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1 **Cumulative and residual effects of two-phase olive mill waste on olive**
2 **grove production and soil properties**

3

4 **A. López-Piñeiro^{1,*}, A. Albarrán², J. M. Rato Nunes³, D. Peña¹, D.**
5 **Cabrera¹**

6

7 ¹Área de Edafología y Química Agrícola, Facultad de Ciencias, Universidad
8 de Extremadura, Avda de Elvas S/N 06071 Badajoz, Spain.

9 ²Área de Producción Vegetal, Escuela de Ingenierías Agrarias, Universidad de
10 Extremadura, Ctra. de Cáceres, 06071 Badajoz, Spain.

11 ³Escola Superior Agraria de Elvas, Apartado 254, 7350 Elvas, Portugal.

12 *Corresponding author:

13 Antonio López Piñeiro

14 Área de Edafología y Química Agrícola.

15 Facultad de Ciencias.

16 Universidad de Extremadura.

17 Avda de Elvas S/N, 06071 Badajoz, Spain.

18 Phone: +34 924289355

19 Fax: +34 924289355

20 E-mail: pineiro@unex.es

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26 **grove production and soil properties**

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ABSTRACT

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31 Modern olive mill extraction techniques generate a large amount of
32 two-phase olive mill waste (OMW) containing up to 90% organic matter.
33 Recycling OMW as a soil amendment may represent a solution for its
34 disposal, especially in Mediterranean areas, where many soils are
35 characterized by very low organic matter levels. A ten-year field study was
36 conducted to evaluate the long-term sustainability of raw OMW disposal as a
37 soil amendment on an olive grove in Elvas, Portugal. The soil was amended
38 with OMW at rates of 0, 30, and 60 Mg ha⁻¹, dry weight equivalent, for eight
39 years, with cumulative and residual effects being assessed in the last year and
40 two years after the last application. Long-term applications of OMW to soil
41 led to cumulative and residual effects on soil physical (aggregate stability and
42 bulk density), chemical (total organic carbon and its humified fractions, total
43 N, available P, and K), and biochemical (dehydrogenase, urease, β-
44 glucosidase, alkaline phosphatase, and arylsulfatase activities) properties, and
45 contributed to an increase in olive yields compared to the control (> 17%;
46 > 1.47 Mg ha⁻¹). However, electrical conductivity rose significantly with
47 OMW application, especially in the residual year, ranging from 0.513 dS m⁻¹
48 for the unamended soil to 2.29 dS m⁻¹ at the 60 Mg ha⁻¹ application rate.
49 Repeated addition of OMW to olive grove soils may be a useful strategy for
recycling this organic amendment to improve soil fertility and increase yields.

50 However, long-term use of OMW increases the risk of soil salinity, especially
51 if application rates are greater than 30 Mg ha⁻¹.

52

53 **Abbreviations:** ARS, arylsulfatase; CHA; total organic carbon associated
54 with FA; CHA, total organic carbon associated with HA; DH, dehydrogenase;
55 EC, electrical conductivity; FA, fulvic acids; GLU, β-glucosidase; HA,
56 humic acids; PG, polymerization grade; PHO, phosphatase; TOC, total organic
57 carbon content; OMW, two-phase olive mill waste; UR, urease; WSOC,
58 water-soluble organic carbon; WSPH, water soluble phenol.

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INTRODUCTION

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74 In most Mediterranean countries olive oil extraction is one of the
75 foremost agricultural industries, with estimated economic impacts of more
76 than € 6102 million yr⁻¹ (FAO, 2008). However, disposal of olive mill waste
77 represents a major environmental problem in those countries. In Spain, the
78 largest olive oil producer in the world, more than 4,000,000 Mg of organic
79 slurry is generated annually from continuous centrifuge two-phase processing
80 (two-phase olive mill waste, OMW), which has to be properly managed to
81 avoid negative environmental impacts. The utilization of most proposed OMW
82 treatments remains uncertain for economic and technical reasons (Hanifi and
83 Hadrami, 2009). Therefore, with the continued generation of OMW, both the
84 proper disposal and the OMW viable management strategies are imperative.

85 Soils in many Mediterranean areas are generally characterized by low
86 organic matter content, which contributes to their limited fertility and
87 productivity (Albaladejo et al., 1994). Therefore, agricultural practices based
88 on periodic inputs of organic amendments are strongly recommended for
89 Mediterranean agro-ecosystems. Since traditional organic soil amendments
90 (i.e., manures) are often scarce, a wide variety of organic wastes from different
91 sources have become useful inputs for the potential improvement and
92 restoration of soil organic matter and nutrients (Fernández et al., 2009). As
93 OMW contains a large amount of organic matter, it might be useful as an
94 amendment for agricultural soils, thereby potentially lowering inputs of N, P,
95 and K fertilizer (López-Piñeiro et al., 2008). Moreover, the slow degradation

96 rate of OMW after application to soil can make the use of this waste an
97 attractive strategy for soil C sequestration (Sánchez-Monedero et al., 2008).

98 Several studies have focused on the use of composted OMW as a soil
99 amendment, although most were carried out under short-term and/or
100 greenhouse conditions (e.g., Madejón et al., 2001; Altieri and Exposito, 2008;
101 Fornes et al., 2009). Direct application of OMW has been found to increase
102 organic C content, improve both nutrient levels (N, P and K) and aggregate
103 stability, and increase wheat yield up to 198% relative to controls (López
104 Piñeiro et al., 2006). In a short-term field experiment, also conducted with a
105 wheat crop, soil amended with raw OMW improved physico-chemical
106 characteristics and increased wheat yield (Brunetti et al., 2005). Nevertheless,
107 other studies have reported that application of OMW may lead to
108 immobilization of the soil's mineral N and P, thereby creating nutrient
109 deficiencies and reducing crop yield (Thompson and Nogales, 1999; Tejada
110 and González, 2004; López-Piñeiro et al., 2006).

