N addition,
366 exchangeable Ca and Mg decreased while extractable micronutrients (Fe, Mn and Cu)
367 increased, which was related to the slight but apparently important decrease in soil pH.
368 Water addition showed no impact on exchangeable Ca, Mg and K, but decreased

369 available Fe and Mn averaging across soil aggregates which partially supported our
370 second hypothesis. Our findings suggest that uncontrolled or long-term N fertilization
371 and irrigation of semi-arid steppe soils cause significant changes in the concentrations
372 of base cations and micronutrients which will ultimately decrease soil fertility,
373 constrain NPP and the nutrient quality of plants. The pathway of the losses (e.g.
374 leaching or uptake into plant tissues followed by harvest) need to be determined in
375 order to manage large and fragile semi-arid grassland systems.

376

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385

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543

544

545 **Tables**

546 **Table 1** The chemical characteristics of bulk soil and soil aggregates without
 547 field-manipulated treatments. SOC, TN, C/N, and DOC represent soil organic carbon,
 548 total nitrogen, the ratio of SOC to TN, and dissolved organic carbon, respectively.
 549 Data are represented as means \pm standard error.

550

	Soil aggregates (mm)			
	Bulk soil	> 2	0.25-2	< 0.25
SOC (g kg soil ⁻¹)	18.9 \pm 1.7	18.2 \pm 1.0	15.1 \pm 2.0	21.6 \pm 1.4
TN (g kg soil ⁻¹)	1.8 \pm 0.2	1.9 \pm 0.1	1.5 \pm 0.2	2.2 \pm 0.1
C/N	10.4 \pm 1.3	9.5 \pm 0.5	10.4 \pm 0.4	10.0 \pm 0.2
pH	7.0 \pm 0.2	7.1 \pm 0.2	7.1 \pm 0.2	7.1 \pm 0.3
DOC	69.8 \pm 5.6	72.5 \pm 6.8	66.6 \pm 1.3	71.2 \pm 1.5
NO ₃ ⁻ -N (mg kg soil ⁻¹)	27.3 \pm 5.9	4.2 \pm 0.1	31.6 \pm 2.2	34.4 \pm 3.8
NH ₄ ⁺ -N (mg kg soil ⁻¹)	9.4 \pm 1.6	17.0 \pm 1.3	17.9 \pm 2.5	22.9 \pm 1.0

551 Data from Wang et al. (2014, 2015a,b)

552

553 **Table 2** Results (*F* values) of ANOVAs with a split-plot design on the effects of N,
 554 water addition (W), and soil fractions (S) on soil electrical conductivity (EC),
 555 exchangeable Ca, Mg, K, Na, ~~total base cations~~ effective cation exchange capacity
 556 (ECEC, sum of exchangeable Ca, Mg, K, and Na , cmol kg aggregate⁻¹), and available
 557 Fe, Mn, Cu, and Zn (mg kg aggregate⁻¹).

558

	EC	Ca	Mg	K	Na	Base- cations ECEC	Fe	Mn	Cu	Zn
W	11.60*	3.07	5.08	0.17	15.6**	4.03	27.8**	14.1*	2.40	3.92
N	14.20**	6.36**	38.7**	0.33	1.71	7.36**	147**	136.3**	44.7**	2.47
S	3.63*	25.0**	168.3**	123.7**	3.49*	30.7**	17.7**	30.1**	33.7**	33.8**
W×N	6.90**	1.01	5.68**	6.81**	1.26	1.25	2.36	3.40*	7.23**	4.34**
W×S	0.28	1.03	3.75*	0.98	0.34	1.03	6.07**	10.8**	6.60**	2.32
N×S	0.77	0.87	0.36	0.88	1.81	0.88	1.59	2.43*	0.85	2.04
W×N×S	0.86	0.44	1.03	1.41	0.61	0.42	0.99	2.54*	1.16	3.84**

559 * and ** indicating the significant level at P<0.05, 0.01, respectively

560

561 **Table 3** Correlation analyses (*R* values) among exchangeable base cations, effective
 562 cation exchange capacity (ECEC), soil organic carbon (SOC), soil pH, and soil
 563 electrical conductivity (EC).

	pH	EC	Ca	Mg	K	Na	Base- <u>cationsECEC</u>
SOC	0.11	0.19	0.42**	0.73**	0.81**	0.17	0.46**
pH		0.66**	0.35**	0.48**	0.04	0.33**	0.37**
EC			0.28**	0.40**	0.15	0.16	0.29**
Ca				0.60**	0.53**	0.23*	0.998**
Mg					0.77**	0.24*	0.65**
K						0.10	0.57**
Na							0.26*

564 * and ** indicating the significant level at $P < 0.05$, 0.01 , respectively

565

566 **Table S1** Chemical characteristics of the irrigation water.

	Irrigation water
EC (us cm ⁻¹)	196.1±6.4
pH	7.7±0.07
Ca (mmol L ⁻¹)	1.6±0.03
Mg (mmol L ⁻¹)	0.8±0.02
K (mmol L ⁻¹)	0.04±0.002
Na (mmol L ⁻¹)	0.6±0.02

567

568 The symbol ‘-’ indicate the ions were not detected.

569

570 **Figure Legends**

571 **Fig.1** The effect of 9-year of water (ambient vs. water addition) and N additions (0
572 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on (a) electrical conductivity (EC, us
573 cm⁻¹) and (b) ~~total base cations~~ effective cation exchange capacity (ECEC, sum of
574 exchangeable Ca, Mg, K, and Na, cmol kg aggregate⁻¹). Data are represented as
575 means ± standard error (n=4). Lowercase letters indicate significant differences
576 between N treatments within a soil aggregate class size and water treatment. The
577 capital letters indicate significant differences between soil aggregate sizes across N
578 and water treatments.

579

580 **Fig.2** The effect of 9-year of water (ambient vs. water addition) and N additions (0
581 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on exchangeable (a) Ca, (b) Mg, (c) K
582 and (d) Na concentrations (cmol kg aggregate⁻¹) in 3 soil aggregate size classes (<0.25
583 mm, 0.25-2 mm, >2 mm). Data are represented as means ± standard error (n=4).
584 Lowercase letters indicate significant differences between N treatments within a soil
585 aggregate size class and water treatment. The capital letters indicate significant
586 differences between soil aggregate sizes across N and water treatments.

587

588 **Fig. 3** The effect of 9-years of water (ambient vs. water addition) and N additions (0
589 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on DTPA-extractable micronutrients (a)
590 Fe, (b) Mn, (c) Cu and (d) Zn in 3 soil aggregate size classes (<0.25 mm, 0.25-2
591 mm, >2 mm (mg kg aggregate⁻¹). Data are represented as means ± standard error

592 (n=4). Lowercase letters indicate significant differences between N treatments within
593 a soil fraction and water treatment. The capital letters indicate significant differences
594 between soil aggregate sizes across N and water treatments.

595

596 **Fig. 4** The relationships between soil pH and DTPA-extractable (a) Fe (mg kg
597 aggregate⁻¹), (b) Mn (mg kg aggregate⁻¹), (c) Cu (mg kg aggregate⁻¹) and (d) Zn (mg
598 kg aggregate⁻¹) across all N and water treatments within the soil aggregate size
599 classes.

