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1 ***Trametes versicolor*: potential for atrazine bioremediation in calcareous clay soil,**
2 **under low water availability conditions**
3

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16 This manuscript includes 2 figures and 3 tables.

17

18 **Scientific relevance:** We investigated the feasibility of *T. versicolor* for actively
19 degrading atrazine (at usual field application rates) in non-sterile calcareous clay soil of
20 South Portugal, under low water availability (-0.7 and 2.8 MPa) and with scarce organic
21 matter content. Results strongly suggested that this species could potentially be used for
22 bioremediation of soil treated with triazine herbicides in semi-arid and Mediterranean-
23 like ecosystems. As far as we are aware, very little work has looked at the influence of
24 soil water potential on triazine biodegradation rates by white rot fungi in non-sterile
25 soil, *T. versicolor* in particular. We therefore consider our work to be an important
26 contribution in the field of applied environmental microbiology.

Abstract

This study has examined the feasibility of *Trametes versicolor* for actively degrading atrazine (0.5 µg g^{-1}) in non-sterile calcareous clay soil (Algarve, Portugal) microcosms for up to 24 weeks (20°C), under low water availability (soil water potentials of -0.7 and -2.8 MPa). Soil respiration, enzymatic (dehydrogenase and laccase) activities and atrazine quantification by high-performance-liquid-chromatography (HPLC) were assessed.

Respiration and dehydrogenase activity (DHA) were significantly ($p<0.05$) enhanced in soil containing the inoculant, particularly in the presence of atrazine, indicating that it remained metabolically active throughout the study. Furthermore, up to 98 and 85% (at -0.7 and -2.8 MPa respectively) of atrazine was degraded in soil containing both atrazine and the inoculant, compared to 96 and 50% in soil containing atrazine only. The contribution of *T. versicolor* to atrazine degradation was only significant ($p<0.005$) under the driest soil treatment conditions. The strategies used for enhancing colonisation and biodegradation capabilities of the inoculant, as well as the selection of sawdust as carrier were thus effective. However, there were no differences ($p>0.05$) in quantified laccase activity in soil containing the inoculant and the control. Overall, this study demonstrates that *T. versicolor* is a strong candidate for atrazine bioremediation in soil with low moisture and organic matter contents, such as that found in semi-arid and Mediterranean-like ecosystems.

Keywords: *Trametes versicolor*; Biodegradation; Atrazine; Soil microcosms; Water potential; Soil respiration; Enzymatic activity.

60 ## 1. Introduction

61

62 The widespread incorporation of herbicides into soil every year is of major concern,
63 since they potentially can pose a threat to our health as well as to the quality of soil,
64 surface water and groundwater resources (Häggblom, 1992; Kearney and Roberts, 1998;
65 Kuo and Regan, 1999; Ashman and Puri, 2002). Atrazine is a chlorinated aromatic
66 herbicide heavily used worldwide for control of broad-leaved weeds in agricultural
67 produce (Ghani *et al.*, 1996; Houot *et al.*, 1998; Ralebitso *et al.*, 2002), as well as in
68 urban and recreational areas (Gadd, 2001).

69

70 Atrazine and related triazines are moderately persistent in soil (Pointing, 2001) with
71 reported half-life values ranging from 35 to 50 days, depending largely on soil
72 environmental conditions (Topp, 2001; Rhine *et al.*, 2003). Microbial metabolism has
73 long been regarded as the most important mechanism of atrazine degradation in soil
74 (Armstrong *et al.*, 1967; Gravilescu, 2005). Nevertheless, in conditions of low moisture
75 and nutrient contents, microbial metabolism is compromised and atrazine persistence
76 may increase (Weber *et al*., 1993). Soil water potential has been widely recognised as a
77 determinant factor controlling soil microbial growth and activity rates. Yet, very little
78 research has looked at atrazine biodegradation in soil under low moisture regimes
79 (Moreno *et al*., 2007).

80

81 The application of white-rot fungi for bioremediation of common environmental
82 contaminants looks promising. Similar to other white-rot species, *T. versicolor* has
83 shown to be able to metabolise a wide range of organic compounds (Bumpus *et al.*,
84 1985; Gadd, 2001). This ability is generally attributed to the production of extracellular
85 ligninolytic enzymes such as laccase, which is non-specific in regard to its substrate
86 (Thurston, 1994; Youn *et al*., 1995; Pointing, 2001; Šašek *et al.*, 2003; Baldrian, 2004).
87 White rot species can also tolerate a broad range of environmental conditions, including
88 temperature, nutrient and moisture contents (Maloney, 2001; Magan, 2007). In previous
89 studies, *T. versicolor* was shown to exhibit good tolerance to water stress conditions
90 (Mswaka and Magan, 1999; Fragoeiro and Magan, 2005) as well as to triazine
91 pesticides (Gadd, 2001; Šašek *et al.*, 2003; Fragoeiro and Magan, 2005). Further, the
92 mycelial growth habit and hyphal extension allow rapid substrate colonisation and

utilization of compounds that are not otherwise readily available to the wider microbial community (Reddy and Mathew, 2001; Magan, 2007).