111 In recent studies, short (2 yr) and medium-term (5 yr) positive effects
112 of applying fresh OMW onto an olive grove soil have been observed (López-
113 Piñeiro et al., 2008). However, successive long-term OMW applications to soil
114 could result in an accumulation of toxic compounds such as salts and phenolic
115 substances such that negative effects might be detectable in olive trees and/or
116 soil. Though olive groves are suitable for OMW application, there is little
117 information available on the agronomic and environmental sustainability of
118 this practice. Moreover, to the best of our knowledge, there are no published
119 studies comparing the cumulative and residual effects of repeated applications

120 of raw OMW on soil properties, nutrient status, and olive yield under long-
121 term field conditions. Such information is needed to ensure sustainable use of
122 this abundant resource.

123 The objectives of this field study were to: (1) evaluate the cumulative
124 effects (8 yr) of repeated applications of OMW on olive tree nutritional status
125 and production; (2) assess the influence of repeated applications of OMW on
126 soil physical, chemical, and biochemical properties; and (3) determine residual
127 effects of OMW application on olive yield and soil properties. We
128 hypothesized long-term application of fresh OMW to olive groves would be
129 an attractive strategy both for its disposal and for the restoration of degraded
130 soils.

131

132 **MATERIALS AND METHODS**

133 **Experimental Design**

134 A field experiment was conducted in Elvas, Portugal (38°53' N; 7°9'
135 W; 290 m a.s.l.) on an olive grove (*Olea europaea* L.) amended or unamended
136 with OMW for eight successive years (from 1999 to 2006). The slope gradient
137 at the field site was < 1%. The sandy loam soil, classified as a Typic
138 Haploxeralf (USDA, 1999) or Cutanic Luvisol (ISSS-ISRIC-FAO, 1994), had
139 an alkaline pH, high K and medium P, low organic matter content, and 19.7%
140 clay, 19.7% silt, and 60.6% sand in the 25 cm depth. Climate at the site is
141 semiarid Mediterranean (Papadakis, 1966) with a mean annual precipitation of
142 500 mm occurring mostly in autumn and spring, and a mean annual
143 temperature of 16.7 °C. The grove was established in 1970 with 1-year old

144 cuttings. Trees are clean cultivated and trained using the central leader system
145 (Fontanazza, 2004).

146 Fresh OMW was obtained from an oil industry located near the town
147 of Portalegre, Portugal which employs a two-phase decanter centrifugation
148 system. Characteristics of the soil and two-phase olive mill waste are given in
149 Table 1.

150 The experimental design consisted of nine contiguous plots established
151 in the olive grove, with amendments made in a complete randomized design.
152 Each plot consisted of 12 trees in a 4 x 3 orientation, from which only the
153 central two trees were used for sampling. The three amendment treatments
154 consisted of 30 (T30) and 60 (T60) Mg OMW ha⁻¹ (dry weight equivalent) and
155 a control (T0) (unamended soil). Each treatment was repeated in triplicate.
156 Amendments were applied annually in February (from 1999 to 2006) by
157 spreading the waste manually on the surface, and then incorporating to a depth
158 of about 15 cm with mouldboard plowing.

159 Every olive grove plot was divided into four quadrants. Soil samples
160 (0-25 cm depth) from each plot were obtained by mixing four randomly
161 collected subsamples (one per quadrant) from each plot in December 2006 and
162 December 2008. Soil samples were taken using a hand auger. Field-moist and
163 air-dried samples passed through a 2-mm sieve and were stored at 4 °C until
164 analysis. Since no OMW amendments were added after 2006, measurements
165 made in 2006 and 2008 represented the “cumulative” (T0C, T30C, and T60C
166 samples) and “residual” (T0R, T30R, and T60R samples) effects, respectively.
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169 **Physical and Chemical Analyses of the Soil and OMW**

170 Soil texture was determined by sedimentation using the pipette method
171 (Gee and Bauder, 1998). Aggregate stability of 1- to 2-mm soil aggregates was
172 determined according to Sun et al. (1995), a method which uses a single 0.250
173 mm sieve and an apparatus with a stroke length of 1.3 cm and a frequency of
174 35 cycles min^{-1} . Soil bulk density was determined using undisturbed soil cores
175 (Blake and Hartge, 1998). The soil was sampled at four locations (one per
176 quadrant) in each plot and at 5 depths (5, 10, 15, 20, and 25 cm) in each
177 location, taking the average of these five depth cores. The pH was measured in
178 1:1 (w/v) soil/water and 1:5 (w/v) OMW/water suspensions using a pH-meter
179 with a combination electrode. Electrical conductivity (EC) was measured in
180 saturated soil samples (USDA, 1954). Total organic C content (TOC) was
181 determined by dichromate oxidation (Nelson and Sommers, 1996). Water-
182 soluble organic C (WSOC) was extracted with de-ionized water at 3:1 (water
183 to soil) and 100:1 (water to OMW) ratios. Humic and fulvic acids (HA and
184 FA) were extracted using a solution of 0.1 M $\text{Na}_4\text{P}_2\text{O}_7 + \text{NaOH}$ and a ratio of
185 extractant to soil sample of 10:1. The supernatant was acidified to pH 2 with
186 H_2SO_4 to precipitate humic acids. The WSOC and the TOC associated with
187 each fraction of HA (CHA) and FA (CFA) were determined by dichromate
188 oxidation at an absorbance of 590 nm (Sims and Haby, 1971). The
189 polymerization grade (PG) was calculated as (CHA/CFA). Total N content
190 was determined by the Kjeldahl method (Bremner and Mulvaney 1982).
191 Mineral N as N-NO_3 was extracted using 2M KCl solution (Keeney and

192 Nelson 1982) and measured by steam distillation with MgO and Devarda's
193 alloy. Available P was determined according to the method of Olsen et al.
194 (1954) using the ammonium molybdate-ascorbic acid method described by
195 Murphy and Riley (1962). Available K was extracted by 1M NH₄OAc at pH 7
196 and was assayed by atomic absorption spectrophotometry.

197 Water content of the OMW was calculated from weight loss after oven
198 drying to a constant weight at 105°C. Total P and K in OMW were extracted
199 by Na₂S₂O₇ fusion (Hossner, 1996). Water soluble phenolic substances were
200 determined by the Folin-Ciocalteu colorimetric method (Box, 1983).
201 Cellulose, hemicellulose, and lignin were determined using the acid and
202 neutral fiber detergent method (Goering and Van Soest, 1970).