600

601 **Fig. S1-5** The relationships between KCl-extractable ammonium (mg kg aggregate⁻¹)
602 and ~~total base cation~~ effective cation exchange capacity (ECEC, sum of exchangeable
603 Ca, Mg, K, and Na , cmol kg aggregate⁻¹) within macroaggregates (a) and
604 microaggregates (b) across all N and water inputs.

1 **Base cations and micronutrients in soil aggregates as affected by enhanced**
2 **nitrogen and water inputs in a semi-arid steppe grassland**

3

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17 **Abstract**

18 The intensification of grassland management by nitrogen (N) fertilization and
19 irrigation may threaten the future integrity of fragile semi-arid steppe ecosystems by
20 affecting the concentrations of base cation and micronutrient in soils. We extracted
21 base cations of exchangeable calcium (Ca), magnesium (Mg), potassium (K), and
22 sodium (Na) and extractable micronutrients of iron (Fe), manganese (Mn), copper
23 (Cu), and zinc (Zn) from three soil aggregate sizes classes (microaggregates, <0.25
24 mm; small macroaggregates, 0.25-2 mm; large macroaggregates, >2 mm) from a
25 9-year N and water field manipulation study. There were significantly more base
26 cations (but not micronutrients) in microaggregates compared to macroaggregates
27 which was related to greater soil organic matter and clay contents. Nitrogen addition
28 significantly decreased exchangeable Ca by up to 33% in large and small
29 macroaggregates and exchangeable Mg by up to 27% in three aggregates but
30 significantly increased extractable Fe, Mn and Cu concentrations (by up to 262%,
31 150%, and 55 %, respectively) in all aggregate size classes. However, water addition
32 only increased exchangeable Na, while available Fe and Mn were decreased by water
33 addition when averaging across all N treatments and aggregate classes. The loss of
34 exchangeable Ca and Mg under N addition and extractable Fe and Mn in soil
35 aggregates under water addition might potentially constrain the productivity of this
36 semi-arid grassland ecosystem.

37

38 **Key words** Nitrogen deposition • Irrigation • Calcium • Magnesium • Manganese •

39 Copper

40 **Introduction**

41 Micronutrient availability constrains net primary productivity (NPP) (Li et al., 2010,
42 2012), and deficiencies in soil micronutrients, including Fe, Mn, Cu, and Zn, are a
43 problem threatening food production worldwide (Jones et al., 2013). Base cations (i.e.,
44 exchangeable Ca, Mg, K, Na) are the predominant exchangeable cations in the
45 calcareous soils (Zhang et al., 2013). They are essential for soil buffering capacity
46 particularly in soils affected by atmospheric acid deposition (Lieb et al., 2011), serve
47 as good indicators of soil fertility (Zhang et al., 2013), and are critical nutrients for
48 both plant and microbial metabolism (Cheng et al., 2010). For instance, Ca regulates
49 plant cell signaling, cell division, and carbohydrate metabolism (McLaughlin and
50 Wimmer, 1999), and Mg is important for photosynthesis and energy storage (Lucas et
51 al., 2011). Biogeochemical processes may be driven by base cation and micronutrient
52 supply; for instance, root-surface phosphatase activity is correlated with available Ca
53 and Mg (Gabbrielli et al., 1989) and Mg and Zn availability are important for litter
54 decomposition (Powers and Salute, 2011).

55 The availability of base cations and micronutrients is influenced by
56 environmental changes, such as altered N and water availability (Treseder, 2008). The
57 availability of base cations varies with edaphic properties, such as soil pH (Katou,
58 2002), organic matter fractions (Oorts et al., 2003) and soil particle sizes (Beldin et al.,
59 2007). Prolonged N inputs generally causes soil acidification and subsequent losses of
60 soil cations (McLaughlin and Wimmer, 1999; Cheng et al., 2010), and micronutrient

61 availability may increase under soil acidification (Malhi et al., 1998) causing toxicity
62 to both plants and soil microorganisms in extreme cases (Bowman et al., 2008;
63 Horswill et al., 2008). Changes in precipitation regime and soil moisture levels may
64 interact with inorganic N affecting soil microbial activities (Wang et al., 2015a)
65 including the decomposition of soil organic matter (SOM) and nutrients release and
66 their subsequent transport in the soil (Dungait et al., 2012; Nielsen and Ball, 2014). In
67 sandy soils, increased precipitation might promote leaching of nitrate and
68 counter-ions (such as base cations) (Ochoa-Hueso et al., 2014).

69 Soil aggregate structure predominantly controls SOM dynamics (Six et al., 2004)
70 and microbial activities (Dorodnikov et al., 2009), and soil aggregate stability can
71 serve as an indicator for grassland ecosystem health (Reinhart et al., 2015). In
72 comparison with macroaggregates, microaggregates provide preferential sites for soil
73 C stabilization (Wang et al., 2015b) and the SOM herein is more microbial-processed
74 as evidenced by natural abundance stable ^{13}C values (Gunina and Kuzyakov, 2014;
75 Wang et al., 2015b). More microbial-processed SOM (i.e. more functional groups)
76 and potentially higher mineral contents within microaggregates (Creamer et al., 2011)
77 would purportedly provide more binding sites for base cations and micronutrients.
78 Therefore, dynamics of soil base cations and micronutrients in aggregate scale would
79 be a good indicator for soil health and for the potential of metal nutrients
80 sustainability. However, studies concerning aggregate-scale distribution of base
81 cations and micronutrients under enhanced N input and precipitation are still rarely
82 seen.

83 Semi-arid steppe grasslands support diverse animal and plant species (Kang et al.,
84 2007) and are experiencing or will experience enhanced atmospheric nitrogen (N)
85 deposition and precipitation (Niu et al., 2009; Bai et al., 2010; Zhang et al., 2014).
86 Previously, we demonstrated that water addition promoted the incorporation of
87 microaggregates into macroaggregates and enhanced decomposition rates within
88 microaggregates compared to macroaggregates, and that the addition of N depressed
89 extracellular enzyme activities within soil aggregates as a result of soil acidification,
90 after 9-years in a field experiment in the semi-arid steppe grasslands of Inner
91 Mongolia (Wang et al., 2015a,b). In this study, we investigated the changes of base
92 cations and micronutrients within the soil aggregates. We hypothesized that (1) both
93 concentrations of base cations and micronutrients would increase in microaggregates
94 because of the increased abundance of adsorption sites provided by greater SOM and
95 mineral contents therein, and (2) that increased N and water inputs would decrease
96 base cations and increase the availability of micronutrients within soil aggregates due
97 to soil acidification and leaching in the free-draining soil.