So far, most studies involving the use of ligninolytic fungi for bioremediation purposes have been carried out in liquid media (e.g. Ryan and Bumpus, 1989), often in bioreactors (Novotný, 2004). *T. versicolor* has been seldom studied in the soil environment although there are reports of its successful application in sterile soil (Lamar, 1993) and soil extract broth (Fragoeiro and Magan, 2005). In non-sterile soil, knowledge is limited on other factors which can influence pesticide degradation, such as competitive interactions between the introduced fungi and native microbial populations (Šašek *et al.*, 2003).

This study aimed to (1) assess the potential of *T. versicolor* for actively degrading atrazine at 0.5 µg g^{-1} (usual field application rates) in non-sterile calcareous clay soil, under low water availability conditions (-0.7 and -2.8 MPa). Soil respiration, dehydrogenase and laccase activities were determined in combination with atrazine quantification by HPLC, under the study conditions. The selection of soil water potentials had the water availability range for microorganisms and plants (i.e. -0.03 MPa, field capacity, to -1.5 MPa, wilting point) as reference.

2. Materials and methods

2.1. Pre-incubation of *T. versicolor* (R26)

Pre-incubation of the fungal inoculum involved growing the isolate in sterile jars on wet sterile sawdust (50% water content, used as carrier) at 25°C for up to 3 weeks, until the substrate was colonised by mycelium. The jar had a vented cap (polypropylene membrane 0.22 µm pore size) allowing adequate aeration. In order to avoid desiccation, the jars were placed inside a polyethylene box, where the equilibrium relative humidity was maintained by a glycerol/water solution (400 ml).

2.2 Soil preparation, conditions and treatments

125 The soil used was a calcareous clay soil (top-soil, 0-20 cm) and was collected from an
126 arable field plot in Lagoa, Algarve, Portugal. The soil had the following main
127 characteristics, analysed by the National Soil Resources Institute (NSRI, Cranfield
128 University) and given as mg g^{-1} oven dried soil: soil organic carbon (SOC), 12.1; water,
129 353; sand, 320; clay, 470; silt, 210; pH 6.8; annual average values of precipitation (mm)
130 and temperature (^{o}C) on site were 400 and 17 respectively; there is no history of
131 pesticide inputs in the last 4 years. Plant residues and stones were removed manually at
132 the time of collection and soil was sieved (2 mm) and air-dried at 20°C for 7 days prior
133 to use. Air-dried soil samples (10 g) were weighed into Universal (25 ml) bottles and
134 target soil water potentials of -0.7 and -2.8 MPa were set by reference to a soil
135 adsorption curve and the addition of sterile reverse osmosis (RO) water (Bastos, 2008).
136
137 *i) Atrazine addition to soil*
138 Atrazine was dissolved in RO water and the solution was sonicated for 1 min until
139 complete dissolution of the herbicide. The amount of water used for dissolution was the
140 same as that required for setting the target soil water potential treatment, calculated by
141 reference to the soil water adsorption curve (Bastos, 2008). The solution was then added
142 to soil (5 g), in order to obtain a final concentration of atrazine of 0.5 μg g^{-1}. This
143 concentration corresponds to usual field application rates of the herbicide (Ghani *et al.*,
144 1996; Abdelhafid, 2000). The fortified soils were thoroughly homogenised and kept for
145 1 day at 4°C allowing microbial activity to stabilise at the required water potential
146 levels, before incubation and analysis.
147
148 *ii) Soil supplemented with sterile sawdust*
149 Wet (50% w w^{-1}) finely chopped sterile sawdust was kept overnight at 4°C. It was then
150 added to air-dried soil (5 g) in order to obtain a concentration of 5% (w w^{-1}) and
151 samples were left equilibrating overnight at 4°C. Conditioning of the treated soil to the
152 required water potentials was then done by reference to a soil-sawdust calibration curve
153 (Bastos, 2008). The procedure followed that described in *i)*.
154
155 *iii) Soil supplemented with sterile sawdust + atrazine*
156 Soil was amended with sterile sawdust as described in *ii)*. Conditioning of the soil to the
157 treatment water potentials was done by reference to a soil-sawdust adsorption curve and
158 the addition of sterile RO water supplemented with atrazine, in order to obtain a final

159 concentration of atrazine in soil of 0.5 μg g^{-1}. The procedure followed that described in
160 *i).*
161
162 *iv) Inoculation of T. versicolor into soil*
163 Sawdust colonised by the test isolate (0.5 g) was added to air-dried soil (5 g) in order to
164 obtain a concentration of 5% (w w^{-1}) and mixed until a homogeneous mixture was
165 obtained. The procedure followed that described in *ii).*
166
167 *v) Incorporation of T. versicolor + atrazine in soil*
168 The preparation of the homogeneous mixture of *T. versicolor* with sawdust (5% w w^{-1})
169 and its incorporation into air-dried soil (5 g) was described previously. The procedure
170 followed that described in *iii).*
171
172 **2.3 Incubation of soil microcosms**
173 Treated soil samples and non-treated controls were incubated at 20°C for up to 24
174 weeks within polyethylene boxes previously thoroughly cleaned. Each box also
175 contained a glycerol/water solution (400 ml), in order to maintain the equilibrium
176 relative humidity within each microcosms the same as that of the soil treatments. All
177 treatments involved in this work are summarised in Table 1.
178
179 **2.4 Temporal evaluation of soil respiration**
180 CO_2 evolved from total soil microbial respiration was determined by gas-
181 chromatography (GC) through static sampling. Following incubation, Universal bottles
182 containing soil samples were sealed and left for 3 h at 20°C prior to analysis, thus
183 ensuring detectable volumes of CO_2 in the soil headspace. Headspace (5 ml) was then
184 injected into a gas chromatographer equipped with a packed column (Porapak Q packed
185 glass column) and a thermal conductivity detector (Carlo Erba Instruments, GC 8000
186 Series MFC 800). Five replicates of each treatment were sampled. The GC settings were
187 the following: column and injector temperatures, 100°C; detector temperature, 180°C;
188 carrier gas (Helium) at a flow rate of 36 ml min^{-1}; the calibration gas consisting of a
189 standard mixture (10.01% v v^{-1} CO_2 in N_2) was injected three times at the beginning and
190 after each set of 15 samples. Soil respiration rate was expressed as μg CO_2 g^{-1} soil h^{-1}.
191
192 **2.5 Temporal evaluation of dehydrogenase activity**