203

204

Enzyme Activities

205 Dehydrogenase (DH) activity was determined by the method of
206 Trevors (1984) modified by García et al. (1993). One gram of soil was
207 incubated for 20 h at 20°C in the dark with 0.2 ml of 0.4% 2-*p*-iodophenyl-3
208 *p*-nitrophenyl-5 tetrazolium chloride (INT) as substrate. At the end of the
209 incubation idonitrotetrazolium formazan was extracted with 10 ml of
210 methanol and the absorbance measured at 490 nm. The DH and other activities
211 were determined in triplicate.

212 To assay urease (UR) activity, 2 ml of 0.1 M pH 7.0 phosphate buffer
213 and 0.5 ml of 1.066 M urea were added to 0.5 g of soil and incubated for 1.5 h
214 at 30°C. The ammonium produced in the hydrolytic reaction was measured

215 spectrophotometrically at 636 nm (Nanniperei et al., 1980; Kandeler and
216 Gerber, 1988).

217 The activity of β -glucosidase (GLU) was determined by incubating 1 g
218 of soil with 4 ml of 25 mM 4-nitrophenyl- β -d-glucanopyranoside in 0.1 M
219 modified universal buffer (MUB) pH 6.0 (Tabatabai, 1982). For the
220 determination of phosphatase (PHO) activity, 4 ml of 25 mM 4-nitrophenyl
221 phosphate MUB pH 11 was added to 1 g of soil (Tabatabai and Bremmer,
222 1969). For the determination of arylsulfatase (ARS) activity, 4 ml of 5 mM 4-
223 nitrophenyl sulfate in 0.5 M acetate buffer pH 5.8 was added to 1 g of soil
224 (Tabatabai and Bremmer, 1970). The soils were incubated for 1h at 37°C. The
225 samples were then cooled to 2 °C for 15 min to stop the reaction, and the *p*-
226 nitrophenol produced in the enzymatic reactions was determined at 400 nm,
227 398 nm, and 410 nm for GLU, PHO, and ARS, respectively. Blank assays
228 without soil and without substrate were performed at the same time as
229 controls.

230 **Crop Measurements**

231 Treatment effects on the nutritional status of olive trees was
232 determined by leaf analysis of a composite sample of 100 leaves per plot
233 (Beutel et al., 1983) collected in July of 2006 and 2008 during the
234 phenological stage of lignification of the stone (Sanz-Cortés et al., 2002). In
235 the laboratory, leaves were washed with 0.03% triton X-100 (Sigma–Aldrich),
236 rinsed with de-ionized water, and oven dried for 72 h at 65 °C. Dried leaf
237 samples were ground and wet-ashed in a block digester using an H₂SO₄-H₂O₂
238 mixture for N, P, and K determination (Lowther, 1980). Nitrogen content was

239 determined by the Kjeldahl method, P was determined colorimetrically using
240 the method described by Murphy and Riley (1962), and K was determined by
241 atomic absorption spectrophotometry. Tree crops were harvested manually
242 (Ravetti, 2008), and olive production was evaluated by weighing the crop
243 (total fresh weight of fruits) from the central two trees for each replicate plot.

244

245 **Statistical Analyses**

246 Statistical analyses were carried out using SPSS 11.5 for Windows
247 (SPSS Inc., 2002). The experimental design was a completely randomized
248 design with three replicates. A repeated measure ANOVA was conducted for
249 selected soil and crop parameters to assess the effects of treatment. The data
250 were analyzed differently by separating out effects following eight yr of
251 OMW application from those observed two yr after the last application. All
252 pairwise multiple comparisons were performed using the Tukey test.
253 Differences between treatment means were considered statistically significant
254 at $P \leq 0.05$.

255

256 **RESULTS AND DISCUSSION**

257 **Olive Tree Yields and Leaf Nutrient Concentrations**

258 In 2006, following eight yr of annual OMW application, olive
259 production was greater in the amended soils despite high concentrations of
260 potentially toxic organic compounds (e.g., phenolic substances) in soil (Table
261 2; Fig. 1). However, this increase (17% compared to the control) was
262 statistically significant only for the lower OMW rate (T30C) (Table 2). In the

263 residual year (2008), yields were significantly greater (>23%) at both OMW
264 rates compared to the control, but were not different between the T30R and
265 T60R treatments. The present results are similar to those reported previously
266 regarding residual effects of OMW application in two Mediterranean soils,
267 although obtained in a greenhouse experiment using wheat as a response crop
268 (López-Piñeiro et al., 2006). Similar results were also obtained by Brunetti et
269 al. (2005 and 2007) using residues from a three-phase decanter centrifugation
270 processing for oil extraction. Those authors concluded that the increased total
271 content of acidic functional groups in the amended soil's humic acids affected
272 wheat grain yield positively in a short-term (1 yr) field experiment. However,
273 in another field study (Altieri and Esposito, 2008), olive yield was not
274 significantly different between amended and unamended plots, though that
275 study was conducted over a shorter time frame (5 yr) with lower OMW
276 application rate (> 3 times less than in our experiment).

277 The sufficient range for N, P, and K concentration in olive trees leaves
278 of have been estimated at 15 g kg⁻¹, 1 g kg⁻¹, and 8 g kg⁻¹, respectively (Marín
279 and Fernández-Escobar, 1997). The nutritional olive tree status in the
280 unamended soil was characterized by leaf N and K contents below the
281 threshold values for the sufficiency range in both cumulative and residual
282 years (Table 2). This result was to be expected since these soils were
283 unfertilized for ten years. In contrast, the leaf N, P, and K concentration for the
284 amended soils were above the sufficiency threshold values in both the
285 cumulative and residual years, indicating OMW may have compensated for
286 the lack of mineral nutrition in soil. After eight years of repeated OMW

287 application, leaf N contents significantly increased ($P<0.05$) with increasing
288 rates of OMW, indicating that N immobilization did not occur at rates
289 impacting plant availability in our study. This positive effect was even more
290 evident in the residual year (Table 2), despite there not being a significant
291 difference in leaf N concentration between the T30R and T60R treatments
292 (Table 2).