98

99 **Materials and methods**

100 *Study sites and experiment design*

101 The experiment was conducted in the Inner Mongolia Restoration Ecological
102 Research Station (IMRERS) in the south of Duolun County, Inner Mongolia, China
103 (42°02'27"N, 116°17'59"E, elevation 1,324 m a.s.l). The topography of the
104 experimental area is flat. The mean annual temperature is 2.1 °C, ranging from -17.8 °C

105 in January to 18.8 °C in July, and mean annual precipitation is 379.4 mm (Xu et al.,
106 2012). The soil is a Haplic Calcisols according to the FAO classification (IUSS
107 Working Group WRB, 2015) with a texture of sandy loam (0-10 cm): 63% sand, 20%
108 silt, and 17% clay (Wang et al., 2014). The chemical characteristics of the 0 – 10 cm
109 depth of whole soil are given in Wang et al. (2014).

110 The experimental design is described in detail in Wang et al. (2014). Briefly,
111 experimental plots were set up and run for 9 years. A split-plot design was applied
112 with water and N addition being the two treatments. In April 2005, twelve 8 m × 8m
113 plots were established in each of seven treatment blocks with 1 m buffer zone
114 between any two adjacent plots. Each block was divided into two main plots with
115 either ambient precipitation or 180 mm water addition) as treatments. Each main plot
116 was divided into six subplots and nitrogen treatments (urea pellets) were applied to a
117 randomly selected subplot (dispersed on the top of the soil) (Xu et al., 2012). Two
118 subplots were phosphorus addition treatments which were not considered in this study.
119 Additional water (approximately 50% of mean annual precipitation) was added to the
120 water addition plots by sprinkling 15 mm weekly during the middle of the growing
121 season from June to August as over 65% of annually total precipitation occurs during
122 this time. The chemical composition of the irrigation water was listed as Table S1.
123 Nitrogen additions were split applications with half applied in early May and the other
124 half in late June at the rates of 0 (control plots, defined as CK), 5 (N₅), 10 (N₁₀), and
125 15 (N₁₅) g N m⁻² yr⁻¹ (Xu et al., 2012). The background N inputs (atmospheric
126 deposition plus fertilizer application) in this area are about 5 g N m⁻² yr⁻¹, so these

127 manipulations represent 100%, 200% and 300% surplus of nitrogen compared to the
128 background N inputs (Wang et al., 2015a).

129

130 *Soil sampling and aggregate-size fractionation*

131 In September 2013, top soils (0 – 10 cm) were sampled by compositing five randomly
132 selected cores within each plot from four out of seven blocks. Fresh soil samples were
133 stored at 4°C during transportation to the laboratory. Soil aggregates were isolated by
134 a dry-sieving method according to Dorodnikov et al. (2009) to minimize the
135 disruption in microbial activities and to prevent leaching of available nutrients by wet
136 sieving. Briefly, fresh bulk soil samples (gravimetric water content of 10-15%) were
137 gently passed through a 5 mm screen and transferred to a nest of sieves (2 and 0.25
138 mm) on a mechanical shaker Retsch AS200 Control (Retsch Technology, Düsseldorf,
139 Germany). The sieves were mechanically shaken (amplitude 1.5 mm) for 2 min to
140 separate the aggregates > 2 mm (large macroaggregates), 2 – 0.25 mm (small
141 macroaggregates), and < 0.25 mm (microaggregates). The mass proportions of large
142 macro-, small macro-, and microaggregates were 30.3-44.8%, 38.3-42.8%, and
143 15.3-31.1%, respectively (Wang et al., 2015b). No correction was made for sand
144 because sand grains can be completely embedded within macroaggregates. The
145 chemical characteristics of bulk soil and soil aggregates (0-10 cm) are given in Table
146 1 (Wang et al., 2014, 2015a,b).

147

148 *Soil physiochemical and biological parameters*

149 Soil aggregate pH and electrical conductivity (EC) was determined in a 1:5 (w/v)
150 soil-to-water extract with a PHS-3G digital pH meter (Precision and Scientific Corp.,
151 Shanghai, China) and a Orion 150A+ conductivity meter (Thermo Fisher Scientific
152 Inc., Beverly, USA), respectively. Soil organic carbon (SOC) within aggregate classes
153 was determined for acid-fumigated soils (to remove carbonates) using elemental
154 analyzer (EA1112, ThermoFisher Scientific, Japan). Concentration of soil aggregate
155 ammonium was determined colorimetrically from 2 M KCl soil extracts (Wang et al.,
156 2015b).

157

158 *Extraction and analysis of base cations and micronutrients*

159 Soil aggregate base cations were extracted and analysed using the method of
160 Ochoa-Hueso et al. (2014). Briefly, 2.5 g of each soil aggregate fraction was extracted
161 by 50 ml 1 M ammonium acetate ($\text{CH}_3\text{COONH}_4$) (pH 7.0). The slurry was orbitally
162 shaken for 30 min at 150 rpm and then filtered through Whatman no. 2V filter paper
163 (quantitative and ash-free). Micronutrients (Fe, Mn, Cu, Zn) within soil aggregates
164 were extracted by diethylenetriamine pentaacetic acid (DTPA) (Lindsay and Norvell,
165 1978). 10 g of soil aggregates was weighted into Erlenmeyer flask to mix with 20 mL
166 0.005 M DTPA + 0.01 M CaCl_2 + 0.1 M TEA (triethanolamine) (pH 7.0). The mixture
167 were shaken at 180 rpm for 2 h and filtered. All of the extractable metal cations were
168 analyzed by atomic absorption spectrometer (AAS, Shimadzu, Japan). The sum of
169 exchangeable Ca, Mg, K, and Na was defined as effective cation exchange capacity
170 (ECEC).

171

172 *Statistical analyses*

173 Three-way ANOVAs with a split-plot design were used to test the effect of water
174 (between subject), N addition (within subject) and soil aggregate sizes on electrical
175 conductivity (EC), the concentrations of base cations (exchangeable Ca, Mg, K, and
176 Na) and available micronutrients (Fe, Mn, Cu, and Zn). Multiple comparisons with
177 Duncan design were performed to determine differences in EC, base cations, and
178 available micronutrients among N rates and run separately for ambient and enhanced
179 precipitation within each soil aggregate. Pearson correlation analysis was used to
180 examine the relationships between base cations and available micronutrients. All
181 statistical analyses were performed in SPSS 16.0 (SPSS, Inc., Chicago, IL, U.S.A.)
182 and statistical significance was accepted at $P < 0.05$.

183

184 **Results**

185 *Responses of electrical conductivity (EC) to N and water addition*

186 Under ambient water conditions, soil EC was significantly reduced in N addition plots
187 from 272.5 to 179.2 $\mu\text{s cm}^{-1}$ in large macroaggregates, from 249.4 to 188.6 $\mu\text{s cm}^{-1}$ in
188 small macroaggregates, and from 295.3 to 173.9 $\mu\text{s cm}^{-1}$ in microaggregates, except
189 for N₅ in large macro- and microaggregates as compared to the control (CK) (Fig. 1a,
190 Table 2). Under elevated water conditions, EC values were significantly smaller in
191 N₁₅ plots across soil aggregate size classes (Fig. 1a). The N₁₅ treatment significantly
192 decreased soil EC from 237.8 to 194.5 $\mu\text{s cm}^{-1}$ in large macroaggregates, from 247.5

193 to $192.9 \mu\text{s cm}^{-1}$ in small macroaggregates, and from 293.5 to $192.7 \mu\text{s cm}^{-1}$ in
194 microaggregates (Fig. 1a). Soil EC values were significantly larger in water addition
195 plots at N application rates of N_{10} in large macroaggregates, N_5 and N_{10} in small
196 macroaggregates, and N_{10} in microaggregates comparing to respective N plots of
197 ambient precipitation (Fig. 1a).