193 The method for DHA quantification was adapted from von Mersi and Schinner (1991).
194 It is based on the incubation of soil with the substrate INT (2 (p-iodophenyl)-3-(p
195 nitrophenyl)-5-phenyl tetrazolium chloride), followed by the extraction and colorimetric
196 estimation of the reduction product INF (iodonitrotetrazolium formazan). The
197 calibration curve (Y = 1.0657x + 0.0061) which was firstly produced using a standard
198 INF solution (100 µg INF ml^{-1}), showed a good correlation (r^2 = 0.998) between the
199 concentration of INF and the optical density of the INF solution.
200
201 The INT solution was prepared by dissolving 500 mg of INT into 2 ml of N,N-
202 dimethylformamide, followed by the addition of 50 ml of RO water. The solution was
203 sonicated (2 min) and the volume was brought up to 100 ml using RO water. The final
204 concentration of the substrate solution was 0.5% (w v^{-1}). For every analysis, fresh INT
205 solution was prepared and stored in the dark until use.
206
207 Soil (0.5 g) at the treatment water potentials was weighed into sterile test tubes and
208 mixed with 740 µl of Tris-HCl buffer (1 M, pH 7.0) and 1 ml of the substrate solution.
209 Test tubes were sealed with sterile sponge stoppers and incubated in the dark at 40°C for
210 2 h. Following incubation, 5 ml of extraction solution (N,N-dimethylformamide:
211 ethanol in a 1:1 ratio) were added to the mixture and samples were kept in the dark for 1
212 h. During this time, every sample was vigorously shaken (using the vortex) at 20 min
213 intervals, ensuring an efficient extraction of the product INF. Aliquots of 2 ml were then
214 transferred to Eppendorf tubes and centrifuged for 2 min. The supernatant (200 µl) was
215 introduced into microplate wells and the INF was determined spectrophotometrically at
216 450 nm using a Microplate reader (Dynex Technologies Ltd., UK).
217
218 Controls were also prepared for estimating the chemical reduction of INT under the
219 same conditions. For each treatment, controls were prepared using autoclaved soil
220 (121°C, 20 minutes) and were treated like samples. Five replicates of each treatment
221 (including respective controls) were sampled. The INT reduction of the control was then
222 subtracted to that of the samples and DHA was expressed as ng INF g^{-1} soil 2 h^{-1}.
223
224 **2.6 Temporal evaluation of laccase activity**
225 Estimating soil laccase activity involved 2 steps: i) extraction of laccase from soil; ii)
226 quantification of enzymatic activity based on the oxidation of the redox substrate ABTS

227 (2,2-azino-bis-ethylbenthiazoline-6-sulphonic acid). The procedure described below
228 was firstly calibrated and optimised using purified commercial laccase from *Rhus*
229 *vernificera* in crude acetone powder (50 Units mg^{-1} solid, minimum) as standard. The
230 calibration curve obtained (Y = 26.33x + 1.643) showed a good correlation ($r^2 = 0.971$)
231 between the concentration of commercial purified laccase (mg ml^{-1}) and laccase activity
232 (U).
233
234 *i) Laccase extraction from soil*
235 The extraction method employed was based on the protocol described by Criquet *et al.*
236 (1999) with adaptations by Fragoeiro and Magan (2005). Sub-samples (2 g) of treated
237 soil and non-treated controls were weighed into sterile test tubes and 8 ml of phosphate
238 buffer in water (10 mM, pH 6.0) were added. The suspension was kept under agitation
239 (incubator shaker, 250 rpm) at 4°C for approximately 1 h. Aliquots of 1 ml were then
240 placed into 1.5 ml Eppendorfs and centrifuged (3800 rpm) for 6 min at room
241 temperature. The supernatant containing the enzyme was stored at -18°C until analyses.
242
243 *ii) Quantification of laccase activity*
244 The method for determining laccase activity using an enzyme extract was based on the
245 protocol described by Buswell *et al.* (1995) with adaptations by Fragoeiro and Magan
246 (2005). The reaction mixture performing a total of 300 µl was contained into a 96 well
247 microtitre plate. It was prepared with 150 µl sodium acetate buffer (0.1 M, pH 5), 50 µl
248 ABTS (0.55 mM) and 100 µl enzyme extract. The procedure was carried out at ambient
249 temperature, although the substrate ABTS and the buffer were at 40°C when added to
250 the reaction mixture. The incubation was performed at 40°C for 1 h. Positive laccase
251 activity was indicated by a green colourisation of the reaction mixture, characteristic of
252 the ABTS oxidised form. The product was determined spectrophotometrically at 405
253 nm using a Microplate reader set in the Endpoint reading mode, with 5 seconds of
254 agitation at the beginning. Control samples were prepared using boiled enzyme (15
255 min). Five replicates of each treatment including the respective controls were used. One
256 enzyme activity unit (U) was defined as the amount of enzyme required for producing a
257 0.001 increase in the optical density of the reaction mixture per minute, under the
258 conditions of the assay. Results were expressed as U g^{-1} soil.
259
260 **2.7 Monitoring atrazine concentration in soil**

