Short-term effect of 915-MHz microwave treatments on soil physicochemical and biological properties

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4 **biological properties**

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11   **Summary**

12   To control the soil seed bank of invasive plants with microwaves represents an alternative

13   method to chemical treatments, but it could alter soil quality. Microwave effects were

14   investigated on 17 soil physicochemical and biological properties. Four 915-MHz

15   microwave treatments combining power and duration of exposure were applied on alluvial

16   soil from a grassland using *Festuca* seeds as an internal standard to evaluate the efficiency

17   of sterilization. Two treatments, 2 kW-8 and 4 kW-4 minutes, completely inhibited *Festuca*

18   seed germination and caused the soil temperature to reach at least 80°C. Conversely, the

19   other treatments, 2 kW-4 and 4 kW-2 minutes, failed to affect seed germination while raising

20   the temperature to 50–60°C. Microbial biomass, the density of culturable heterotrophic

21   bacteria and fluorescent Pseudomonads as well as fluorescein di-acetate hydrolysis

22   significantly decreased by 2.7-, 1.1-, 1.4- and 5.1-fold compared with the control,

23   respectively, following the 2 kW-8 and 4 kW-4 minutes treatments. These treatments also

24   increased dissolved organic carbon and inorganic phosphorus contents by 1.6- and 1.2-fold,

25   respectively. In contrast, nitrate increased only (about 140%) in the soils under the 2 kW-4

26   and 4 kW-2 minutes conditions. Furthermore, the growth of *Medicago truncatula* Gaertn.

27   seedlings transplanted in microwave-treated soils under the 2 kW-8 and 4 kW-4 minutes

28   conditions was not impaired despite total nitrogen and microbial population reduction and

29   lower numbers of nodules. These results suggest that soil seed bank sterilization by

30   microwaves that is 100% effective alters soil microbial indicators more strongly than

31   chemical treatments without compromising the early growth stages of transplanted

32   *Medicago* plants.

33

34   **Keywords**: Invasive plant species, soil seed bank, microbial indicators, *in situ* catabolic

35   potential, microwave sterilization, soil quality

Highlights

- Effects of microwave seed sterilization on soil physicochemical and biological properties.

- Microbial properties were more sensitive than chemical ones.

- Microbial biomass, bacterial density, hydrolase activity and root nodulation were reduced.

- Soluble carbon, nitrate and phosphorus increased depending on treatment effectiveness.

Introduction

Invasive plants can colonize certain areas strongly and cause serious damage to the environment and human health. Controlling their spread and reducing their effects are stated objectives of European policy and legislation (EU Regulation 1143/2014 on invasive alien species). Traditional methods such as fire, flaming or herbicide treatments are applied to control and reduce established invasive species (Heap, 1997; Vitousek *et al.*, 1997; Vitelli & Madigan, 2004). The effectiveness of these methods is limited because only vegetative plants are affected, whereas seed survival is not. Microwave-based treatment of infested soils represents an alternative method to help control plant invasiveness (Bebawi *et al.,* 2007; Brodie *et al.,* 2012).

Microwaves cause dielectric heating of water in soils and organism cells, and rapidly increase temperatures to 60–90°C; the range needed to decrease seed viability (Barker & Craker, 1991; Brodie *et al.,* 2007; Sahin, 2014). They have a non-poisoning environmental effect compared to chemical methods under which transfer of chemical residues to adjacent ecosystems or induced herbicide resistance can occur (Heap, 1997). However, specific drawbacks of microwave methods have been identified, e.g. the large amount of energy

61 required to sterilize seeds effectively (Nelson, 1996), which also depends on soil

62 physicochemical properties such as moisture content or texture, including the presence of

63 stones (Brodie *et al.,* 2007; Sahin, 2014).

64   In addition to the energy cost and the need to adjust the microwave method to the local

65 soil environment, the temperature increase induced by microwaves could also trigger side

66 effects on soil biological and chemical properties (Zagal, 1989; Cooper & Brodie, 2009;

67 Shamis *et al.,* 2012; Brodie *et al.*, 2015). Several investigations have revealed various

68 susceptibilities among microbial populations to microwaves (Woo *et al.,* 2000; Tahir *et al.,*

69 2009). In particular, 2.45-GHz microwave treatment caused bacterial densities to decline in

70 the top layers of soil, whereas fungal, ciliate, amoeba and flagellate densities were not

71 affected. *Escherichia coli* populations declined by 10^5 in the top layer of soil treated with

72 500 J cm^{-2} (i.e. 500 W) of microwave energy (Brodie *et al.,* 2015). Microwave treatment

73 (625 W heating power, 2.45 GHz for 150 s) eliminated specific populations of fungi such as

74 *Pythium* and *Fusarium* together with non-cyst-forming nematodes, whereas *Rhizoctonia*,

75 cysts of *Heterodera glycines* and mycorrhizal fungi survived better (Ferriss 1984). Cooper

76 & Brodie (2009) investigated the effects of the length of exposure to microwaves (750 W

77 heating power, 2.45 GHz for 2–16 minutes) in relation to soil depth (down to 40 cm) on

78 plant nutrients and bacterial populations. They revealed small effects on nitrate, potassium

79 and sulphate availability whatever the duration and the depth, whereas significant reductions

80 of nitrite contents and bacterial populations (by 78%) were observed only in the topsoil as a

81 function of treatment duration. Conversely, microwave treatment (600 W heating power,

82 2.45 GHz for 120 s) increased soil total organic carbon and nitrogen mineralisation (Zagal

83 1989).

84   Such chemical and biological modifications of soil induced by microwave treatments

85 might exert long-lasting effects and jointly influence the germination and development of

reintroduced indigenous plant species or selected species chosen to engineer a new plant cover (Brodie *et al.*, 2015). Against this background, the objectives of this study were to evaluate the effects of four distinct 915-MHz microwave treatments on a set of 13 chemical (total nitrogen, ammonium, nitrate, inorganic phosphorus and total organic carbon contents), physical (moisture) and microbiological (microbial biomass, bacterial density and enzyme activities) properties of a grassland soil. The rates of germination of introduced *Festuca rubra* L. seeds were evaluated to control of microwave effectiveness on seeds. Moreover, seedlings of the leguminous species *Medicago truncatula* Gaertn. were grown in the five experimental soil batches (including untreated soil) to assess the influence of the modified soil properties on its development and rate of nodulation.