198

199 *Response of ECEC, exchangeable Ca, Mg, K, and Na and DTPA-extractable*
200 *micronutrients to N and water addition*

201 The ECEC (sum of exchangeable Ca, Mg, K, and Na) in soil aggregates increased in
202 the order: small macroaggregates < large macroaggregates < microaggregates
203 averaging across all N and water treatments (Table 2, Fig. 1b). Soil base cations
204 decreased with N addition in both large and small macroaggregates under both
205 ambient and elevated water conditions (Fig. 1b).

206 When averaging all the N and water treatments, the greatest concentrations of
207 exchangeable Ca, Mg, and K were all detected in microaggregates, followed by large
208 macroaggregates (Fig. 2a, b, c). Microaggregates contained significantly more
209 exchangeable Na than the large macroaggregates averaging across all the treatments
210 (Fig. 2d). Under ambient precipitation, exchangeable Ca was significantly less in
211 N-addition plots with N_5 , N_{10} , and N_{15} in large macroaggregates, and in N_{10} and N_{15}
212 plots in small macroaggregates as compared to control plots by as much as 33 %
213 (between CK and N_{15} treatments) (Fig. 2a). Under elevated water conditions, N
214 addition significantly decreased exchangeable Ca in both large (N_5 , N_{10} , and N_{15}) and

215 small macroaggregates (N_{10} and N_{15}) by as much as 26 % (Fig. 2a). Under ambient
216 water conditions, exchangeable Mg was significantly lower in N_{15} in large
217 macroaggregates, in N_5 and N_{15} in small macroaggregates, and in N_{15} in
218 microaggregates by 4 - 27 % (Fig. 2b). Under increased water inputs, N addition
219 decreased exchangeable Mg in N_{10} and N_{15} plots in both large and small
220 macroaggregates and in N_5 , N_{10} , and N_{15} in microaggregates by 2 - 19 % (Fig. 2b).
221 Both exchangeable K and Na showed no response to N addition across all
222 aggregate-size classes (Fig. 2c, d). Significant positive water effects were only seen
223 on the concentration of exchangeable Na when comparing the means of all N
224 treatments and aggregate classes between ambient precipitation and water addition
225 (Table 2).

226 DTPA-extractable micronutrients were distributed differently across
227 aggregate-size classes (Fig. 3, Table 2). Significantly less extractable Fe and Mn were
228 detected within small macroaggregates compared to large macro- and
229 microaggregates (Fig. 3a, b). Extractable Cu increased in the order large macro- >
230 micro- > small macroaggregates when averaging all the N and water treatments (Fig.
231 3c). The largest extractable Zn was observed in microaggregates, while no significant
232 difference was found for Zn between large and small macroaggregates (Fig. 3d).
233 Following N additions, DTPA-extractable Fe, Mn, and Cu significantly increased
234 within all soil aggregates under both ambient and elevated water conditions by as
235 much as 262 %, 150 %, and 55 %, respectively (Fig. 3a, b, c). No overall N effects
236 were detectable on extractable Zn (Table 2), but Zn decreased significantly with N_{10}

237 and N₁₅ treatments under water addition within microaggregates (Fig. 3d). Extractable
238 Fe and Mn significantly decreased with water addition under N addition rates of 5, 10,
239 and 15 g N m⁻² yr⁻¹ within large macroaggregates (Fig. 3a,b, Table 2).

240

241 *Correlations between soil physicochemical parameters, base cations and*
242 *micronutrients*

243 Significant positive correlations were observed between SOC and soil exchangeable
244 Ca, Mg, and K, and between pH and EC, exchangeable Ca, Mg, and Na (Table 3).

245 There were significant positive relationships between exchangeable Ca vs. Mg, Ca vs.
246 K, Ca vs. K, Mg vs. K, and Mg vs. Na (Table 3). Micronutrients Fe, Mn, and Cu were
247 negatively correlated with soil pH across all soil aggregate size classes (Fig. 4a, b, c).

248 Within macroaggregates, ECEC significantly and negatively correlated with
249 extractable ammonium (Fig. 5a).

250

251 **Discussion**

252 *Distribution of base cations and micronutrients within soil aggregates*

253 Relatively increased clay mineral and SOM contents are commonly recognized to be
254 associated with the smallest aggregate size fractions (< 0.25 mm), both of which play
255 an essential role in retaining soil base cations especially in sandy, free-draining soils
256 similar to our experimental field plot (Oorts et al., 2003; Beldin et al., 2007). In line
257 with our initial hypothesis, a significant positive correlation was detected between
258 SOC (the largest component of SOM) and exchangeable Ca, Mg, K, and ECEC (Table

259 3), and their distribution was similar to the distribution pattern of SOC and total
260 nitrogen with the highest concentrations in microaggregates (Wang et al., 2015b). Soil
261 organic matter was more decomposed within microaggregates (Wang et al., 2015b)
262 and thus may have provided more binding sites (e.g. more carbonyl functions;
263 Golchin et al., 1994) for exchangeable base cations. It is widely reported that
264 available micronutrients correspond to both SOM and clay content which are both
265 usually more abundant in microaggregates (e.g. Sharma et al., 2004; Six et al., 2004).
266 Although we observed greater available Zn in microaggregates compared to
267 macroaggregates, there was no significant relationship of SOC with Fe, Mn, and Cu,
268 which had quite different distribution patterns from SOC within soil aggregate-size
269 classes and was similar to the findings by Głab and Gondek (2014) in bulk soil. Due
270 to the fact that large macroaggregates are responsible for providing nutrients for
271 plants and the early stages of SOM formation (Six et al., 2004), plant cycling (uptake
272 and biomass decomposition) might contribute to the higher concentrations of Fe, Mn
273 and Cu in large macroaggregates (Jobbágy and Jackson, 2001).

274

275 *Response of EC and base cations to nitrogen and water addition*

276 Protons released when soils acidify may exchange with base cations making them
277 available for take-up by plants or transport (and loss) by water in soils (Lucas et al.,
278 2011; Malhi et al., 1998; Sharma et al., 2004). As EC represents salt concentration in
279 soil solution (Zhou et al., 2011), significantly lower EC values might suggest the loss
280 of ions, in line with the decline of ECEC under higher N rates (Fig. 1a vs. Fig. 1b).