293 A significant ($P\leq 0.05$) positive effect on leaf P and K concentration
294 was also observed after repeated raw OMW applications. At the 60 Mg OMW
295 ha^{-1} rate, relative increases in leaf P were 14% and 12% for the cumulative and
296 residual years, respectively, compared with the control. This suggests not all P
297 was immobilized during the experiment, despite OMW possessing a high C/P
298 ratio. Leaf K content also increased after repeated applications of OMW,
299 although this increase was significant ($P\leq 0.05$) only for the residual year
300 (Table 2). A simple correlation analysis was used to identify relationships
301 between olive yields and leaf nutrient status. This relationship was only
302 significant for leaf N content ($r=0.676$, $P\leq 0.05$), thereby confirming N as a
303 major nutritional factor affecting olive growth in our study.

304 **Physical and Chemical Soil Properties**

305 In comparison with the control, repeated 30 and 60 Mg ha^{-1} OMW
306 application rates significantly increased soil TOC by factors of 2.3 and 3.3,
307 respectively (Table 3). Two years after the last OMW application, TOC
308 remained relatively constant in OMW-amended plots (T30R and T60R,
309 treatments), suggesting moderate to high amounts of OMW were capable of
310 building stable organic matter pools resistant to decomposition. Similarly,

311 repeated raw OMW applications led to significant positive effects on HA, FA,
312 and WSOC (Table 3). Compared to the control, amended soils had greater
313 HA, FA, and WSOC, with higher percentages attained at the 60 Mg ha⁻¹
314 application rate (74%, 94%, and 244% greater for HA, FA, and WSOC,
315 respectively). Two years after the last OMW application HA content
316 significantly increased, while FA and WSOC contents decreased (Table 3).
317 Compared with the control, HA increased by factors of 1.7 and 3.2 for the
318 cumulative and residual years, respectively, at the higher rate of OMW
319 application. Conversely, FA content was much lower in the residual year at
320 both rates of OMW, and may have been attributed to its higher degradability
321 and/or to its transformation into more complex molecules such as HAs (De
322 Nobili and Petrusi, 1988; Fernandez et al., 2007). Moreover, the
323 polymerization grade (PG) of OMW-amended soils increased significantly in
324 the residual year. These results suggest repeated raw OMW amendments can
325 lead to an increase in the native soil organic matter stability by increasing the
326 humified organic matter fraction, which represents a positive outcome in the
327 context of beneficial recycling of OMW. For that reason, it is possible OMW
328 amendments would contribute to improving soil properties and controlling
329 degradation processes. Indeed, many authors argue that erosion is the principal
330 problem associated with olive production (Fleskens and Stroosnijder, 2007),
331 thereby emphasizing the importance of increasing organic matter and
332 improving soil properties.

333 In the residual year, a sharp decrease in WSOC was observed in the
334 OMW treated soils. The T60R treatment maintained WSOC values

335 significantly higher than the control. Therefore, OMW could result in an
336 increased risk of aquifer contamination, even two years after the last
337 application. Consequently, safe OMW application should be restricted to areas
338 not directly connected to drinking water aquifers.

339 Eight years of continued application of OMW (Table 3) led to a
340 significant linear increase in total N ($r=0.914$, $P\leq 0.001$). Similarly, inorganic
341 N concentrations increased with increasing OMW rates compared to the
342 control. This result is in agreement with that reported following OMW
343 addition to the same soil in a short-term greenhouse and field experiment
344 (López-Piñeiro et al., 2006 and 2008). Indeed, similar trends were detected in
345 the residual year, supporting observations of increased N concentration in the
346 leaves following OMW treatment. Furthermore, the increase in inorganic N
347 suggests immobilization of mineral N did not occur during labile C
348 degradation of OMW compounds. Nevertheless, while increased inorganic N
349 can result in improved yields, it can negatively affect the quality of both olive
350 oil (Fernández-Escobar et al., 2006) and groundwater (Fernández-Escobar et
351 al., 2004).

352 Application of OMW led to a significant linear increase in available P
353 in the cumulative ($r=0.925$, $P\leq 0.001$) and residual ($r=0.939$, $P\leq 0.001$) years.
354 The available P content increased more in the residual year than in the
355 cumulative year. In particular, the increase for the 30 Mg ha⁻¹ treatment was
356 from 14.0 (T0C) to 37.6 mg kg⁻¹ in the cumulative year, but from 10.7 (T0R)
357 to 52.9 mg kg⁻¹ in the residual year. For the 60 Mg ha⁻¹ treatment, the increase
358 was from 14.0 (T0C) to 60.3 mg kg⁻¹ in the cumulative year, but from 10.7

359 (TOR) to 75.8 mg kg⁻¹ in the residual year (Table 3). The trend in available P
360 was consistent with the increase in leaf P concentration following the OMW
361 application. The increase in soil available P content with OMW application
362 may not only provide agronomic benefits but also help resolve problems
363 related to the P fixation frequently observed on calcareous soils (Sharpley et
364 al., 1989). However, accumulation of labile P in soil may increase the
365 concentration of dissolved P in surface run-off (Ajiboye et al., 2004).

366 Compared to the control, increases of available K in the cumulative
367 year were 2.2 and 4.0 times greater at the 30 and 60 Mg ha⁻¹ OMW rates,
368 respectively (Table 3). The increases in available K are consistent with results
369 of short-term studies using treated and untreated residues from three-phase
370 (Madejón et al. 2003; Montemurro et al. 2004) and two-phase decanter
371 processes (Tejada and González, 2004; López-Piñeiro et al., 2006 and 2008).
372 Increased available K was also detectable two years after the last OMW
373 application, confirming that this residue could act as an alternative for K
374 fertilizer. Moreover, the observed increase in available K could improve the
375 tolerance of the olive trees to various stress situations, including drought
376 (Tisdale et al., 1999).

377 Soil pH was only significantly affected by OMW at the higher
378 application rate, decreasing from 8.0 to 7.5 after eight years of repeated
379 applications (Table 3). In the residual year, unamended and OMW-amended
380 soils showed similar pH values, reflecting the strong buffering capacity of the
381 soil under study (Tisdale et al., 1999). These results are consistent with those
382 obtained in previous short-term studies (López-Piñeiro et al., 2006), where a

383 significant ($P \leq 0.05$) relationship between the application rate of OMW and pH
384 was only found in acidic soils.