Materials and methods

Soil sampling

Around 50 kg of soil were collected on April 11th 2017 at Laudun-l'Ardoise (Gard, France) in a grassland field (44° 6'46.88"N / 4°41'30.28"E) located between two rivers (La Cèze and La Tave): the upper layer (0.5 cm) was discarded, and soil was taken from the 0.5-15 cm layer (bulk density = 1.37 g cm^{-3}). Plant debris and stones were removed prior to sieving at 4 mm. Samples were stored at 4°C. The soil is classified as a Fluvisol (World reference base for soil resources, FAO 2015) and characterized as an alkaline (pH 8.6) sandy loam (clay 12.8%, silt 22.3%, sand 64.9%). It contained limestone (14.4%) and had a mean moisture content of 8.0%.

Microwave system

The AMW200 batch microwave system (SAIREM SAS, Neyron, France, http://www.sairem.com) was described previously by De Wilde *et al.* (2017). This system

111 was designed for experimental purposes, not for industrial use. The microwave oven was a

112 stainless-steel 304-l chamber equipped with a rotating table (840 mm × 620 mm). It could

113 hold a maximum volume of 154 mm × 400 mm × 250 mm and a maximum weight of 30 kg.

114 The magneton that produced 915 MHz consisted of two 5-kW generators with power rating

115 between 1 to 10 kW. It was cooled by chilled water circulation.

116

117 *Experimental design*

118 Sieved soil samples (500 g) were placed in plastic bags (Milhe & Avons, France). To avoid

119 treatment heterogeneity from oven border effects described by De Wilde *et al.* (2017), the

120 soil bags were placed into larger plastic bags that also contained 500 g of soil to surround

121 smaller bags with soil (Figure S1, Supporting Information). Four microwave treatments

122 (power × duration couple) were applied with four-fold replication according to previous

123 research on microwave treatment on the rates of germination of seeds from three invasive

124 species (De Wilde *et al.,* 2017): two couples found to be ineffective for total seed sterilization

125 (2 kW for 4 minutes and 4 kW for 2 minutes) and two 100% effective couples (2 kW for 8

126 minutes and 4 kW for 4 minutes) were chosen. During the microwave treatments, the bags

127 remained open to prevent pressure from steam, and were sealed only after the treatments to

128 limit contamination. The control samples, again with four-fold replication received no

129 microwave treatment. The total load in the oven was 10 kg (10 soil units) per pass. To

130 evaluate the efficacy of the microwaves on seed germination, one paper bag containing 30

131 seeds of *Festuca rubra* was introduced into each soil bag at 5-cm depth before microwave

132 treatments. The soil temperature was measured with a mercury thermometer inserted 5-cm

133 deep in the centre of one replicate soil bag, before and immediately after microwave

134 radiation. The initial soil temperature was 9.5°C. Soil samples submitted to the two

135 ineffective treatments for total seed sterilization (2 kW-4 and 4 kW-2 minutes) reached

136 temperatures of 53.2 and 58.3°C, respectively, while those submitted to the two effective

137 treatments (2 kW-8 and 4 kW-4 minutes) reached temperatures of 95.9 and 83.2°C,

138 respectively.

139

140 *Germination test*

141 The content of each *F. rubra* seed bag (30 seeds per bag) was collected and dispersed on

142 filter paper (Whatman N°1) soaked with distilled water, and then placed into sterile Petri

143 dishes. The rate of germination was determined after three weeks of incubation in a growth

144 chamber (14 hours day, 10 hours night; 22, 15°C day, night; 60-70% relative moisture).

145

146 *Soil physicochemical characterization*

147 We determined the soil moisture content (mass of water/dry mass of soil) by drying sub-

148 samples in triplicate at 105°C for 48 hours. Total organic carbon (TOC) and total nitrogen

149 (TN) were determined by the Anne method based on $K_2Cr_2O_7$ oxidation (AFNOR standard

150 NF ISO 14235) and by the total Kjeldahl nitrogen (TN) method (AFNOR standard NF ISO

151 11261), respectively (Aubert, 1978). Inorganic phosphorus was determined by the Olsen

152 method (OIP) (Olsen *et al.,* 1954).

153 To analyse the soluble nutrient contents of the samples, 20 g of sieved soil were shaken

154 at 22°C for 2 hours in 100 ml of distilled water. Soil extracts were obtained after

155 centrifugation at 900 *g* for 4 minutes and filtered with filter paper. Dissolved organic carbon

156 (DOC), ammonium (NH_4^+), nitrate (NO_3^-) and water-soluble inorganic phosphorus (WIP)

157 were measured in the soil water extracts. Dissolved organic carbon (DOC) was measured by

158 a TOC-analyser (TOC-V CSH, Shimadzu Europa GmbH, Marne la Vallée, France) in

159 accordance with the AFNOR standard NF ISO 1484. Ammonium (NH_4^+) and NO_3^-

160 concentrations were determined on a continuous-flow auto analyser (Skalar-40, Skalar

161   Analytical B.V., Breda, The Netherlands), with a colorimetric method (Keeney & Nelson,

162   1982) after filtration at 0.2 µm. Analyses were all done by the Celesta Lab (France;

163   www.celesta-lab.fr), except for WIP which was determined by the malachite green method

164   (Ohno & Zibilske, 1991) after filtration at 0.2 µm (Millipore). Results were expressed as g

165   kg^{-1} of dried soil.

166

167   *Diversity of the* in situ *catabolic potentials (ISCPs) of the soil microbial community*

168   The diversity of the *in situ* catabolic potentials (ISCPs) of the soil microbial community was

169   assessed according to Dieng *et al.* (2014). The oxidation amounts (CO_2 emissions) of 30

170   organic substrates were estimated, including 11 carbohydrates, six carboxylic acids and 13

171   amino acids, as previously described. The acidity of the carboxylate stock solutions was

172   adjusted to pH 6.5 with NaOH to minimize chemical artefacts from carbonate-derived CO_2

173   (Bérard *et al.*, 2011).

174

175   *Fluorescein di-acetate (FDA) hydrolysis*

176   Fluorescein di-acetate (FDA) hydrolysis was determined according to Schnurer & Rosswall

177   (1982). One g of soil was suspended in 10 ml of sodium phosphate buffer (60 mM, pH 7.6)

178   and further incubated with 50 µl of FDA substrate solution (stock solution at 2 mg ml^{-1} in

179   acetone) for 1 hour at 24°C. After incubation, 10 ml of acetone were added to stop the

180   reaction. Each soil suspension was centrifuged (8000 *g* for 5 minutes) and the supernatant

181   was transferred to a colorimeter tube to measure its absorbance at 490 nm. Measurements

182   were made in duplicate for each soil sample. The amounts of fluorescein released in the

183   supernatant were calculated from a standard curve drawn from standard solutions containing

184   0.5, 1, 2, 3, 4 and 5 µg of FDA ml^{-1}. Standard solutions were prepared in sodium phosphate

185 buffer and boiled for 30 minutes to perform FDA hydrolysis. Values were expressed as µg

186 of fluorescein released per g of dried soil.