261 A two-step procedure was involved in assessing the rate of atrazine degradation in soil
262 microcosms: i) pesticide extraction from soil and ii) HPLC analysis of soil extracts. The
263 method employed for atrazine extraction and quantification was adapted from that of
264 Elyassi (1997) and Fragoeiro and Magan (2005).
265
266 *i) Atrazine extraction from soil*
267 Soil samples corresponding to the SA and SAT treatments (at -0.7 and -2.8 MPa) were
268 weighed (2 g) into test tubes. Aliquots (3 ml) of methanol (100%) were added to soil,
269 the tubes were sealed and shaken at 300 rpm in a circular motion shaker for 24 hours in
270 the dark at room temperature. Following agitation soil was allowed to settle until a clear
271 supernatant was obtained (30 min aprox.). Aliquots of supernatant (extract) was then
272 withdrawn with a syringe and filtered using a nylon 0.22 µm syringe filter.
273
274 *ii) HPLC analysis of soil extracts*
275 Extracts were diluted with acetonitrile (75% sample: 25% acetonitrile). A volume of 50
276 µl was injected into a Gilson HPLC system equipped with a Gilson 117 UV detector
277 operating at 215 nm, a Gilson 231XL sampling injector, Gilson 306 pump, Gilson 811
278 C dynamic mixer and an Altima C18 5 mm column (4 mm x 250 mm x 4.6 mm). The
279 column operated at ambient temperature with a flow rate of 1.5 ml min^{-1}. An isocratic
280 mobile phase system was established using acetonitrile:water at a ratio of 70:30.
281 Atrazine eluted at approximately 9.8 min. The limit of detection
282
283 **2.8 Data handling and statistical treatment**
284 For comparison between means of treatments in respect to respiration, enzymatic
285 activities and atrazine quantification, analysis of variance (ANOVA) was performed
286 using STATISTICA (Version 7) at a significance level $p = 0.05$. Standard error of
287 means are shown as vertical bars in figures and indicated in Tables as ± SE.
288
289 # 3. Results
290
291 **3.1. Temporal evaluation of microbial respiration**
292 Figure 1 shows the respiration rate for the clay soil under different soil treatments
293 incubated at (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Generally, soil

294 treated with atrazine was shown to produce over 40% more CO_2 than non-treated soil
295 (SS). Throughout the study, soil containing the inoculum alone (ST) showed a
296 significant ($p<0.01$) increase in respiratory rates compared to un-inoculated soil (S).
297 Surprisingly, differences between respiration rates of soil treated with atrazine (SA),
298 sawdust (SS) and *T. versicolor* (ST) individually, were often not significant
299 ($0.05<p<0.16$) under the treatment soil conditions. Overall, the highest CO_2 evolution
300 rates ($0.01<p<0.04$) were achieved by soil containing both atrazine and the inoculum
301 (SAT). These were at least 20% higher than in the absence of the inoculant. Maximal
302 respiration rates occurred generally after week 6, and were followed by a slow but
303 consistent decrease. Under drier conditions and throughout the study, respiration rates
304 were generally not statistically different ($0.05<p<0.27$) between treatments.
305
306 **3.2. Temporal evaluation of dehydrogenase activity**
307 Figure 2 shows the DHA for the clay soil under different soil treatments incubated at
308 (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Regardless of soil treatment,
309 the highest DHA levels were achieved under the wettest conditions ($p<0.001$). Non-
310 treated soil had the lowest DHA but sawdust and atrazine supplements (individually or
311 combined) enhanced this enzymatic activity by over 40% at both water potentials
312 ($p<0.001$) over the first 6 weeks. However, after 12 weeks and from then onwards, soil
313 carrying the inoculum alone (ST) showed over 20% higher DHA compared to sawdust-
314 treated soil whether atrazine was present or not. Activity rates peaked after 6 weeks but
315 overall, they remained high throughout the study, even under the driest soil conditions.
316 Nevertheless, irrespective of water potential, there was no significant ($p>0.09$)
317 difference between soil inoculated with *T. versicolor* in the presence (SAT) and absence
318 (ST) of atrazine from week 6 onwards.
319
320 **3.3. Temporal evaluation of fungal laccase activity**
321 Table 2 shows ABTS oxidation levels in the clay soil under different soil treatments
322 incubated at (A) -0.7 MPa and (B) -2.8 MPa (20°C) for up to 24 weeks. Interestingly,
323 substrate oxidation was found to occur in non-treated clay soil in the absence of the
324 fungus at -0.7 MPa. Further, there was enhanced substrate oxidation ($p<0.001$) as a
325 response to atrazine (SA) and sawdust (SS) alone under both water regimes. Differences
326 between laccase activity in soil containing sawdust only and that carrying the inoculant
327 were generally only significant ($p<0.03$) at -2.8 MPa, with the second treatment having

over 96% higher laccase production than the first. Under the wettest conditions, the SAT treatment had only minimal levels of this enzyme, when comparing to the remaining treatments, including that of soil carrying the inoculum alone (ST). This was most evident after 6 weeks. Surprisingly, the opposite was observed under drier soil conditions, with the SAT treatment having nearly 40% higher ($p < 0.0004$) laccase activity than that of ST, although differences between SAT and SA were not statistically significant ($p > 0.06$). Very little activity was found after 24 weeks, independent of the treatment conditions.