385 Application of OMW significantly increased water soluble phenol
386 (WSPH) content (Fig. 1). In the cumulative year, WSPH was 1.8 and 3.5 times
387 greater for the T30C and T60C treatments, respectively, relative to the control.
388 Two years following the last OMW application, WSPH content decreased in
389 the amended soils, a result likely caused by microbial degradation and/or
390 organic matter incorporation (Sierra et al., 2007). This finding was consistent
391 with data reported by Mekki et al. (2007), where phenol compounds decreased
392 rapidly in soils amended with OMW. Despite the decrease in WSPH in the
393 residual year, WSPH content was greater in the T60R treatment compared to
394 the T30R or non-amended control.

395 Plots amended with the higher OMW rate possessed electrical
396 conductivity (EC) values significantly greater than control plots (Fig. 2).
397 However, differences in EC between the amended soils and the control
398 increased significantly in the residual year. The increase in the residual year
399 could be attributable to the release of soluble organic and inorganic species
400 during the humification of OMW (Table 3). The EC increases with OMW
401 application may affect yields of salt-sensitive crops, and therefore may be a
402 cause for concern.

403 The application of raw OMW significantly increased the percentage of
404 water stable aggregates (WSA) (Table 3). Similar improvements in WSA were
405 also observed after addition of fresh OMW in greenhouse and short-term field
406 experiments (Tejada and González, 2004; López-Piñeiro et al., 2006). This

407 effect was maintained in the field as it was still evident two years after the last
408 OMW application. For both cumulative and residual treatments, WSA was
409 greater at the higher OMW application rate. Good soil structure is important
410 for sustaining long-term crop production because it influences water status,
411 nutrient availability, crop growth and development, and resistance to erosion
412 (Piccolo and Mbagwu, 1999). The increased aggregate stability may be
413 attributed to organic matter input from OMW application (Tejada and
414 González, 2004; García-Orenes et al. (2004).

415 The effectiveness of OMW in enhancing WSA was significantly
416 greater in the residual than the cumulative year. Average increases in WSA
417 over the unamended plots were 64% and 99%, for the T60C and T60R
418 treatments, respectively. Water stable aggregates were correlated positively
419 with HA ($r=0.964$, $P\leq 0.001$) and TOC ($r=0.873$, $P\leq 0.01$), suggesting humic
420 substances may be involved in soil structural stability. This finding was
421 consistent with previous reports indicating that humic acids play a significant
422 role in soil aggregate stability through the formation of clay-organic
423 complexes (Piccolo et al., 1997; Pikul et al., 2009; Tejada et al., 2009).

424 Compared with the control, soil bulk density (BD) decreased by 27%
425 and 31% at the higher OMW rate in the cumulative (T60C) and residual
426 (T60R) years, respectively (Table 3). A significant ($P\leq 0.001$) negative
427 relationship was observed between BD and TOC ($r=0.914$) and WSA
428 ($r=0.941$), suggesting the observed decrease in BD was associated with higher
429 organic matter content and better aggregation due to OMW application.

430 Several authors have also reported a decrease in BD with the addition of
431 organic amendments (e.g., Celik et al., 2004; Hati et al., 2007).

432 **Enzyme Activities**

433 Dehydrogenase (DH) is involved in redox soil reactions and is
434 considered to be a measure of the soil's microbiological activity (Nannipieri et
435 al., 2003; Moreno et al., 2009). The DH activity increased significantly
436 following application of OMW (Fig. 3A). Compared to the control, DH
437 activity increased by about 70% and 115% in the cumulative year and by
438 about 29% and 49% in the residual year, at the 30 and 60 Mg ha⁻¹ OMW rates,
439 respectively. The addition of a readily-available C source from OMW could
440 promote the growth of indigenous microorganisms, and therefore result in
441 increased synthesis of DH. Similar responses in enzyme activities have been
442 observed upon addition of other organic amendments (Herrero et al., 1998;
443 Kotsou et al., 2004). Although DH activity was always greater in amended
444 than in unamended soils, it decreased significantly at the higher application
445 rate two years after the last OMW application. The decrease in DH activity
446 may have been due to a lack of labile organic matter, which had most likely
447 been decomposed by the residual year.

448 Beta-glucosidase (GLU) plays an important role in hydrolytic
449 processes during organic matter decomposition (Acosta-Martínez et al., 2008;
450 Stott et al., 2010). Beta-glucosidase activity increased with increasing OMW
451 application rate (Fig. 3B). Furthermore, as GLU is mainly produced by fungi
452 (Perucci, 1992), its increased activity suggests the presence of OMW caused a
453 shift in the relative proportions of fungi and bacteria. These results are in

454 agreement with those reported by Mechri et al. (2007), who found a significant
455 increase in fungi after OMW water application.

456 Olive mill waste application significantly increased urease (UR)
457 activity compared with the control (Fig. 3C), although residual effects were
458 less pronounced than cumulative effects. In particular, UR activity was greater
459 than the unamended soil by factors of 4.8 and 3.4 at 60 Mg ha⁻¹ for the
460 cumulative and residual years, respectively. The high concentration of
461 available substrate coupled with the demand for nutrients by vegetation or
462 microorganisms could lead to a high activity of these enzymes during OMW
463 mineralization (García-Gil et al., 2000; Fernández et al., 2009). Indeed, UR
464 activity was significantly correlated with total N ($r=0.943$, $P\leq 0.001$), TOC
465 ($r=0.969$, $P\leq 0.001$), and leaf N content ($r=0.908$, $P\leq 0.001$). The UR increases
466 were similar to those reported by Fernández et al. (2009) in a short-term study
467 where a Mediterranean soil was amended with two types of sewage sludge.
468 However, in a laboratory study using OMW water amended soils (Piotrowska
469 et al., 2006), UR activity increased much less than in our study due to less
470 total N applied.

471 Similarly to UR, phosphatase (PHO) activity increased by factors of
472 2.7 and 2.8 at 60 Mg ha⁻¹ compared to the control for the cumulative and
473 residual years, respectively (Fig. 3D). Likewise, PHO activity was
474 significantly correlated with available P ($r=0.886$, $P\leq 0.001$), and TOC
475 ($r=0.977$, $P\leq 0.001$).