187

188 In vitro *enumeration of total culturable heterotrophic bacteria and fluorescent*

189 *Pseudomonads*

190 Soil (4 g) was shaken at 150 revolutions per minute (rpm) in 36 ml of NaCl 0.9% for 30

191 minutes at 28°C. Serial decimal dilutions were prepared in NaCl 0.9%, and 100-µl aliquots

192 were spread-plated in four replicates on to R2A and King's B agar media to enumerate total

193 bacteria and fluorescent Pseudomonad populations, respectively. Petri dishes were incubated

194 at 28°C, and colonies were counted for one week. Fluorescent colonies on King's B medium

195 were identified under UV light (366 nm). Results were expressed as $\log_{10}$ of colony-forming

196 units (CFU) g^{-1} of dried soil.

197

198 *Microbial biomass*

199 Microbial biomass was assessed by the chloroform fumigation–extraction method (Brookes

200 *et al.,* 1985) in accordance with the AFNOR standard NF ISO 14240-2. Measurements were

201 performed by the Celesta Laboratory. Results were expressed as mg of organic carbon (OC)

202 kg^{-1} dried soil.

203

204 Medicago truncatula *growth response*

205 *Medicago truncatula* seeds were pre-germinated, and 10 seedlings were transplanted per pot

206 containing 100 g of microwave-treated or control soil (one pot per experimental soil bag),

207 and then grown for 7 weeks under controlled conditions (14 hours day, 10 hours night,

208 22,15°C day, night). Soil moisture was adjusted with sterile distilled water. At harvest, root

209 nodules were counted and chlorophyll content was evaluated with a SPAD52 (Minolta Soil-

210   Plant Analyses Development, Roissy, France). Fresh and dried (65°C for 2 weeks) shoot and

211   root (nodules included) biomass values were evaluated.

212

213   *Statistical analyses*

214   We used the Rcmdr package in R Software (R Foundation for Statistical Computing,

215   Vienna) for out statistical analyses of variances and XLSTAT (version 2018.1.49150) for

216   multivariate analyses and graphs. The effects of microwave and control treatments were

217   tested on soil physicochemical and biological characteristics first by one-way analysis of

218   variance (ANOVA). The normality of the residuals and homoscedasticity of residual variances

219   were checked by graphical analyses of the standardized residuals considering histograms

220   and the quantile–quantile (Q–Q) plots. The significance of the difference between means

221   was evaluated for ANOVA by Fisher's least significant difference (LSD), with $P < 0.05$ as

222   the criterion of significance. When the residuals of a variable did not satisfy the assumptions

223   of ANOVA, diverse data transformations were tested until the normality of new residuals was

224   reached. When the assumptions of ANOVA could not be met, the non-parametric Kruskal–

225   Wallis (KW) test was used.

226   The ISCP profiles were compared by principal component analyses (PCA): as no

227   groupings congruent with some microwave treatments could be visualized (data not shown),

228   ISCPs were not considered in the further statistical analyses below.

229   Effects of microwave treatments on properties of soil (except ISCP) and *Medicago*

230   plants growing in the soils were then analysed separately by PCA (Pearson's correlation

231   matrix) to (1) explore the sample relationships and possible groupings that could be related

232   to some microwave treatments and (2) to identify major soil or plant variables contributing

233   to distribution variability along the principal components of the PCAs.

234   Lastly, to identify and measure the associations among the two types of variables (soil or

235   plant) analysed by PCA separately, correlations between the soil and plant datasets were

236   investigated by CCA (canonical correlation analysis, Pearson's correlation matrix). This

237   could figure out how the two datasets were related to one another and to identify the most

238   strongly correlated properties. This approach may help to hypothesize that some soil

239   properties impacted by microwave treatments may have further influenced development of

240   *Medicago* plants.

241

242   **Results**

243   *Effects of microwaves on* Festuca rubra *seed germination*

244   The rate of germination was completely inhibited by the 2 kW-8 and 4 kW-4 minutes

245   treatments that both caused the soil temperature to reach 80°C or more (Figure S2,

246   Supporting Information). The 2 kW-4 and 4 kW-2 minutes treatments did not significantly

247   affect the rate of germination. Consequently, the 2 kW-8 and 4 kW-4 minute combinations

248   were designated as effective treatments, and the 2 kW-4 and 4 kW-2 minutes combinations

249   were designated as ineffective treatments.

250

251   *Effects of microwaves on soil physicochemical properties*

252   The mean initial soil moisture content was 8.01% before microwave treatment. The final

253   moisture contents were statistically identical among microwave-treated soil samples (7.07

254   to 8.02%); only the 2 kW-8 minutes-treated soil was significantly drier (–12%) than the

255   control (Table 1, Table S1, Supporting Information).

256   Soil total organic carbon (TOC) and NH_4^+ contents were not significantly affected by the

257   microwave treatments, whereas the total nitrogen (TN) content decreased somewhat

258   following the 2 kW-8 and 4 kW-4 minutes treatments (Table 1, Table S1, Supporting

259 Information). Inorganic phosphorus measured by Olsen's method (OIP) and dissolved

260 organic carbon (DOC) values increased significantly after the two effective treatments (+18,

261 +30% for OIP and +88, +38% for DOC values, respectively for the two treatments, Table

262 1). Intriguingly, these two effective treatments did not alter NO_3^- and water-soluble inorganic

263 phosphorus (WIP), whereas the other microwave treatments did. The NO_3^- content was

264 greatly and significantly increased by the two ineffective treatments (+154 and +127%),

265 whereas the WIP content increased slightly following the 2 kW-4 minutes treatment (+14%).