3.4. Monitoring atrazine concentration in soil microcosms

Table 3 shows the remaining atrazine ($\mu g\ g^{-1}$) in clay soil incubated for up to 24 weeks at (A) -0.7 and (B) -2.8 MPa (20°C) in the absence (SA, SSA) and in the presence (SAT) of *T. versicolor*. Two controls were used in order to reduce bias in respect to the contribution of the sawdust supplement for atrazine degradation under the study conditions. The amount of atrazine present in the soil decreased with the incubation period in all treatments, and this was more rapid during the first 6 weeks.

In the absence of the fungus, 0.071 $\mu g\ g^{-1}$ of atrazine was recovered from sawdust supplemented soil after 6 weeks, corresponding to around 14% of its initial concentration. For the same time period, only 0.023 $\mu g\ g^{-1}$ of atrazine (i.e. 4% of the initial concentration) was extracted from soil containing the inoculum. By the end of the study, residues of the herbicide in soil were decreased to 0.019 and 0.011 in the absence (SSA) and presence (SAT) of the inoculum, corresponding to 96 and 98% degradation respectively. At -0.7 MPa, the impact of *T. versicolor* on atrazine breakdown in soil was only significant ($p < 0.003$) within the first 12 weeks. In contrast, at -2.8 MPa, there was still a significant ($p < 0.01$) difference between both treatments at the end of the study.

4. Discussion

In this study, *T. versicolor* was inoculated into non-sterile soil containing atrazine at usual field application rates for up to 24 weeks under low water regimes. Atrazine quantification by HPLC, combined with the assessment of soil microbial respiration and dehydrogenase activity allowed estimating the feasibility of this white-rot species to

361 remain metabolically active and degrade atrazine under the study conditions. Laccase
362 activity was also determined as an indicator of *T. versicolor* relative activity, in order to
363 evaluate the contribution of this enzyme in the degradation process. An optimal
364 performance of *T. versicolor* in terms of growth and enzymatic activity is dependent on
365 its capability to compete with native microflora in contaminated soil (Bumpus, 1993;
366 Levanon, 1993; Baldrian, 2004). According to Šašek *et al.* (2003), this is an important
367 aspect since the interaction between both parts can result in either inhibition or
368 cooperation in the degradation process. In order to enhance *T. versicolor* colonisation
369 and activity under such conditions, two strategies were employed: pre-incubation of the
370 fungus on a ligninolytic substrate (wet sawdust) prior to inoculation into soil; use of
371 sawdust as carrier (5 g inoculant to 95 g soil) but also as nutrient source selective for the
372 fungus. Other authors have used different carriers and inoculant/soil ratios, ranging
373 from 5% woodchips-based *T. versicolor* inoculum (Fragoeiro and Magan, 2005) to 50%
374 straw-based inoculum (Novotnỳ et al., 2003).
375
376 *Temporal soil respiration*
377 Soil respiration was used as an indicator of overall microbial activity and pesticide
378 breakdown. Increased respiratory activity following incorporation of sawdust and
379 atrazine (individually or combined) was not surprising as they provide nutrient sources
380 suitable for native microorganisms (Mandelbaum *et al.*, 1993; Haney *et al.*, 2002;
381 Moreno *et al.*, 2007). Our results were thus comparable to those in other studies which
382 used atrazine at similar concentrations (Dzantor and Felsot, 1991; Moreno *et al.*, 2007).
383 Further, sawdust addition may have also improved aeration throughout soil, favouring
384 microbial activity in ways equivalent to that reported by Boyle (1995) using alfalfa and
385 bran.
386
387 Respiratory activity was also enhanced in soil containing the inoculum, indicating that
388 the test isolate was able to remain metabolically active throughout the study. However,
389 few significant differences were found between that and soil containing sawdust alone,
390 which suggests competitive interactions between the inoculant and native microflora.
391 The highest CO_2 evolution rates were obtained from soil containing both atrazine and
392 the inoculant, indicating atrazine breakdown by the test isolate, even under limiting
393 water potentials of -2.8 MPa. Comparable results were obtained by Fragoeiro and
394 Magan (2008), who employed *T. versicolor* for bioremediation of a pesticide mixture