281 Deficiencies in soil base cations caused by N deposition could pose a threat to
282 ecosystems through their effect on soil microbial activity (Vitousek et al., 1997;
283 Treseder, 2008). Many studies on the effects of chronic N addition to soil contribute
284 shifts in soil microbial community structure and activity to the reduction of soil pH
285 (Ramirez et al., 2010; Wang et al., 2014), and we previously reported soil enzyme
286 depression under N addition attributed to soil acidification (Chung et al., 2007; Wang
287 et al., 2014). Consistent with our hypothesis, we observed a decrease in Ca and Mg,
288 which are the major base cations in macroaggregates (Fig. 2). This decrease
289 corresponded to a negative correlation between NH_4^+ and ECEC across soil
290 macroaggregates (Fig. 5a). Nitrogen addition increased NH_4^+ concentrations across all
291 three soil aggregate size classes (Wang et al., 2015b) which may displace soil
292 aggregate base cations and bind strongly to soil surfaces (Matschonat and Matzner,
293 1996). We did not measure leaching rates in the field experiment, but these are likely
294 to be substantial given the sand content of the soil (> 60%). Increased leaching under
295 water addition that caused NO_3^- loss was previously reported (Wang et al., 2015b) and
296 may indicate a route for the loss of Ca and Mg within soil aggregates, as observed by
297 Currie et al. (1999) and Lucas et al. (2011). Our results contradict those of
298 Ochoa-Hueso et al. (2014) who reported an increase of extractable base cations under
299 N deposition in a highly buffered, limestone soil in the semi-arid Mediterranean
300 region. However, where N addition increases NPP in an N-limited soil, such as those
301 of semi-arid steppe grasslands (Xu et al., 2014) a coincident uptake of base cations by
302 vigorous plant growth may occur (Tomlinson, 2003). But we did not investigate

303 changes in the cation content of plant tissues in this experiment to confirm this flux.
304 Furthermore, no effect of N addition was observed on soil aggregate K and Na
305 concentrations (although Ca and Mg decreased; Fig. 2, Table 2) indicating the
306 selective depletion of base cations (Lu et al., 2014). The depletion of base cations
307 within soil aggregate can accelerate a reduction in the buffering capacity of soils and
308 accelerate soil acidification (with increased N at ambient water soil pH 6.97 decreased
309 to 6.10, and with added water, 7.17 to 6.42; Wang et al., 2015a); the dynamics of the
310 base cations were affected substantially. Water addition increased EC and
311 exchangeable Na but did not affect the other base cations (Table 2; Fig. 1a, 2). The
312 positive effect of water on soil EC and exchangeable Na might be caused by the
313 relatively high EC value and Na concentration in irrigative water in this calcareous
314 grassland (Table S1). We previously reported that water addition increased soil
315 microbial activity at the study site (Wang et al., 2014, 2015a): if litter decomposition
316 rates were promoted as a consequence (e.g. Liu et al., 2006) Ca, Mg and K may have
317 been released from this secondary biological source to supplement the soil pools.

318

319 *Response of micronutrients to nitrogen and water addition*

320 Micronutrient cycling relies on the balance between input (mineral weathering
321 release), recycling (plant biomass turnover), retention (sorption reactions), and
322 removal (biomass harvesting, fire, and hydrologic leaching) (Li et al., 2008).

323 Long-term N addition caused large increases in extractable Fe, Mn and Cu
324 concentrations in all soil aggregate size classes (by up to 262%, 150%, and 55 %,

325 respectively; Fig. 3a, b, c), presumably due to the decrease in soil pH which may
326 increase micronutrient release through weathering and desorption from soil minerals
327 and SOM (Malhi et al., 1998). Acidification caused by the long-term addition of N
328 negatively affected microbial activity previously observed as the depression of
329 enzyme activity in these experimental soils (Chung et al., 2007; Wang et al., 2014).
330 The mechanism underlying this biological response was not explored but is likely to
331 be complex and due in part to the effect of the changes in micronutrient supply.
332 Long-term irrigation had the opposite effect on extractable Fe and Mn within soil
333 aggregates where increases in soil aggregate pH (which usually decrease the
334 mobilization of micronutrients) were detected compared to ambient precipitation plots
335 (Wang et al., 2014). Our previous study suggested that water addition increased soil
336 pH from 7.1 to 7.3 in CK and from 5.6 to 6.1 in N₁₅ plots for large macroaggregates,
337 from 7.1 to 7.5 in CK and from 5.8 to 6.2 in N₁₅ plots for small macroaggregates, and
338 from 7.1 to 7.4 in CK and from 5.9 to 6.2 in N₁₅ plots for microaggregates (Wang et
339 al., 2015). A previous study carried out at the same plots showed that water addition
340 significantly increased NPP (Xu et al., 2010), suggesting enhanced uptake of growing
341 plants may have reduced concentrations of Fe and Mn in the soil aggregates. This is
342 consistent with the findings of Li et al. (2008) who reported that Mn and boron uptake
343 by plants outpaced their resupply by mineral weathering. However, Fe and Mn may
344 be toxic to plants (Brown and Jones, 1977), so losses by increased leaching may be
345 the most likely route for their decrease in the irrigated plots. However, neither
346 leaching nor plant performance was measured in this experiment.

347

348 **Conclusions**

349 Consistent with our first hypothesis, microaggregates had greater base cation
350 concentrations, which was ascribed to a proportion of sorption provided by increased
351 SOM and clay contents associated with the smallest aggregate size class. However,
352 extractable micronutrients (except Zn) did not follow the same concentration pattern
353 as base cations across three soil aggregate size classes. Under N addition,
354 exchangeable Ca and Mg decreased while extractable micronutrients (Fe, Mn and Cu)
355 increased, which was related to the slight but apparently important decrease in soil pH.
356 Water addition showed no impact on exchangeable Ca, Mg and K, but decreased
357 available Fe and Mn averaging across soil aggregates which partially supported our
358 second hypothesis. Our findings suggest that uncontrolled or long-term N fertilization
359 and irrigation of semi-arid steppe soils cause significant changes in the concentrations
360 of base cations and micronutrients which will ultimately decrease soil fertility,
361 constrain NPP and the nutrient quality of plants. The pathway of the losses (e.g.
362 leaching or uptake into plant tissues followed by harvest) need to be determined in
363 order to manage large and fragile semi-arid grassland systems.

364

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373

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531

532

533 **Tables**

534 **Table 1** The chemical characteristics of bulk soil and soil aggregates without
535 field-manipulated treatments. SOC, TN, C/N, and DOC represent soil organic carbon,
536 total nitrogen, the ratio of SOC to TN, and dissolved organic carbon, respectively.
537 Data are represented as means \pm standard error.