266

267 *Effects of microwaves on soil microbial properties*

268 All soil microbial properties were negatively affected by microwave radiation to different

269 extents (Table 2), except the diversity of the ISCP of the soil microbial community (data not

270 shown). The two effective microwave treatments systematically triggered similar significant

271 and major declines in all properties, whereas the two ineffective treatments had no such

272 effect. A decrease of more than 60% in microbial biomass was induced by the two effective

273 treatments. In addition, smaller but significant reductions of microbial biomass were also

274 observed following the ineffective treatments (–21 and –35%). The greatest reductions in

275 the soil microbial biomass were mirrored by significant decreases in the culturable densities

276 of heterotrophic bacteria by almost 10 times of CFU. For fluorescent Pseudomonads, the

277 corresponding density losses even exceeded 100 times of CFU. The decrease in bacterial

278 densities was correlated to the increase in soil temperature measured immediately after

279 microwave radiation (Pearson' coefficient r = –0.52 (total bacteria) and –0.48

280 (Pseudomonads), Table S2 ($P < 0.05$), Supporting Information) to the increase in the soil

281 temperature.

282 The global hydrolytic activity revealed by the FDA enzyme assay also showed a marked

283 loss following the two effective treatments (–86 and –75%, Table 2). The two ineffective

284   treatments also triggered significant reductions in this enzyme activity (–37 and –49%). The

285   decrease in FDA hydrolysis was also correlated ($r = –0.52$, Table S2) to the increase of the

286   soil temperature.

287

288   *Development of* Medicago truncatula *in microwave-treated soils*

289   Surprisingly, the mean plant survival rate following plantlet transplantation to experimental

290   soils (Table 3) was less in the control soil (29 out of 40 plants) than in all microwave-treated

291   soils (34 to 38 out of 40 plants). Dried shoot and root biomass values were not directly

292   affected by microwave treatments, except the 2 kW-8 minutes treatment that went together

293   with significantly more root biomass (+71%, Table 3, Table S1, Supporting Information).

294   Nodule numbers were significantly affected only in the soil submitted to the effective 2 kW-

295   8 minutes treatment. This last treatment shows the largest effect (–76%), and did not

296   significantly differed from the result obtained with the 4 kW-4 minutes treatment (–39% of

297   reduction compared to the control). In contrast, leaf chlorophyll (SPAD) contents decreased

298   significantly only with the soils submitted to the two ineffective treatments (–17 and –11%).

299

300   *Multivariate statistical analysis*

301   The PCA with the soil variables (fluorescein di-acetate hydrolysis, microbial biomass, $\log_{10}$

302   of soil density of culturable fluorescent Pseudomonads, $\log_{10}$ of soil density of culturable

303   bacteria, total nitrogen, total organic carbon; inorganic phosphorus measured by Olsen's

304   method, dissolved organic carbon, water-soluble inorganic phosphorus) revealed a particular

305   distribution pattern of the 20 soil samples in the plane of the two leading components that

306   accounted for more than 63% of total inertia (Figure 1a). Three groups could be delineated

307   mainly along the first principal component F1 referring to the three main experimental

308   conditions, namely group I (effective microwaves), group II (ineffective microwaves) and

309   group III (untreated soil) (Figure 1a). The last two groups were further resolved along the

310   second principal component F2. On the first axis, group II is closed to group III containing

311   the soil samples of the control which is also ineffective. The 4 kW-2 and 2 kW-4 minutes

312   treatments were not fully resolved within group II, whereas the 2 kW-8 and 4 kW-4 minutes

313   treatments were fully resolved within group I. All microbial variables (microbial biomass,

314   bacterial densities and hydrolase activity) were strongly correlated with one another

315   according to the Pearson correlations (Table S2, Supporting information). The eigenvectors

316   show that these microbial variables together with the total N, OIP and DOC variables

317   contribute strongly to the definition of the first component in the correlation circle

318   (F1=45.3%, Figure 1b). Thus, soil replicates in Group I could be distinguished from those

319   of Group II and III, mostly on the basis of that subset of variables (e.g. effective treatments

320   caused soil OIP and DOC contents to increase while diminishing the total N content and

321   microbial properties, in particular the microbial biomass, Pseudomonads density and enzyme

322   FDA hydrolysis (see also Tables 1 and 2)). Soil NO_3^-, TOC and NH_4^+ variables were

323   preponderant in the definition of the second principal component (Figure 1b). Yet, only NO_3^-

324   was useful to distinguish soil replicates from Groups II and III as confirmed in Table 1.

325   In contrast, the PCA ordination of *Medicago* seedling properties (Figure 2) did not show a

326   similar pattern congruent with the treatment groups previously defined with soil properties

327   in Figure 1. Indeed, soil replicates of the 2 kW-4 treatment were the only ones that could be

328   unambiguously distinguished from the other soil samples and treatments along the first

329   principal plan (Figure 2a). All *Medicago* plant properties, except the nodule number,

330   contributed strongly to the definition of the first principal component (F1=53.9% Figure 2b).

331   Thus the relative distribution of the 2 kW-4 minutes treatment in this plane (Figure 2a)

332   seemed to be explained mainly by diminished shoot and root biomasses, and lower SPAD

333   unit values (Figure 2b) compared with all the other microwave treatments and the control.

334 Correlations between soil and *Medicago* plant properties were further analysed by CCA

335 (Figure 3, Table 4 and Table S3 Supporting Information). The first two canonical

336 correlations calculated between the two sets of variables were almost maximal (0.96 and

337 0.91, Table 4) revealing that soil and plant properties datasets were highly correlated

338 according to the first two canonical components and legitimating the interpretation of their

339 relationships in this plane. This analysis essentially revealed that plant properties (except

340 nodules) were mostly positively correlated with soil DOC and to a lesser extent with soil

341 OIP (see also Table S2, Supporting Information). By contrast, plant SPAD, shoot and root

342 biomass were negatively correlated with nodule number and most of the other soil variables,

343 especially microbial densities and enzyme FDA hydrolysis.

344

345 **Discussion**

346 The effects of 915-MHz microwave treatment of a soil to eradicate the seed bank of invasive

347 species were investigated under four radiation conditions that combined power level and

348 exposure time. The effectiveness of the four conditions chosen according to De Wilde *et al.*

349 (2017), related to the rates of seed germination of three invasive plant species, was confirmed

350 here on *Festuca rubra* seed germination. More precisely, the 2 kW-8 and 4 kW-4 minute

351 conditions caused soil temperature to reach at least 80°C leading to complete inhibition of

352 *Festuca* seed germination and were designated effective treatments. By contrast, the 2 kW-

353 4 and 4 kW-2 minute treatments failed to affect the germination rate of *Festuca* seeds

354 significantly (50–60°C soil temperature) and were considered ineffective treatments. The

355 effectiveness of our microwave-based seed sterilization thus appeared to be positively

356 related to the final soil temperature, which has been reported previously by several authors

357 (Sartorato *et al.,* 2006; Brodie *et al.,* 2007; Sahin, 2014). In addition to temperature, we

358 measured chemical (total nitrogen, NH_4^+, NO_3^-, inorganic phosphorus and organic carbon

contents), physical (moisture) and microbiological (ISCP signatures, microbial biomass, bacterial density and hydrolytic enzyme activity) features to delineate the effects of effective and ineffective microwave treatments on soil quality.