395 (simazine, dieldrin and trifluraline at 5 ppm) in soil microcosms. Using a sandy loam
396 soil under similar water potentials, they have also reported maximal CO_2 evolution from
397 soil containing both the pesticide mixture and *T. versicolor*.
398
399 Overall, respiration rates peaked at around 6 weeks, followed by a consistent decrease
400 towards the end of the study due to nutrient exhaustion (Balba et al., 1998). There was
401 evidence that water potential was the limiting factor for soil respiration, as respiratory
402 activity between treatments was generally not statistically different under the driest soil
403 conditions. This is consistent with Conant et al. (2004), who found that drier (-1.0 and -
404 1.5 MPa) soils have substantially lower respiration rates than those moist (-0.03 to -0.05
405 MPa), partially due to severely restricted bacterial activity.
406
407 *Temporal dehydrogenase activity*
408 Biological dehydrogenation (oxidation) of organic matter under aerobic conditions is
409 ultimately linked to the respiratory chain and the synthesis of adenosine triphosphate
410 (ATP) (Trevors, 1982; von Mersi and Schinner, 1991) and is catalysed by
411 dehydrogenases (Harris and Steer, 2003; Nannipieri *et al.*, 2002, 2003). Besides organic
412 matter decomposition, intracellular dehydrogenase activity has also been associated
413 with other key soil functions such as xenobiotic degradation (Min *et al.*, 2001; Acosta-
414 Martinez *et al.*, 2003). This enzymatic activity has been widely recognised as a good
415 indicator of microbial activity, since it is linked to viable cells only and has shown to be
416 positively correlated to respiration under different soil conditions (e.g. von Mersi and
417 Schinner, 1991; Garcia et al., 1994; Jimenez et al., 2002). In this study, DHA was
418 determined in order to assess the overall soil oxidative status and this enzymatic activity
419 in relation to atrazine biodegradation.
420
421 Regardless of soil treatment, the highest DHA levels were achieved under the wettest
422 conditions, which is supported by previous studies (Quilchano and Maranon, 2002).
423 Further, supplement addition to soil (sawdust and atrazine, individually or combined)
424 generally enhanced DHA regardless of soil water potential. In contrast, the low DHA
425 levels in soil containing the inoculum alone over the first 6 weeks, is likely to reflect
426 competitive interactions between the inoculant and native microorganisms, agreeing
427 with respiration data. However, increased DHA from then onwards indicated that the
428 inoculum remained metabolically active, even under the driest soil conditions.

429 Surprisingly, unlike that observed in the remaining treatments, DHA in the SAT
430 treatment was the highest over 24 weeks, which is inconsistent with respiratory activity.
431 This adding to the fact that the SAT treatment did not show improved levels of this
432 enzyme (when comparing to ST), suggested that atrazine degradation by this white-rot
433 species may not be coupled to the oxidative metabolism of the fungus. It is possible that
434 under the treatment environmental conditions, atrazine was not being used by *T.*
435 *versicolor* for generation of ATP (Haney *et al.*, 2002). This enzymatic activity has been
436 scarcely studied in relation to atrazine biodegradation by white-rot fungi in soil.
437
438 Previous work has linked single pesticide degradation and DHA activity in soil (e.g.
439 Min et al., 2001; Moreno *et al.*, 2007). Moreno *et al.* (2007) reported enhanced DHA in
440 soil containing atrazine in the range of 0.2 to 1000 mg kg^{-1} at 28°C. In contrast,
441 McGrath and Singleton (2000) monitored pentachlorophenol (PCP) biodegradation in a
442 clay loam. While PCP concentration was found to decrease (from 250 to 2 mg kg^{-1}) in
443 just 6 weeks, levels of DHA remained minimal throughout the study. They suggested
444 that the generation of toxic PCP biodegradation products may have been inhibitory to
445 DHA (McGrath and Singleton, 2000). However, it is unlikely for that to explain the low
446 DHA obtained in this study in soil containing the inoculant. Previous work has shown
447 that very few toxic simazine breakdown products were originated by this inoculant in
448 soil extract broth at -0.7 and -2.8 MPa, using the luminescent bacterium *Vibrio fischerie*
449 (standard toxicity assay) (Fragoeiro, 2005).
450
451 *Temporal laccase activity*
452 Since it is difficult to directly assess fungal growth in soil (Novotný *et al.*, 1999, 2004),
453 colonisation of white-rot fungi is usually determined indirectly through enzymatic
454 activity. The ability of such fungi to degrade pesticides has been largely associated with
455 the production of the glycoprotein laccase (polyphenol oxidase) in the presence of
456 adequate ligninolytic substrates (Häggblom, 1992; Paszczynski and Crawford, 2000;
457 Novotný *et al.*, 1999, 2004). Such enzymes have broad substrate specificity towards
458 aromatic compounds containing hydroxyl and amine groups. ABTS is considered to be
459 a primary mediator for laccase and therefore its oxidation is generally regarded as an
460 indication of laccase activity (Youn *et al.*, 1995; Podgornik *et al.*, 2001).
461