538

	Soil aggregates (mm)			
	Bulk soil	> 2	0.25-2	< 0.25
SOC (g kg soil ⁻¹)	18.9 \pm 1.7	18.2 \pm 1.0	15.1 \pm 2.0	21.6 \pm 1.4
TN (g kg soil ⁻¹)	1.8 \pm 0.2	1.9 \pm 0.1	1.5 \pm 0.2	2.2 \pm 0.1
C/N	10.4 \pm 1.3	9.5 \pm 0.5	10.4 \pm 0.4	10.0 \pm 0.2
pH	7.0 \pm 0.2	7.1 \pm 0.2	7.1 \pm 0.2	7.1 \pm 0.3
DOC	69.8 \pm 5.6	72.5 \pm 6.8	66.6 \pm 1.3	71.2 \pm 1.5
NO ₃ ⁻ -N (mg kg soil ⁻¹)	27.3 \pm 5.9	4.2 \pm 0.1	31.6 \pm 2.2	34.4 \pm 3.8
NH ₄ ⁺ -N (mg kg soil ⁻¹)	9.4 \pm 1.6	17.0 \pm 1.3	17.9 \pm 2.5	22.9 \pm 1.0

539 Data from Wang et al. (2014, 2015a,b)

540

541 **Table 2** Results (*F* values) of ANOVAs with a split-plot design on the effects of N,
 542 water addition (W), and soil fractions (S) on soil electrical conductivity (EC),
 543 exchangeable Ca, Mg, K, Na, effective cation exchange capacity (ECEC, sum of
 544 exchangeable Ca, Mg, K, and Na , cmol kg aggregate⁻¹), and available Fe, Mn, Cu,
 545 and Zn (mg kg aggregate⁻¹).

546

	EC	Ca	Mg	K	Na	ECEC	Fe	Mn	Cu	Zn
W	11.60*	3.07	5.08	0.17	15.6**	4.03	27.8**	14.1*	2.40	3.92
N	14.20**	6.36**	38.7**	0.33	1.71	7.36**	147**	136.3**	44.7**	2.47
S	3.63*	25.0**	168.3**	123.7**	3.49*	30.7**	17.7**	30.1**	33.7**	33.8**
W×N	6.90**	1.01	5.68**	6.81**	1.26	1.25	2.36	3.40*	7.23**	4.34**
W×S	0.28	1.03	3.75*	0.98	0.34	1.03	6.07**	10.8**	6.60**	2.32
N×S	0.77	0.87	0.36	0.88	1.81	0.88	1.59	2.43*	0.85	2.04
W×N×S	0.86	0.44	1.03	1.41	0.61	0.42	0.99	2.54*	1.16	3.84**

547 * and ** indicating the significant level at P<0.05, 0.01, respectively

548

549 **Table 3** Correlation analyses (*R* values) among exchangeable base cations, effective
 550 cation exchange capacity (ECEC), soil organic carbon (SOC), soil pH, and soil
 551 electrical conductivity (EC).

	pH	EC	Ca	Mg	K	Na	ECEC
SOC	0.11	0.19	0.42**	0.73**	0.81**	0.17	0.46**
pH		0.66**	0.35**	0.48**	0.04	0.33**	0.37**
EC			0.28**	0.40**	0.15	0.16	0.29**
Ca				0.60**	0.53**	0.23*	0.998**
Mg					0.77**	0.24*	0.65**
K						0.10	0.57**
Na							0.26*

552 * and ** indicating the significant level at $P < 0.05$, 0.01 , respectively

553

554 **Table S1** Chemical characteristics of the irrigation water.

	Irrigation water
EC (us cm ⁻¹)	196.1±6.4
pH	7.7±0.07
Ca (mmol L ⁻¹)	1.6±0.03
Mg (mmol L ⁻¹)	0.8±0.02
K (mmol L ⁻¹)	0.04±0.002
Na (mmol L ⁻¹)	0.6±0.02

555

556 The symbol ‘-’ indicate the ions were not detected.

557

558 **Figure Legends**

559 **Fig.1** The effect of 9-year of water (ambient vs. water addition) and N additions (0
560 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on (a) electrical conductivity (EC, us
561 cm⁻¹) and (b) effective cation exchange capacity (ECEC, sum of exchangeable Ca, Mg,
562 K, and Na, cmol kg aggregate⁻¹). Data are represented as means ± standard error (n=4).
563 Lowercase letters indicate significant differences between N treatments within a soil
564 aggregate class size and water treatment. The capital letters indicate significant
565 differences between soil aggregate sizes across N and water treatments.

566

567 **Fig.2** The effect of 9-year of water (ambient vs. water addition) and N additions (0
568 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on exchangeable (a) Ca, (b) Mg, (c) K
569 and (d) Na concentrations (cmol kg aggregate⁻¹) in 3 soil aggregate size classes (<0.25
570 mm, 0.25-2 mm, >2 mm). Data are represented as means ± standard error (n=4).
571 Lowercase letters indicate significant differences between N treatments within a soil
572 aggregate size class and water treatment. The capital letters indicate significant
573 differences between soil aggregate sizes across N and water treatments.

574

575 **Fig. 3** The effect of 9-years of water (ambient vs. water addition) and N additions (0
576 [CK], 5 [N₅], 10 [N₁₀], 15 [N₁₅] g N m⁻² yr⁻¹) on DTPA-extractable micronutrients (a)
577 Fe, (b) Mn, (c) Cu and (d) Zn in 3 soil aggregate size classes (<0.25 mm, 0.25-2
578 mm, >2 mm (mg kg aggregate⁻¹). Data are represented as means ± standard error
579 (n=4). Lowercase letters indicate significant differences between N treatments within

580 a soil fraction and water treatment. The capital letters indicate significant differences
581 between soil aggregate sizes across N and water treatments.

582

583 **Fig. 4** The relationships between soil pH and DTPA-extractable (a) Fe (mg kg
584 aggregate⁻¹), (b) Mn (mg kg aggregate⁻¹), (c) Cu (mg kg aggregate⁻¹) and (d) Zn (mg
585 kg aggregate⁻¹) across all N and water treatments within the soil aggregate size
586 classes.

587

588 **Fig. 5** The relationships between KCl-extractable ammonium (mg kg aggregate⁻¹) and
589 effective cation exchange capacity (ECEC, sum of exchangeable Ca, Mg, K, and Na ,
590 cmol kg aggregate⁻¹) within macroaggregates (a) and microaggregates (b) across all N
591 and water inputs.