Soil biological properties were the most responsive to microwave treatments. Microbial biomass, has been used traditionally as a bioindicator of soil quality (Andrews *et al.*, 2004). In this study it was affected considerably by microwaves, especially under the effective treatments (–60%). The high temperatures recorded (80°C) probably weakened biological membranes, e.g. by denaturing their proteins, leading to their poration and disruption. Local temperatures in some soil micro-niches might even have reached >100°C leading to boiling of the cellular contents. Banning & Murphy, 2008) reported that the direct heating of field soil microcosms in an oven at 120°C for 20 minutes resulted in a similar loss of microbial biomass (–50%). Our soil density of total heterotrophic culturable bacteria and especially fluorescent Pseudomonads were also affected by effective treatments (about 10-fold and 100-fold less CFU, respectively). It can be speculated that Pseudomonads could be more easily lysed because they are Gram-negative bacteria and that Gram-negative bacteria show lower resistance towards microwaves than Gram-positive bacteria, as previously published (Woo *et al.,* 2000, Gedikli *et al.,* 2008, Tahir *et al.,* 2009). Moreover, certain Gram-positive bacteria can tolerate heat shocks better because of their sporulation ability, which could have contributed to the smaller decrease we observed in our total bacteria counts. In addition to loss in cell vitality, microwaves might also have decreased bacterial culturability and our subsequent CFU counts. Against this background, it would be interesting to refine the analysis by monitoring the proportion of culturable sporulating bacteria and evaluating the density of viable but non-culturable bacteria from microscopic counts combined with vital staining.

Similar to microbial biomass, FDA hydrolysis largely decreased following microwave radiation, especially following the effective treatments that sustained the highest temperature increases. As FDA hydrolysis is catalysed by a cocktail of hydrolytic enzymes, the significant loss of activity probably resulted from the thermal denaturation of a fraction of the enzyme pool. The soil's FDA hydrolysis activity had already been described as sensitive to elevated temperature: soil samples dried at 40°C for 4 days were associated with a loss of FDA hydrolysis of nearly 90% (Daou *et al.,* 2016). Several studies also demonstrated a negative effect of microwave radiation on the activity of bacterial enzymes like protease and urease. Total protease activity decreased in *Aeromonas hydrophila* (–33%) and urease activity ceased completely in *Staphylococcus aureus* after 2 minutes of microwave treatment (90 W heating power, 2.45 GHz) (Dholiya *et al.* 2012). Unlike the FDA assay, the variation in our ISCP signatures between replicates appeared larger than the treatments effects (data shown), therefore, ISCP could not be used as a variable to separate between treatments. The ISCP catabolic activities also rely on enzyme activities, therefore, we could deduce that there was no preferential thermal denaturation of enzymes related to the oxidation of specific ISCP substrates, whatever the treatment. All enzyme types were probably equally affected by the increase in temperature. Nevertheless, the ISCP and FDA assays were done under different conditions: microplates of remoistened soil were preincubated three days prior to their amendment with ISCP substrates. This could have facilitated rapid recovery of enzyme pools and partly sheds light on the opposite indications from FDA and ISCP. Overall, deleterious effects of microwave radiation have been recorded for major features of the soil microbial component. However, they were not completely destructive and probably enabled resilience mechanisms to be triggered. Mid- or long-term monitoring should be implemented to assess the legacy of the initial microwave disturbance on fundamental microbial functions and diversity.

408   The microwave treatments we applied, had less effect on soil chemical properties than

409   microbiological traits, as already observed by Cooper & Brodie (2009). These authors

410   reported that microwave treatment (750 W heating power, 2.45 GHz) had no effect on soil

411   pH or nitrate, phosphorus, potassium and sulphate availability: they found that only the size

412   of the bacterial community was reduced by 78% in the top layer of the soil. Nevertheless in

413   our experiment, soil DOC and OIP contents increased significantly following the two

414   effective treatments. The increase in DOC we recorded (+38-88%) might have been fueled

415   largely by the decrease in microbial biomass ($r = -0.82$, Table S2) probably resulting from

416   disruption of the plasma membrane and leading to the release of cytosolic soluble organic

417   compounds into the soil. Furthermore, some soil organic compounds might have undergone

418   abiotic hydrolysis with increased temperatures and thus might have taken part in the increase

419   in DOC. The release or accumulation of DOC had also been reported before with the

420   temperature increase induced by microwave treatment (625 W heating power, 2.45 GHz)

421   (Ferriss 1984). The dissolved organic carbon DOC is traditionally described as a tool for

422   monitoring adverse and rapid effects of management on soil quality (Silveira, 2005) and is

423   therefore useful for assessing unusual soil management techniques such as microwave

424   treatments. In our assay, the soil OIP content evolved in the same way as DOC in response

425   to the increased soil temperature. Again, this extra supply of OIP might have originated from

426   microbial biomass lysis and from hydrolysis of insoluble phosphorus forms. Applying

427   microwave treatment (1000 W heating power, 2.45 GHz for 5 minutes) has already been

428   shown to induce the release of soluble phosphorus into sewage sludge solution (+76% under

429   all temperature settings from 60 to 110°C) (Liao *et al.,* 2005). While our soil DOC and OIP

430   contents were solely reactive to effective treatments, there was a significant increase in the

431   NO_3^- content under the two ineffective treatments only. Both ammonium and nitrate are

432   commonly used by microbial communities, especially nitrifiers that sequentially oxidize

ammonia into nitrate. The first step of nitrification is carried out by ammonia-oxidizing-archaea (AOA) and ammonia-oxidizing-bacteria (AOB). Surprisingly, Li *et al.,* 2015 showed that the AOA activity increased when temperature rose from 50 to 70°C, whereas above 80°C the expression of ammonia monooxygenase (*amo*A), a key nitrification enzyme, was inactivated. Our study possibly suggests that the increase of NO_3^- in our two ineffective treatments (50–70°C) could be linked to an increase in nitrification activity that was, however, inhibited by the two effective ones (up to 80°C).