476 A significant increase in arylsulfatase (ARS) activity was observed in
477 OMW amended soils in the cumulative and residual years, independent of

478 application rate (Fig. 3E). The residual effect of OMW application on ARS
479 activity was more pronounced than the cumulative effect. Compared with the
480 control, ARS activity increased by about 120% and 254% in the cumulative
481 and the residual years, respectively. Arylsulfatase activity was positively
482 correlated with HA ($r=0.891$, $P\leq 0.001$), PG (0.790 , $P\leq 0.001$), and TOC
483 ($r=0.760$, $P\leq 0.01$). Significant correlations between enzyme activities and
484 humic substances have been previously reported (Nannipieri et al., 1996;
485 Cayuela et al., 2008), suggesting that the relationship between ARS activity
486 and humified organic matter could be attributed to the protection of enzymes
487 by humic-like substances.

488 According to Verstraete and Voest (1977), positive relationships
489 between soil enzyme activities and crop yields are to be expected. In our study
490 olive yields were significantly correlated with DH ($r= 0.513$, $P<0.05$), GLU
491 ($r=0.587$, $P<0.05$), PHO ($r=0.712$, $P<0.01$), and UR ($r=0.753$, $P<0.01$),
492 confirming that a measurable association existed between crop yield and soil
493 microbial activity (Antolín et al., 2005). These relationships may be
494 considered indicative of improved soil conditions for crop growth (Madejón et
495 al., 2003).

496

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CONCLUSIONS

498 Long-term application of raw OMW to soil had positive effects on
499 olive yields and soil physical, chemical, and biochemical properties. In
500 particular, OMW led to an increase of total organic carbon and humic
501 fractions, which may contribute to improved soil quality and fertility.

502 Therefore, use of OMW on olive groves as an organic amendment may be
503 considered a sound management strategy for its disposal and for restoration of
504 degraded olive grove soils. However, results from this study suggest OMW
505 application should not exceed 30 Mg ha⁻¹ in order to mitigate concerns
506 associated with soil salinity.

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Table 1. Initial characteristics of the soil and two-phase olive mill waste (OMW).[†]

Properties	Unit	Soil	OMW
Gravimetric water content (w/w)	(g kg ⁻¹)		480.20
Water stable aggregates (1-2 mm)	(%)	26.2	
Organic carbon	(g kg ⁻¹)	13.3	535
pH		8.10	5.70
Total N	(g kg ⁻¹)	1.49	16.0
Electrical conductivity	(dS m ⁻¹)	0.513	5.02
P	(g kg ⁻¹)	0.013 [‡]	2.75 [¶]
K	(g kg ⁻¹)	0.200 [§]	15.0 [#]
Water-soluble phenols	(g kg ⁻¹)	0.016	7.3
Lignin	(g kg ⁻¹)		19.6
Hemicellulose	(g kg ⁻¹)		19.1
Cellulose	(g kg ⁻¹)		22.8

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[†]All concentrations are on an air-dried basis except gravimetric water content which is on an oven-dried basis.

[‡]Available P.

[§]Available K.

[¶]Total P.

[#]Total K.

847 Table 2. Cumulative and residual effects of repeated applications of two-phase olive mill waste (OMW) on
 848 yield and leaf N, P, and K concentrations.

	Units	----- Year 2006 -----			----- Year 2008 -----			Analysis of variance		
		T0C [†]	T30C [‡]	T60C [§]	T0R [¶]	T30R [#]	T60R ^{††}	T ^{‡‡}	S ^{§§}	T×S
Yield	(Mg ha ⁻¹)	8.55a ^{¶¶} (1.00) ^{###}	10.2b (0.69)	9.11ab (0.15)	7.13a (0.88)	8.60b (0.40)	8.83b (0.32)	**	**	NS
N concentration	(g kg ⁻¹)	12.3a (0.41)	15.2b (0.21)	15.9c (0.60)	12.0a (1.12)	15.3b (0.55)	16.2b (0.87)	***	*	NS
P concentration	(g kg ⁻¹)	1.24a (0.27)	1.36ab (0.05)	1.42b (0.02)	1.75a (0.18)	1.93b (0.07)	1.97b (0.04)	**	***	*
K concentration	(g kg ⁻¹)	7.83a (0.35)	8.21a (0.95)	8.19a (1.04)	7.58a (0.44)	9.23b (0.64)	9.54b (0.77)	*	*	NS

849 [†]T0C= unamended soil, year 2006.

850 [‡]T30C= amended soil at rate of 30 Mg (dry weight) ha⁻¹ yr⁻¹ for eight years.

851 [§]T60C= amended soil at rate of 60 Mg (dry weight) ha⁻¹ yr⁻¹ for eight years.

852 [¶]T0R= unamended soil, year 2008.

853 [#]T30R= amended soil at rate of 30 Mg (dry weight) ha⁻¹ yr⁻¹, two years after the last application.

854 ^{††}T60R= amended soil at rate of 60 Mg (dry weight) ha⁻¹ yr⁻¹, two years after the last application.

855 ^{‡‡}T= treatment.

856 ^{§§}S= sampling time.

857 ^{¶¶}Values with the same letter within a row, for a given sampling date, are not significantly different at
 858 P≤0.05 level of probability.

859 ^{###}Standard deviation of the mean.

860 *, **, *** significant at 0.05, 0.01, and 0.001 probability levels, respectively; NS is not significant.

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86Table 3. Cumulative and residual effects of repeated applications of two-phase olive mill waste (OMW) on
863selected soil properties (0-25 cm depth).