Fig. 1

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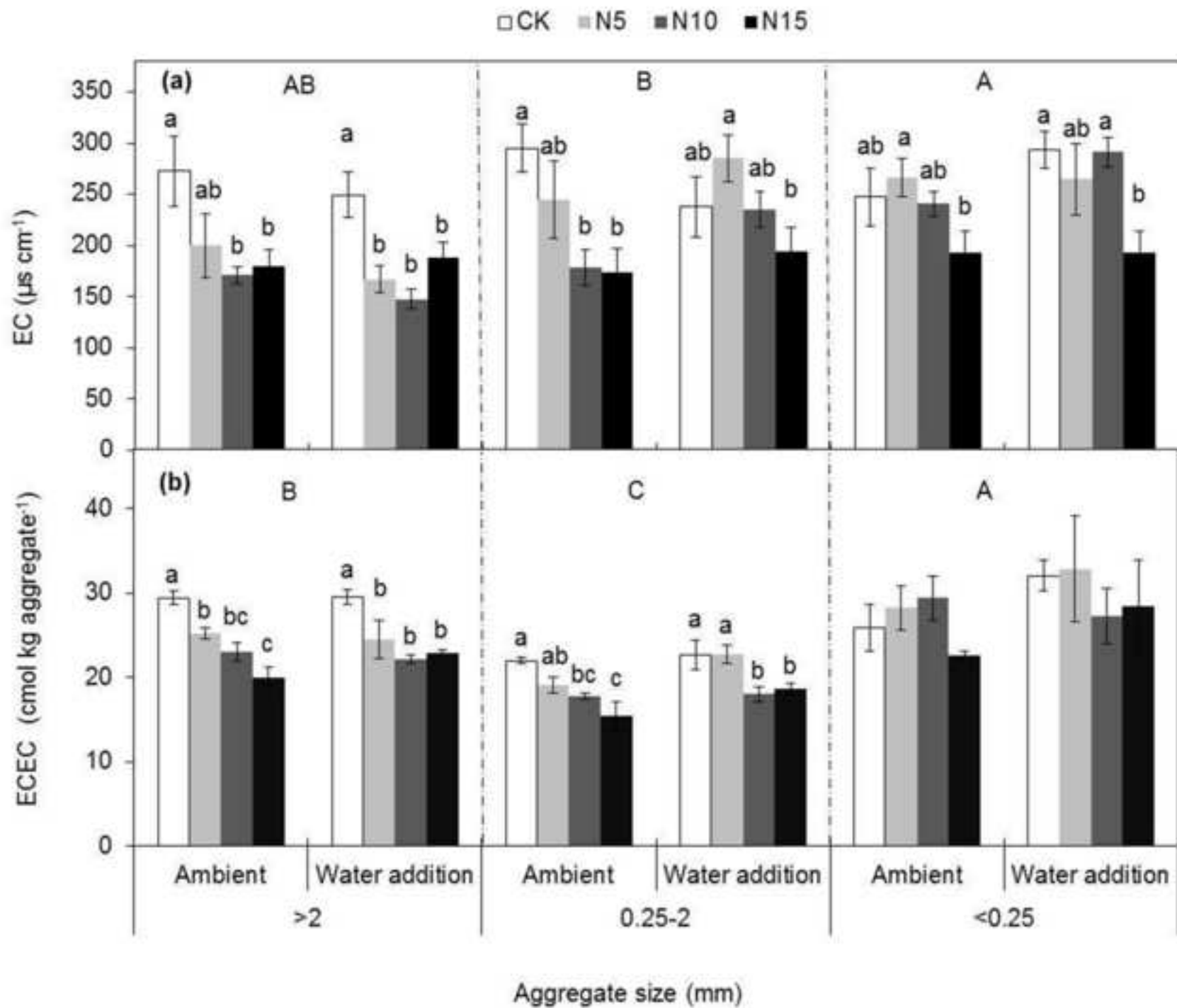


Fig. 2

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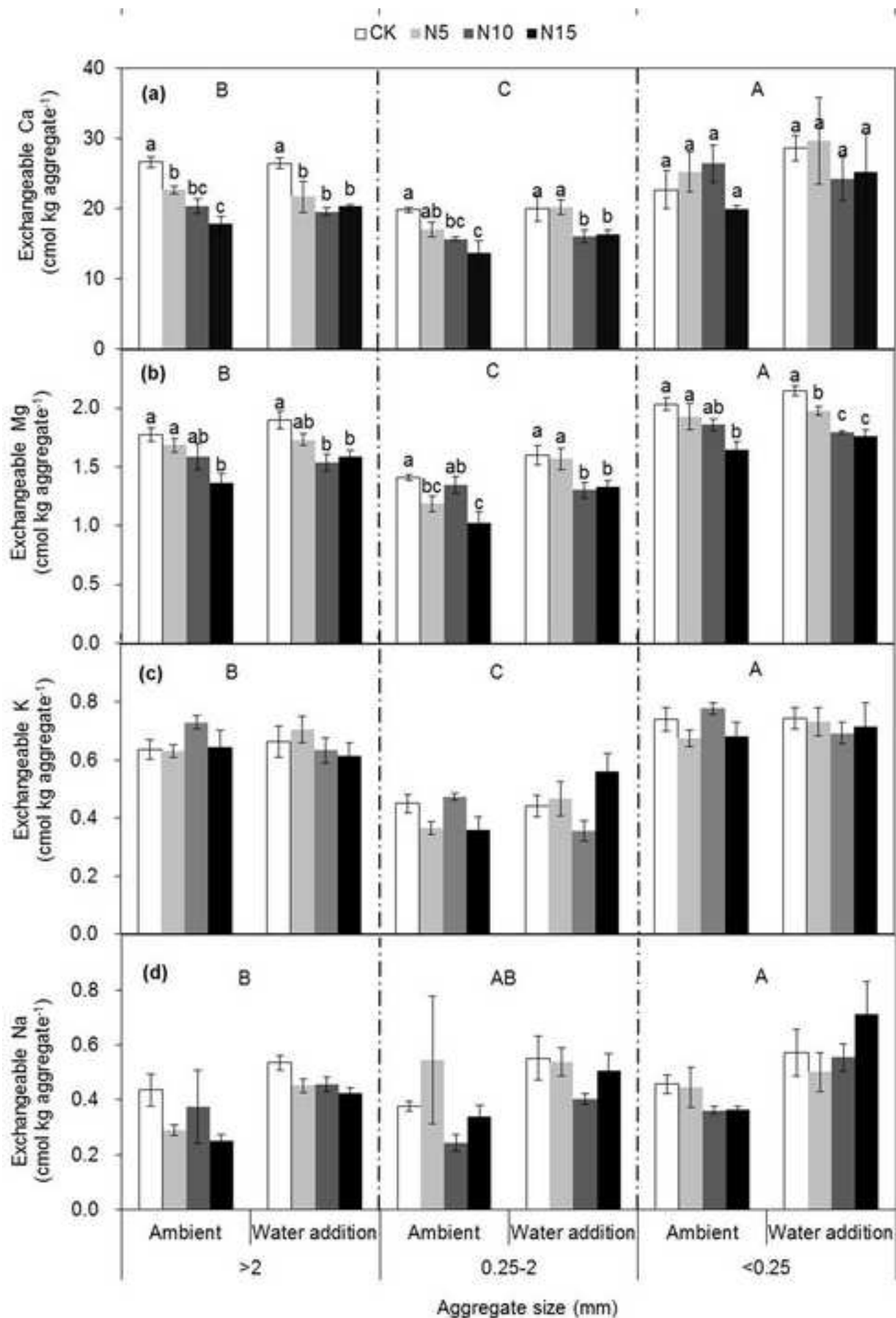