We could postulate, for some treatment conditions at least, that the increased availability of some nutrients (NO_3^- or phosphorus, or both) stimulated plant growth in the treated soils concomitantly with weaker competition for nutrients because of the decrease in microbial biomass. Khan *et al.* (2016) hypothesized improved nutrient availability after reporting that microwave treatment of soil (1100 W heating power, 2.45 GHz for 120 s) enhanced the growth and yield of wheat (+175% in dry biomass and +96% in grain yield). This hypothetical phytostimulatory scenario could also be strengthened by a potential reduction of phytoparasites (e.g. phytophagous nematodes) or of phytopathogen populations. The larger survival rates of our *Medicago* plantlets following their transfer into the treated soils could be explained in relation to the phytopathogenic–parasitic hypothesis. However, the initial positive effect we observed did not lead to larger biomass yields, except for root biomass under the 2 kW-8 minutes treatment. In addition, the chlorophyll contents known to be related to N supply were significantly and slightly less following the ineffective treatments, although soil NO_3^- contents were the largest of all. Consequently, increased availability of certain nutrients and weaker microbial competition in treated soils were not especially beneficial to *Medicago* plant growth. Alternatively, as plant biomass was not limited, microwave radiation probably did not generate phytotoxic compounds. Against this background, the number of nodules was significantly reduced under the two effective

458   treatments only without affecting plant growth, suggesting efficient rates of nitrogen (N)

459   fixation and mineral N uptake. The higher soil temperatures generated by the effective

460   treatments might have greatly affected the soil density of the *Medicago* symbiont *Ensifer*

461   *meliloti* and consequently the rate of nodulation. Furthermore, the increased soil NO_3^-

462   concentrations recorded under the two ineffective treatments were still below the

463   concentration threshold that inhibits root nodulation because the number of nodules per plant

464   was similar in the control. Because the effects of microwave radiation on soil NO_3^- and

465   nodulation patterns were strong, the effect of this seed-sterilizing procedure on rhizobial

466   symbiosis deserves further investigation. The diversity of nodulating bacteria should be

467   assessed in parallel with their N-fixing efficiency to determine whether their functional

468   diversity could be modified and alter the growth and biomass yield of legumes. Mycorrhizal

469   symbiosis, an almost universal plant–fungus symbiosis, should also be investigated.

470

471   **Conclusion**

472   Microwave radiation of soil belongs to the 'tool box' used to eradicate seed banks of invasive

473   species. However, such physical treatments can induce side effects on soil physicochemical

474   and biological properties that need to be anticipated to prevent ecological risks and enable

475   soil regeneration. Among the 16 properties we analysed to delineate the effects of

476   microwaves on soil, eleven were significantly affected by at least one of the effective

477   microwave treatments (microbial biomass, abundance of culturable bacteria – total bacteria

478   and fluorescent Pseudomonads, FDA hydrolysis, *Medicago* root nodules and root biomass,

479   DOC, TN and OIP contents, moisture). In addition, several effects revealed by the effective

480   treatments were correlated to the induced temperature increase (up to 83–95°C), as indicated

481   by correlation analysis. The deleterious effects on the soil microbial component were strong

482   but still far from complete soil sterilization, suggesting that the resilience of bacteria was

483 unaltered. However, the smaller nodulation of *M. truncatula* raises concern about the future

484 establishment of plant–microbe symbioses and calls for a thorough interpretation before

485 engineering new plant covers in such microwave-treated soils.

Figure S1 Diagram showing the experimental device used before the microwave treatment of soil samples.

Figure S2 Means of germination rate of *Festuca rubra* seeds ($n = 4$) inserted into untreated soil (control) and into microwave-treated soils before microwave radiation.

Table S1 Effect of five treatments (four microwave conditions and a control) on properties of soil and plants grown in the soil, tested using ANOVA from data presented in Tables 1 (physico-chemical soil data) and 3 (plant data).

Table S2 Correlation coefficients (r) of Pearson between all soil and plant properties (except ISCP data).

Table S3 Canonical correlation coefficients between the soil variables, the plant variables and the four principal components (PCs named from F1 to F4) after canonical correlation analysis (CCA).

FIGURE CAPTIONS

Figure 1 Ordination by principal component analysis (PCA) of soil properties (except ISCP data) in relation to microwave treatments. (a) Ordination in the plane of the two leading components of the control and microwave treatments; square, circle and triangle symbols stand for the mean coordinates ($n = 4$) with associated standard deviation (b) Correlation circle of the soil properties. FDA, fluorescein di-acetate hydrolysis; MB, microbial biomass; CFU-Pseudo, $\log_{10}$ of soil density of culturable fluorescent Pseudomonads; CFU-Tot. bact, $\log_{10}$ of soil density of culturable bacteria; TN, total nitrogen; TOC, total organic carbon; OIP, inorganic phosphorus measured by Olsen's method; DOC, dissolved organic carbon; WIP, water-soluble inorganic phosphorus

511

Figure 2 Ordination by principal component analysis (PCA) of *Medicago* seedlings properties in relation to their development in control and microwave-treated soils. (a) Ordination in the principal plan of the control and microwave treatments; square, circle and triangle symbols stand for the mean coordinates (n=4) with associated standard deviation (b) Correlation circle of the *Medicago* seedlings properties; SPAD, chlorophyll content; nodules, nodule number per plant.

518

Figure 3 Results of the canonical correlation analysis (CCA) of all soil and plant properties (except ISCP) displaying the strength of the correlations between *Medicago* growth parameters (in gray) and soil properties (in black). Canonical correlation analysis (CCA) was performed on soil and plant data (except ISCP). DOC, dissolved organic carbon; TN, total nitrogen; WIP, water-soluble inorganic phosphorus; MB, microbial biomass; FDA, fluorescein di-acetate hydrolysis activity; CFU-Pseudo, $\log_{10}$ of soil density of culturable fluorescent Pseudomonads; CFU-Tot. bact, $\log_{10}$ of soil density of culturable bacteria; SPAD, chlorophyll content; OIP, inorganic phosphorus measured by Olsen's method.