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Properties	Units	----- Year 2006 -----			-----Year 2008 -----			Analysis of variance		
		T0C [†]	T30C [‡]	T60C [§]	T0R [¶]	T30R [#]	T60R ^{††}	T ^{‡‡}	S ^{§§}	TxS
Organic carbon	(g kg ⁻¹)	11.07a ^{¶¶} (0.55) ^{###}	26.16b (1.51)	36.42c (2.45)	10.26a (0.30)	23.93b (3.46)	33.83c (2.00)	***	*	NS
Total N	(g kg ⁻¹)	1.53a (0.07)	2.77b (0.10)	3.33b (0.42)	1.12a (0.20)	2.57b (0.51)	3.39b (0.36)	**	NS	NS
NO ₃ -N	(mg kg ⁻¹)	6.79a (0.34)	16.8b (0.57)	21.2c (0.59)	5.91a (0.32)	17.3b (0.37)	20.7c (0.60)	***	**	***
WSOC ^{†††}	(mg kg ⁻¹)	142a (5.3)	224b (31.5)	489c (39.7)	122a (12.6)	152a (11.7)	189a (22.8)	***	**	**
Humic acid	(g kg ⁻¹)	1.35a (0.14)	1.89b (0.15)	2.35c (0.09)	1.27a (0.10)	2.53b (0.17)	4.03c (0.20)	***	***	***
Polymerization grade		1.54b (0.12)	1.24a (0.06)	1.37a (0.04)	1.52a (0.07)	3.06b (0.19)	3.71b (0.40)	*	**	*
pH		8.00a (0.03)	7.80a (0.08)	7.51b (0.19)	8.03a (0.10)	8.01a (0.10)	7.91a (0.11)	*	***	*
Available P	(mg kg ⁻¹)	14.0a (1.1)	37.6ab (1.6)	60.3b (2.3)	10.7a (1.5)	52.9b (2.3)	75.8c (2.5)	**	*	*
Available K	(mg kg ⁻¹)	351a (22.5)	780b (32.1)	1404c (156)	376a (35.4)	858b (82.5)	1386c (101.4)	**	NS	NS
Bulk density	(Mg m ⁻³)	1.45c (0.05)	1.25b (0.05)	1.06 ^a (0.06)	1.47b (0.10)	1.06a (0.01)	1.01a (0.02)	***	**	**
WSA ^{†††}	(%)	31.6a (3.8)	45.4ab (6.9)	51.9b (1.97)	32.7a (5.70)	50.1b (1.70)	65.2b (3.20)	*	*	*

865 [†]T0C= unamended soil, year 2006.

866 [‡]T30C= amended soil at rate of 30 Mg (dry weight) ha⁻¹ yr⁻¹ for eight years.

867 [§]T60C= amended soil at rate of 60 Mg (dry weight) ha⁻¹ yr⁻¹ for eight years.

868 [¶]T0R= unamended soil, year 2008.

869 [#]T30R= amended soil at rate of 30 Mg (dry weight) ha⁻¹ yr⁻¹, two years after the last application.

870 ^{††}T60R= amended soil at rate of 60 Mg (dry weight) ha⁻¹ yr⁻¹, two years after the last application.

871 ^{‡‡}T= treatment.

872 ^{§§}S= sampling time.

873 ^{¶¶}Values with the same letter within a row, for a given sampling date, are not significantly different
874 at $P \leq 0.05$ level of probability.

875 ^{##} Standard deviation of the mean.

876 ^{†††}WSOC= water soluble organic carbon.

877 ^{†††}WSA= water stable aggregates.

878 *, **, *** significant at 0.05, 0.01, and 0.001 probability levels, respectively; NS is not significant.

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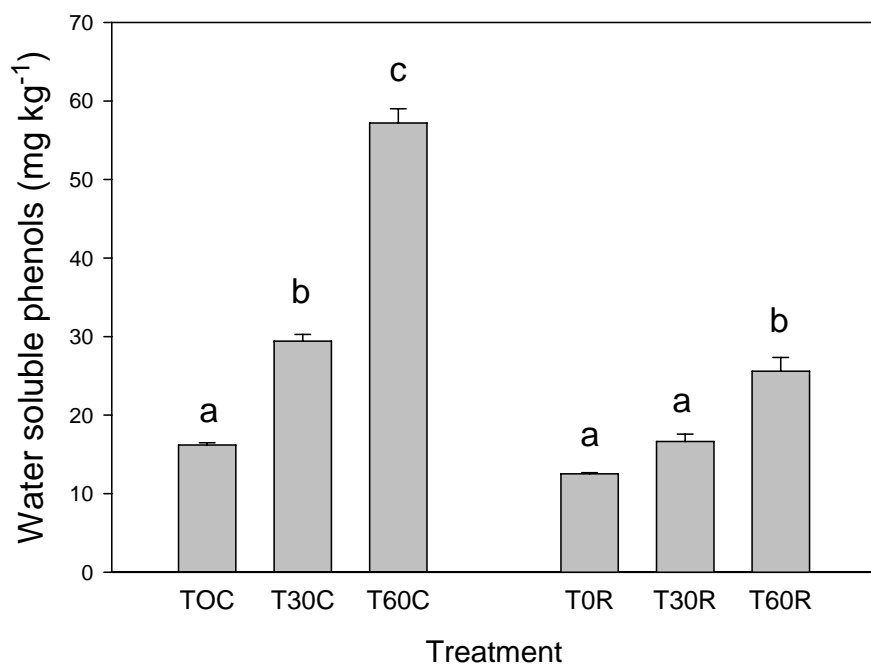
List of Figures

Fig. 1. Cumulative and residual effects of repeated applications of two-phase olive mill waste on water soluble phenols. (T0C) unamended soil year 2006; (T30C) and (T60C) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹ for eight years, respectively; (T0R) unamended soil, year 2008; (T30R) and (T60R) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹, respectively, two years after the last application. Bars with the same letter are not significantly different at P≤0.05 level of probability. Error bars represent one standard error of the mean.

Fig. 2. Cumulative and residual effects of repeated applications of two-phase olive mill waste on electrical conductivity. (T0C) unamended soil year 2006; (T30C) and (T60C) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹ for eight years, respectively; (T0R) unamended soil, year 2008; (T30R) and (T60R) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹, respectively, two years after the last application. Bars with the same letter are not significantly different at P≤0.05 level of probability. Error bars represent one standard error of the mean.