462 ABTS oxidation did occur in non-treated clay soil under the study water potentials,
463 contrary to that found by Fragoeiro and Magan (2008) using a sandy loam. This may be
464 because other genera of fungi (e.g. *Aspergillus*, *Rhizopus*), actinomycetes (e.g.
465 *Streptomyces*) and also some bacteria (e.g. *Pseudomonas, Bacillus*) are known to
466 express laccase activity at some extent (Kearney and Roberts, 1998). It suggests that
467 this enzymatic activity may not be suitable for assessing *T. versicolor* relative activity in
468 non-sterile soil. The incorporation of sawdust (individually or combined with atrazine)
469 has shown to stimulate LAC production, which might be a reflection of an active fungal
470 and actinomycete communities in such soil types (Brown, 1979; Wilson and Griffin,
471 1975; Harris, 1981; Magan, 1988, 1997; Halverson *et al*., 2000).
472
473 Very little research has looked at the implications of soil water potential on LAC
474 activity. In this study, whereas the incorporation of *T. versicolor* into soil did not
475 resulted in enhanced laccase activity at -0.7 MPa, that enhancement was obtained under
476 drier soil conditions. This indicates that *T. versicolor* had ligninolytic activity under -2.8
477 MPa, similarly to that reported by Boyle (1995) and later by Fragoeiro and Magan
478 (2008). Further, that result suggests that LAC production by the inoculant may be
479 influenced by competitive interactions with native microflora (White and Boddy, 1992)
480 at -0.7 MPa, when the wider fraction of the microbial community was metabolically
481 active. For example, there is evidence of total inhibition of ligninolytic activity in *T.*
482 *versicolor* when co-inoculated in soil with species of the genus *Trichoderma* (Freitag
483 and Morrell, 1992). According to Novotný (1999), such interactions may explain why
484 *T. versicolor* generally produces relatively low levels of laccase in non-sterile soil.
485
486 Under wetter soil conditions, soil containing both atrazine and the inoculant has shown
487 minimal levels of laccase, compared to soil containing sawdust and atrazine. In contrast,
488 the opposite was found at -2.8 MPa. Overall, evidence suggests that atrazine
489 degradation in this soil by *T. versicolor* may have had little or no contribution of laccase
490 activity under the conditions studied. It is therefore likely that other enzymes may have
491 been involved at a larger scale. For example, Podgornik and co-workers (2001)
492 defended that ABTS is also a good substrate for manganese peroxidase (MnP) in *P.*
493 *chrysosporium* and therefore there is the possibility of this ligninolytic enzyme to have
494 been equally responsible for ABTS oxidation to a certain degree. Additionally, those
495 same authors confirmed that Mn(III) complex formation during cultivation of *P.*

496 *chrysosporium* can provide a false-positive for laccase, when ABTS is used as substrate.
497 Further research is needed on the link between ABTS oxidation and laccase activity
498 from *T. versicolor*, as well as between such enzymatic activity and pesticide degradation
499 by this species in non-sterile soil. Similarly, although it is known that *T. versicolor*
500 produces both MnP and lignin peroxidise (LiP) in culture (reviewed by Tuor *et al*.,
501 1995), much remains to be done in order to evaluate the contribution of these enzymes
502 in atrazine biodegradation by *T. versicolor* in the soil environment.
503
504 Contradictory evidence has led to the role of laccase production in the co-metabolism of
505 pesticides with lignin by white-rot fungi not yet to be well understood (Youn *et al*.,
506 1995). In this study, laccase production has shown to be highly impacted by soil
507 treatment, particularly by soil water potential. Bending *et al.* (2002), who used *T.*
508 *versicolor* for biodegradation of atrazine in liquid culture for up to 42 days, have
509 reached similar conclusions. Similarly, Mougin *et al*. (1996) have also reported that the
510 degradation of lindane in soil by *Phanerochaete chrysosporium* was independent of
511 laccase production by the fungus. In contrast, Fragoeiro and Magan (2008) reported
512 extremely high laccase activity (797.8 units ml^{-1}) by *T. versicolor* in a sandy loam soil
513 (25°C, -0.7 MPa) treated with a pesticide mixture (simazine, dieldrin and trifluralin, 5-
514 30 ppm). Besides having been associated also with interspecific interactions of the
515 fungus (White and Boddy, 1992), Novotný (1999) has further suggested that laccase
516 production may be influenced by the nature and concentration of the potential
517 contaminant and soil environmental conditions (Tuor *et al*., 1995). It is also likely to
518 vary with the white-rot strain and perhaps with the carrier used (Mougin *et al*., 1996;
519 Boyle, 1997) or be dependent on the combination of the aforementioned factors.
520
521 *Monitoring atrazine concentration in soil microcosms*
522 Pesticide degradation in soil was estimated by determining the amount of herbicide
523 extracted from soil after 6-24 weeks (20°C, -0.7 and -2.8 MPa), compared to its initial
524 concentration. The decrease in recovered atrazine in the SA and SSA treatments can be
525 explained by the presence of active native microbial populations, capable of degrading
526 the herbicide under the study conditions. This was consistent with the enhanced
527 microbial metabolic activity found for the same time period. Other studies (Haney *et al*.,
528 2002; Moreno *et al*., 2007) have reported similar results on the capability of native soil
529 populations to degrade atrazine added at low concentrations. For example, Moreno *et al*.