23

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627

628 **TABLES**

629 **Table 1** Means values of soil physicochemical properties in relation to microwave treatments and variance analysis (ANOVA) results

Microwave treatments	TN	TOC	OIP	N-NH$_4^+$	N-NO$_3^-$	DOC	WIP	Moisture content
	/g kg^{-1} DS	/g kg^{-1} DS	/µg^{-1} g DS	/µg g^{-1} DS	/µg g^{-1} DS	/mg g^{-1} DS	/µg g^{-1} DS	/% DS
2 kW-4 minutes	0.84	10.14	9.33	1.75	**3.61**	0.08	**0.91**	8.02
4 kW-2 min	0.85	9.95±	9.52	1.97	**3.23**	0.08	0.85	7.90
2 kW-8 min	**0.78**	9.74	**10.53**	1.72	1.44	**0.15**	0.85	**7.07**
4 kW-4 min	**0.81**	9.97	**11.58**	1.84	1.51	**0.11**	0.76	8.00
Control	0.90	9.74	8.9	1.39	1.42	0.08	0.80	8.01
P	0.047	0.618	0.008	0.731	<0.0001	<0.0001	0.026	0.015
SE	0.014	0.088	0.33	0.13	0.24	0.006	0.016	0.11
LSD	0.078	ns	1.44	ns	0.75	0.017	0.087	0.59

630 The values shown in bold face are significantly different from the control ($P \leq 0.05$). SE, standard error; LSD, least significant differences. The

631 value of LSD was not listed if differences were not significant. DS, dried soil; min, minutes; TN, total nitrogen; TOC, total organic carbon; OIP,

632 inorganic phosphorus measured by Olsen's method; DOC, dissolved organic carbon; WIP, water-soluble inorganic phosphorus.

633

634 **Table 2** Mean values of soil microbial properties in relation to microwave treatments and

635 variance analysis (ANOVA) results.

Microwave treatments	Microbial biomass	Density of total heterotrophic bacteria	Density of fluorescent Pseudomonads	FDA hydrolysis
	/mg OC kg^{-1} DS	/log$_{10}$ CFU g^{-1} DS	/log$_{10}$ CFU g^{-1} DS	/µg g^{-1} DS
2 kW-4 min	**223.76**	7.0	6.5	**14.37**
4 kW-2 min	**269.69**	7.1	6.6	**11.57**
2 kW-8 min	**114.21**	**6.7**	**4.4**	**3.24**
4 kW-4 min	**135.29**	**6.6**	**4.7**	**5.63**
Control	342.73	7.3	6.5	22.67

636

637 ANOVA

Properties	Source	Mean square MS	F	P	SE	LSD
Microbial biomass	Between treatments	35871.9	103.20	<0.0001	19.8	28.1
	Within treatments	347.6				
log10 (CFU of total bacteria)	Between treatments	0.301	5.28	0.007	0.074	0.36
	Within treatments	0.057				
log10 (CFU of Pseudomads)	Between treatments	4.937	13.87	<0.0001	0.26	1.61
	Within treatments	0.356				
FDA	Between treatments	235.56	74.66	<0.0001	0.90	2.68
	Within treatments	3.155				

638
639 The values shown in bold face are significantly different from the control ($P \leq 0.05$). DS, dried

640 soil; min, minutes; OC, organic carbon; FDA, fluorescein di-acetate hydrolysis. SE, standard

641 error; LSD, least significant differences. Degrees of liberty (df) = 4 and 15 for Between

642 treatments, and Within treatments, respectively.

643

644 **Table 3** *Medicago truncatula* development in microwave-treated soils and variance analysis

645 (ANOVA) results.

Microwave treatments	Number of surviving plants on 40 plants tested	Dried shoot biomass /mg plant⁻¹	Dried root biomass /mg plant⁻¹	Number of nodules per plant	Chlorophyll contents /SPAD units)
2 kW-4 min	38	8.14	1.37	6.48	**21.0**
4 kW-2 min	34	10.37	1.90	6.33	**22.5**
2 kW-8 min	36	11.29	**2.50**	**1.85**	24.2
4 kW-4 min	37	9.87	2.14	4.51	25.9
Control	29	10. 03	1.46	7.00	25.30
P	NA	0.077	0.035	0.025	0.002
SE	NA	0.37	0.14	0.61	0.51
LSD	NA	ns	0.77	3.26	2.22

646 The values shown in bold face are significantly different from the control ($P \leq 0.05$). SE, standard

647 error; LSD, least significant differences. The value of LSD was not listed if differences were not

648 significant. NA, not applied.

649

650 **Table 4** Eigenvalue, variance accounted for and cumulative variance from principal component

651 analysis (PCA) or canonical correlation analysis (CCA).

Data source and multivariate analysis	PC number	F1	F2	F3	F4	F5	F6
Soil properties, PCA (Figure 1)	Eigenvalue	5.4	2.2	1.4	0.7	0.6	0.6
	Explained variance / %	45.3	18.0	12.0	6.1	5.0	4.6
	Cumulative variance / %	45.3	63.3	75.3	81.4	86.4	91.1
Plant properties, PCA (Figure 2)	Eigenvalue	2.2	0.9	0.6	0.3	NA	NA
	Explained variance / %	53.9	22.4	15.4	8.3	NA	NA
	Cumulative variance / %	53.9	76.2	91.6	100.0	NA	NA
Plant and soil properties, CCA (Figure 3)	Eigenvalue	0.92	0.82	0.56	0.55	NA	NA
	Explained variance / %	32.3	28.8	19.6	19.3	NA	NA
	Cumulative variance / %	32.3	61.1	80.7	100.0	NA	NA
	Canonical correlation	0.96	0.91	0.77	0.70	NA	NA