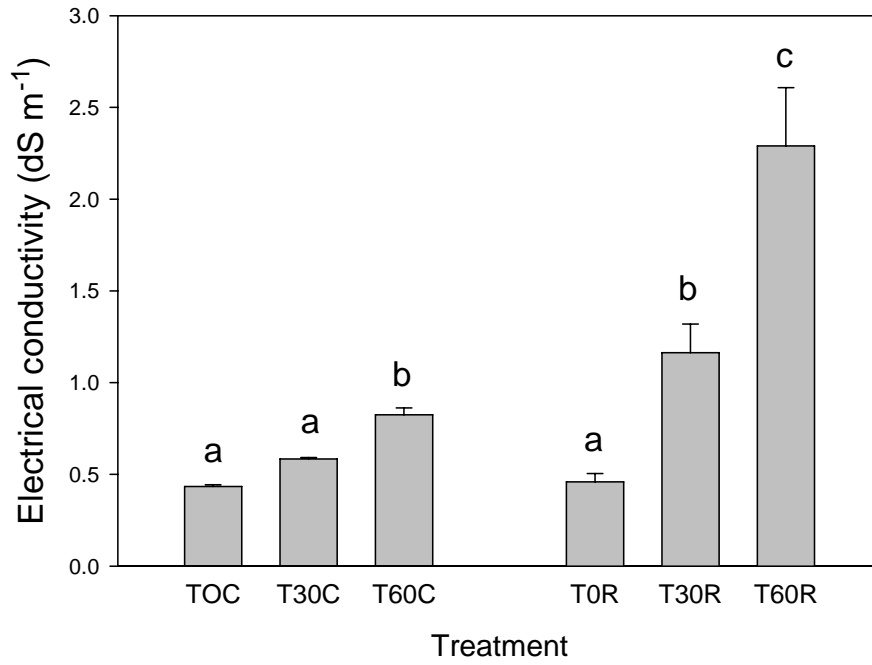
Fig 3. Cumulative and residual effects of repeated applications of two-phase olive mill waste on dehydrogenase (A), β-glucosidase (B), urease (C), alkaline phosphatase (D), and arylsulfatase (E). (T0C) unamended soil year 2006; (T30C) and (T60C) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹ for eight years, respectively; (T30R) unamended soil, year 2008; (T30R) and (T60R) amended soils at rates of 30 and 60 Mg ha⁻¹ yr⁻¹, respectively, two years after the last application. Bars with the same letter are not significantly different at P≤0.05 level of probability. Error bars represent one standard error of the mean.

918 Fig. 1.
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935 Fig. 2.



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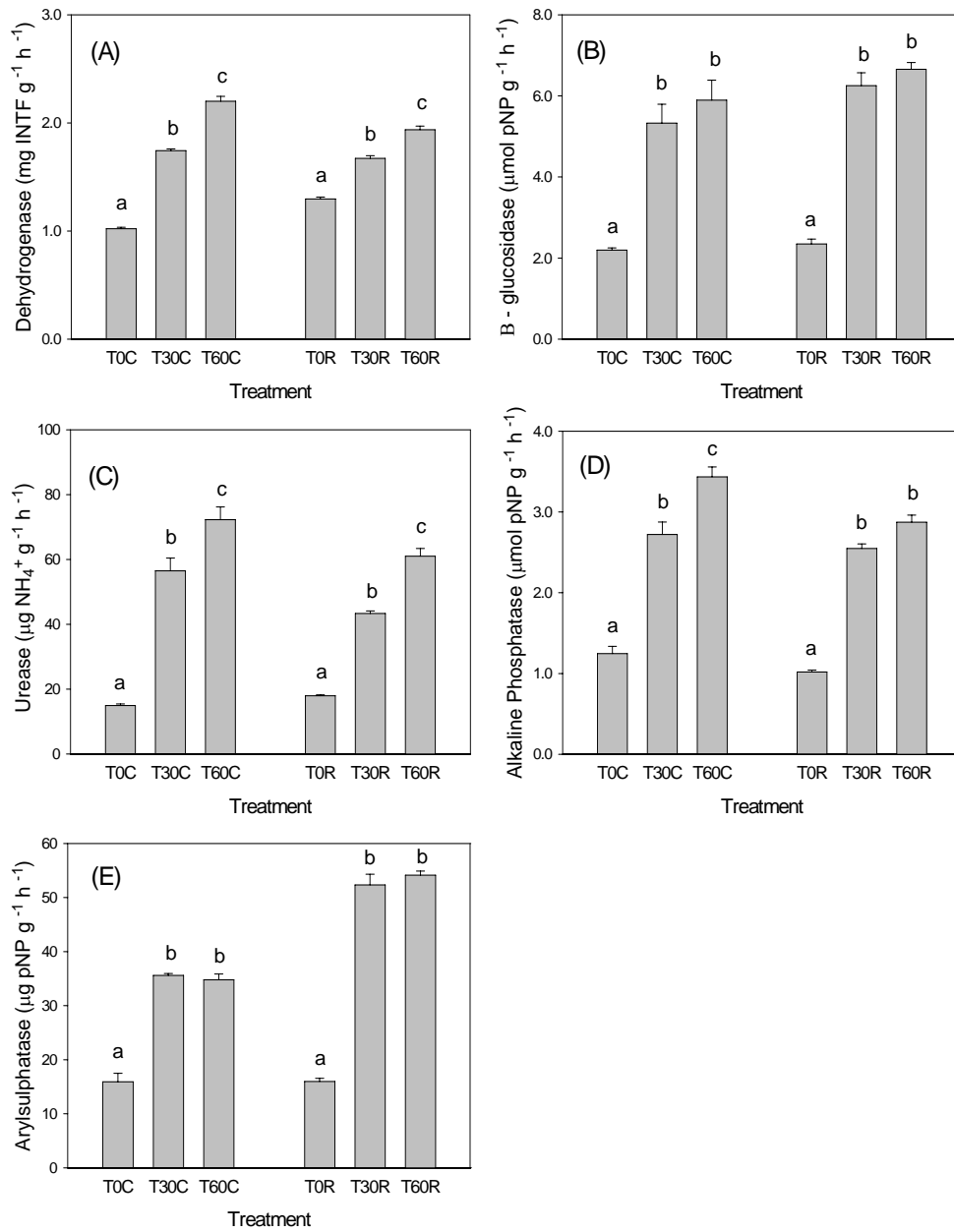
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950 Fig. 3.
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