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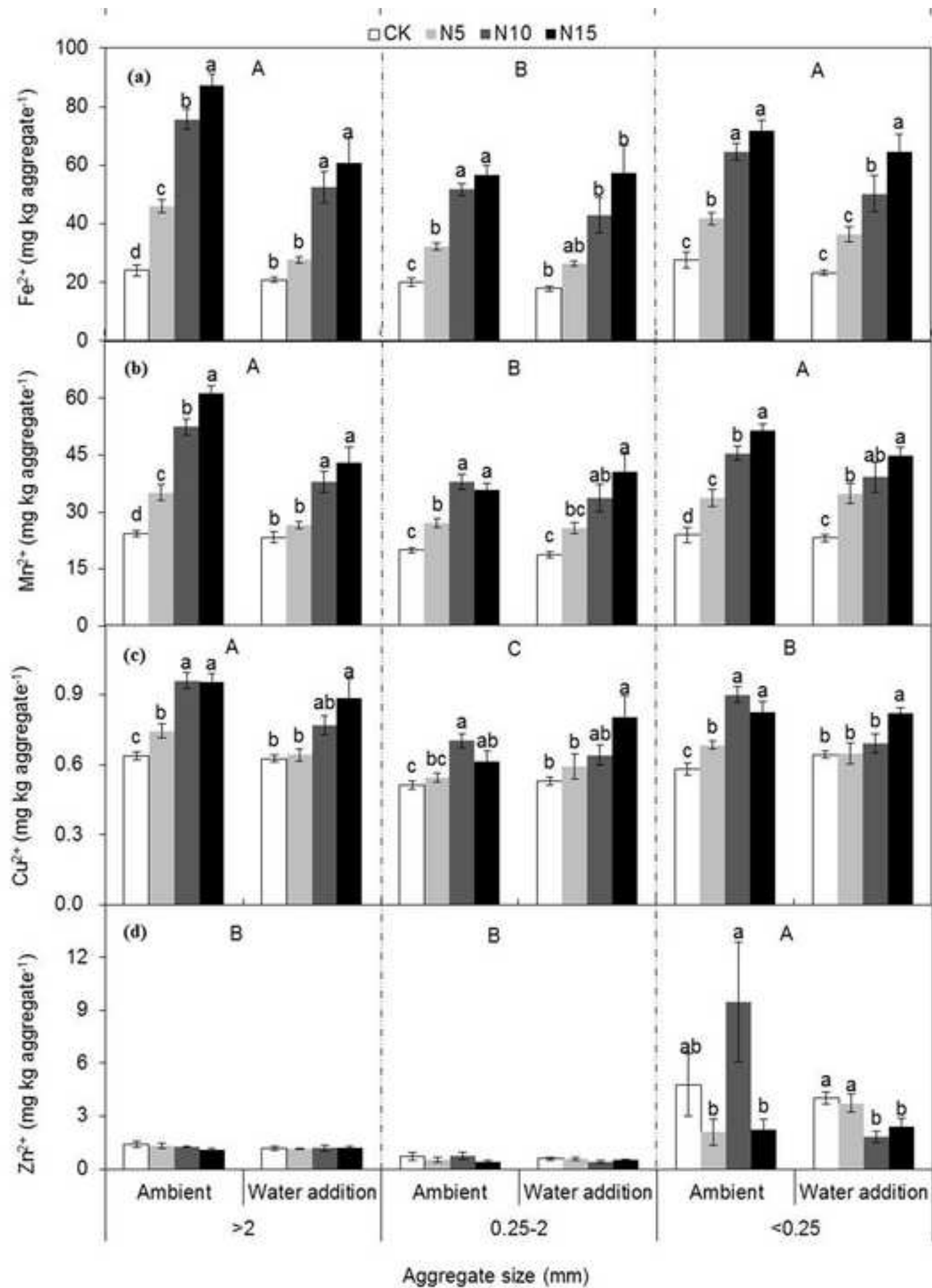


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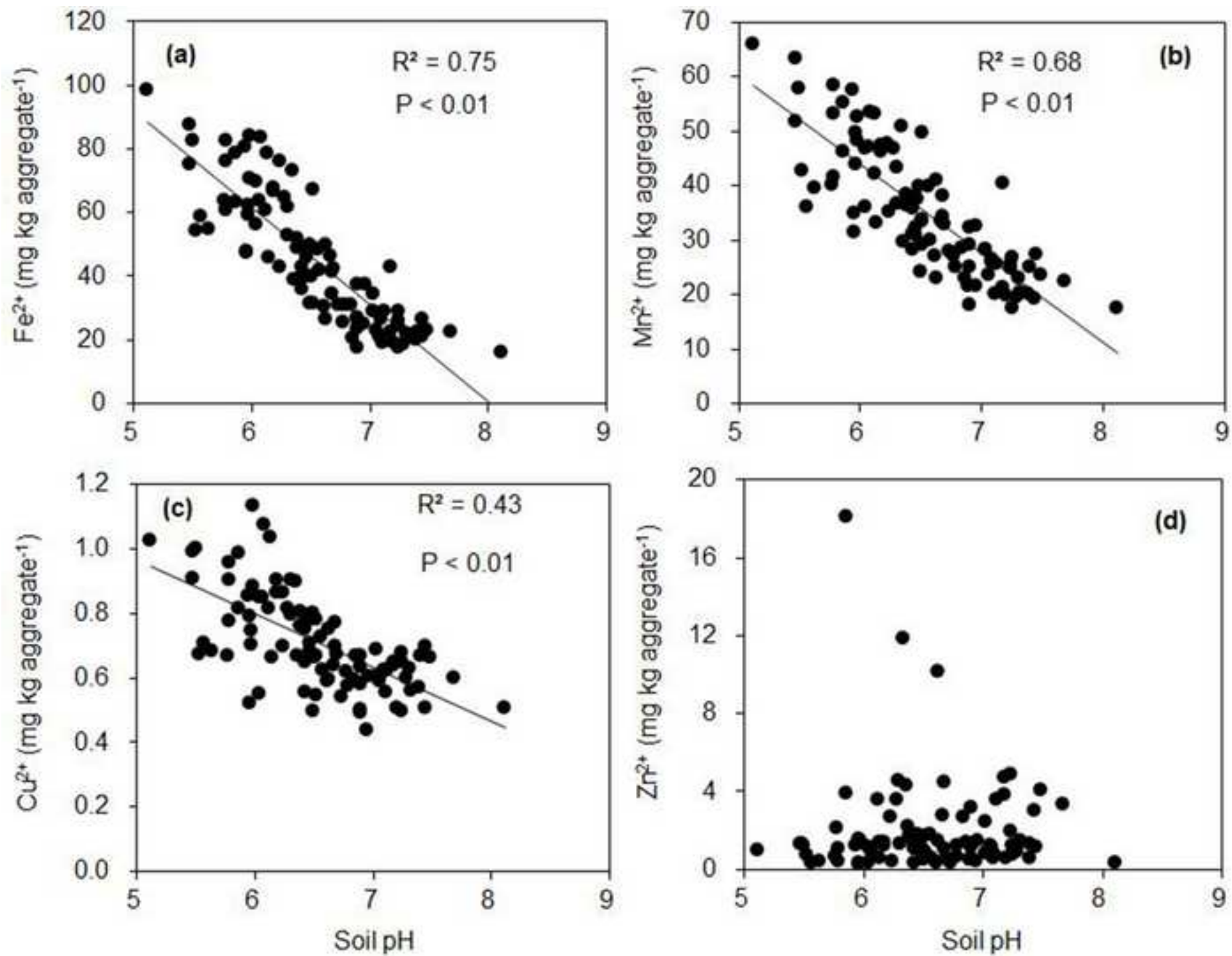


Fig. 5

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