652 PC, principal component; the first three principal components explain more than 75% of the
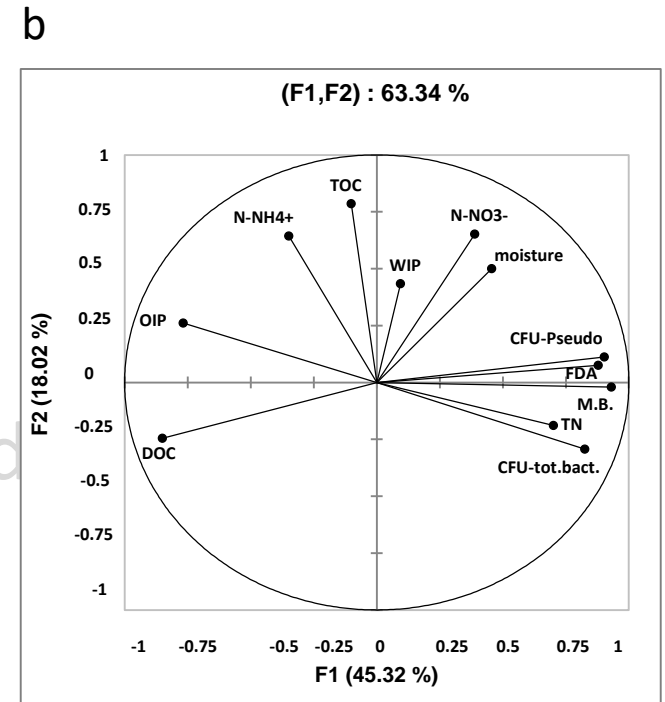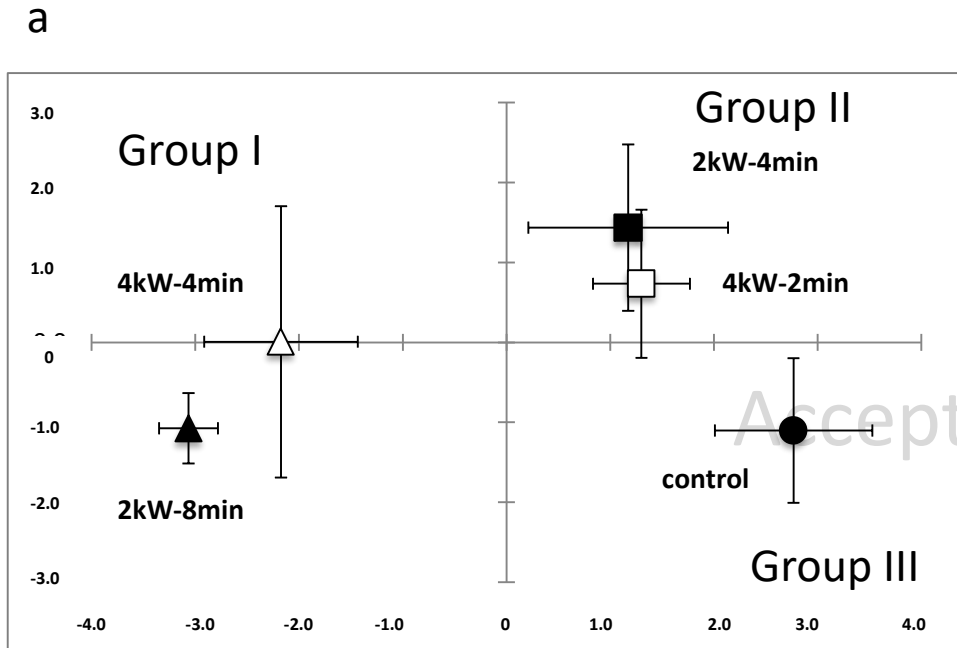
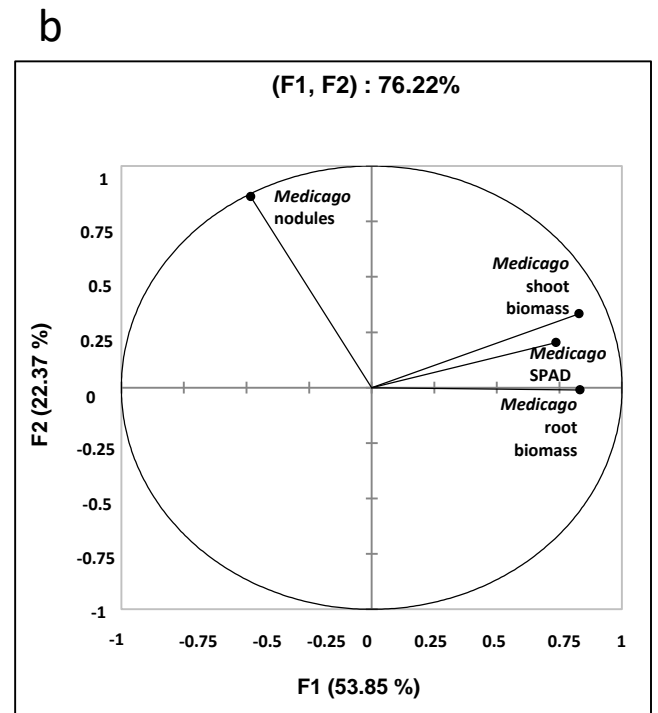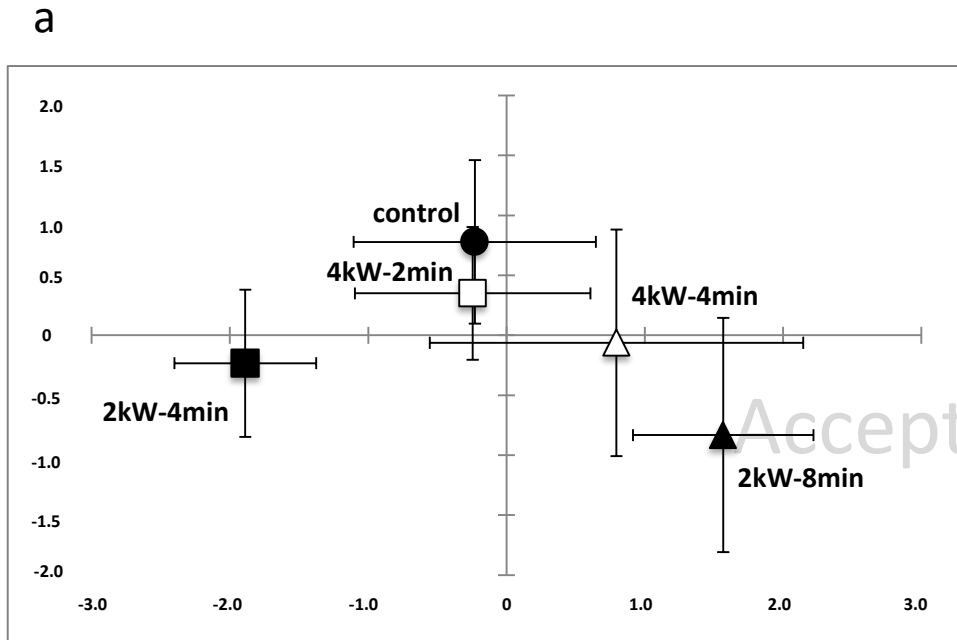653 variation in the data. NA: not applicable.

Fig. 1

a

b

(F1,F2) : 63.34 %

Group I

Group II

2kW-4min

4kW-4min

4kW-2min

Accepted

control

2kW-8min

Group III

Fig. 2

Fig. 3

(F1 ,F2) : 61.07 %