530 (2007) have recently demonstrated that 50% of the atrazine added (5 ppm) to clay loam
531 with freely available water had been degraded by day 16 (28°C) and that no herbicide
532 was recovered after 45 days.
533
534 However, herbicide breakdown was substantially enhanced in soil containing the
535 inoculum, particularly within the first 12 weeks. It provides evidence that *T. versicolor*
536 was able to grow and actively degrade atrazine in non-sterile soil under low water
537 availability conditions. It also suggests that the pre-incubation of the test isolate and the
538 use of sawdust as carrier were effective for this species. In this case, it is likely that the
539 relationship established between the inoculum and native degraders was mainly
540 cooperative, agreeing with earlier findings by Boyle (1995). In contrast, Tornberg *et al.*
541 (2003) reported that this species failed to remain viable once inoculated in non-sterile
542 soil.
543
544 Over 24 weeks, the contribution of *T. versicolor* was found to be no longer significant at
545 -0.7 MPa. In contrast, surprisingly, its contribution was still significant at -2.8 MPa,
546 when the remaining fraction of the microbial community had their metabolic activity
547 limited by water restriction. This clearly shows that this white-rot species is able to
548 actively biodegrade potential contaminants under environmental conditions, which do
549 not promote biodegradation by soil native microflora. It is likely to be partially
550 explained by the mycelial growth habit, allowing rapid and efficient colonisation of soil
551 while maximising interactions between extracellular enzymatic activity and the
552 surrounding environment (Maloney, 2001; Reddy and Mathew, 2001). Atrazine
553 breakdown was less pronounced towards the end of the study, whether *T. versicolor* was
554 present or not, due to nutrient exhaustion.
555
556 **5. Conclusion**
557
558 This study has shown that *T. versicolor* has the potential to be used as a bioremediation
559 agent for atrazine and related triazine compounds in non-sterile calcareous clay soil,
560 under low water availability conditions. This may be particularly relevant for
561 bioremediation strategies in semi-arid and Mediterranean-like ecosystems.
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833 Table 1. Summary of the soil treatments involved in this work. Key to treatments: WP, water potential; SD, sawdust; Atra, atrazine., Tv, *T.versicolor*.

835		Ref	WP (-MPa)	Atra (ug)	SD (%)	SD+*Tv*
836	Soil	S	-0.7; -2.8	-	-	-
837	Soil + Atra	SA	-0.7; -2.8	0.5	-	-
838	Soil + SD	SS	-0.7; -2.8	-	0.5	-
839	Soil + SD + Atra	SSA	-0.7; -2.8	0.5	0.5	-
840	Soil + *Tv*	ST	-0.7; -2.8	-	-	0.5
841	Soil + Atra + *Tv*	SAT	-0.7; -2.8	0.5	0.5	0.5

842

843 Table 2. Temporal laccase activity (U g/soil) based on ABTS oxidation in clay soil
844 incubated for up to 24 weeks at 20oC at (A) -0.7 and (B) -2.8 MPa as a response to
845 different soil amendments.

846 **A)**

	Incubation time (weeks)			
	0	**6**	**12**	**24**
S	0.30 ± 0.29	2.55 ± 1.06	1.88 ± 0.88	0.67 ± 0.41
SA	9.04 ± 1.41	12.0 ± 3.53	7.92 ± 1.57	0
SS	9.50 ± 0.77	24.5 ± 9.60	16.4 ± 3.04	0
SSA	10.3 ± 1.12	28.7 ± 0.79	19.9 ± 0.32	0
ST	8.49 ± 3.53	21.2 ± 9.11	13.3 ± 2.89	0
SAT	18.5 ± 5.66	0.51 ± 0.22	0.50 ± 0.51	0

847

848 **B)**

	Incubation time (weeks)			
	0	**6**	**12**	**24**
S	0.72 ± 0.11	1.97 ± 0.80	0.86 ± 0.23	0
SA	0	21.5 ± 4.32	15.7 ± 2.03	0
SS	6.50 ± 0.51	11.1 ± 3.88	0	0
SSA	8.57 ± 1.01	13.4 ± 0.90	6.20 ± 0.56	0
ST	10.0 ± 1.06	14.6 ± 3.63	9.88 ± 1.54	0
SAT	14.2 ± 1.90	23.1 ± 5.69	13.4 ± 2.01	0

849

850 **Table 3. Remaining atrazine (ug/g) in clay soil at (A) -0.7 and (b) -2.8 MPa at 20oC**
851 **in the absence (SA, SSA) and presence (SAT) of *T.versicolor*.**
852 **(A)**

Incubation (weeks)	Treatment	Remaining atrazine (µg g^{-1})
0	SA	0.495 ± 0.01
	SSA	0.500 ± 0.03
	SAT	0.500 ± 0.02
6	SA	0.080 ± 0.01
	SSA	0.071 ± 0.02
	SAT	0.023* ± 0.02
12	SA	0.036 ± 0.03
	SSA	0.034 ± 0.02
	SAT	0.016* ± 0.03
24	SA	0.022 ± 0.06
	SSA	0.019 ± 0.04
	SAT	0.011 ± 0.05

853 **B)**

Incubation (weeks)	Treatment	Remaining atrazine (µg g^{-1})
0	SA	0.480 ± 0.03
	SSA	0.495 ± 0.03
	SAT	0.490 ± 0.01
6	SA	0.262 ± 0.04
	SSA	0.255 ± 0.01
	SAT	0.102* ± 0.03
12	SA	0.258 ± 0.02
	SSA	0.242 ± 0.02
	SAT	0.084* ± 0.02
24	SA	0.254 ± 0.06
	SSA	0.237 ± 0.04
	SAT	0.076* ± 0.03

854 * Statistically different from both controls (SA and SSA) at $p < 0.05$.
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856

857 Figure 1. Respiration rates for the clay soil under different soil treatments for up to 24
858 weeks at 20oC under (A) -0.7 and (B) -2.8 MPA. For key to treatments see Table 1.
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