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

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Short title: Effect of microwaves on soil properties

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Summary

To control the soil seed bank of invasive plants with microwaves represents an alternative method to chemical treatments, but it could alter soil quality. Microwave effects were investigated on 17 soil physicochemical and biological properties. Four 915-MHz microwave treatments combining power and duration of exposure were applied on alluvial soil from a grassland using *Festuca* seeds as an internal standard to evaluate the efficiency of sterilization. Two treatments, 2 kW-8 and 4 kW-4 minutes, completely inhibited *Festuca* seed germination and caused the soil temperature to reach at least 80°C. Conversely, the other treatments, 2 kW-4 and 4 kW-2 minutes, failed to affect seed germination while raising the temperature to 50–60°C. Microbial biomass, the density of culturable heterotrophic bacteria and fluorescent Pseudomonads as well as fluorescein di-acetate hydrolysis significantly decreased by 2.7-, 1.1-, 1.4- and 5.1-fold compared with the control, respectively, following the 2 kW-8 and 4 kW-4 minutes treatments. These treatments also increased dissolved organic carbon and inorganic phosphorus contents by 1.6- and 1.2-fold, respectively. In contrast, nitrate increased only (about 140%) in the soils under the 2 kW-4 and 4 kW-2 minutes conditions. Furthermore, the growth of *Medicago truncatula* Gaertn. seedlings transplanted in microwave-treated soils under the 2 kW-8 and 4 kW-4 minutes conditions was not impaired despite total nitrogen and microbial population reduction and lower numbers of nodules. These results suggest that soil seed bank sterilization by microwaves that is 100% effective alters soil microbial indicators more strongly than chemical treatments without compromising the early growth stages of transplanted *Medicago* plants.

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

required to sterilize seeds effectively (Nelson, 1996), which also depends on soil physicochemical properties such as moisture content or texture, including the presence of stones (Brodie *et al.*, 2007; Sahin, 2014).

In addition to the energy cost and the need to adjust the microwave method to the local soil environment, the temperature increase induced by microwaves could also trigger side effects on soil biological and chemical properties (Zagal, 1989; Cooper & Brodie, 2009; Shamis *et al.*, 2012; Brodie *et al.*, 2015). Several investigations have revealed various susceptibilities among microbial populations to microwaves (Woo *et al.*, 2000; Tahir *et al.*, 2009). In particular, 2.45-GHz microwave treatment caused bacterial densities to decline in the top layers of soil, whereas fungal, ciliate, amoeba and flagellate densities were not affected. *Escherichia coli* populations declined by 10^5 in the top layer of soil treated with 500 J cm^{-2} (i.e. 500 W) of microwave energy (Brodie *et al.*, 2015). Microwave treatment (625 W heating power, 2.45 GHz for 150 s) eliminated specific populations of fungi such as *Pythium* and *Fusarium* together with non-cyst-forming nematodes, whereas *Rhizoctonia*, cysts of *Heterodera glycines* and mycorrhizal fungi survived better (Ferriss 1984). Cooper & Brodie (2009) investigated the effects of the length of exposure to microwaves (750 W heating power, 2.45 GHz for 2–16 minutes) in relation to soil depth (down to 40 cm) on plant nutrients and bacterial populations. They revealed small effects on nitrate, potassium and sulphate availability whatever the duration and the depth, whereas significant reductions of nitrite contents and bacterial populations (by 78%) were observed only in the topsoil as a function of treatment duration. Conversely, microwave treatment (600 W heating power, 2.45 GHz for 120 s) increased soil total organic carbon and nitrogen mineralisation (Zagal 1989).

Such chemical and biological modifications of soil induced by microwave treatments 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

Analytical B.V., Breda, The Netherlands), with a colorimetric method (Keeney & Nelson, 1982) after filtration at 0.2 µm. Analyses were all done by the Celesta Lab (France; www.celesta-lab.fr), except for WIP which was determined by the malachite green method (Ohno & Zibilske, 1991) after filtration at 0.2 µm (Millipore). Results were expressed as g kg^{-1} of dried soil.

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

The diversity of the *in situ* catabolic potentials (ISCPs) of the soil microbial community was assessed according to Dieng *et al*. (2014). The oxidation amounts (CO_2 emissions) of 30 organic substrates were estimated, including 11 carbohydrates, six carboxylic acids and 13 amino acids, as previously described. The acidity of the carboxylate stock solutions was adjusted to pH 6.5 with NaOH to minimize chemical artefacts from carbonate-derived CO_2 (Bérard *et al.*, 2011).

Fluorescein di-acetate (FDA) hydrolysis

Fluorescein di-acetate (FDA) hydrolysis was determined according to Schnurer & Rosswall (1982). One g of soil was suspended in 10 ml of sodium phosphate buffer (60 mM, pH 7.6) and further incubated with 50 µl of FDA substrate solution (stock solution at 2 mg ml^{-1} in acetone) for 1 hour at 24°C. After incubation, 10 ml of acetone were added to stop the reaction. Each soil suspension was centrifuged (8000 *g* for 5 minutes) and the supernatant was transferred to a colorimeter tube to measure its absorbance at 490 nm. Measurements were made in duplicate for each soil sample. The amounts of fluorescein released in the supernatant were calculated from a standard curve drawn from standard solutions containing 0.5, 1, 2, 3, 4 and 5 µg of FDA ml^{-1}. Standard solutions were prepared in sodium phosphate

buffer and boiled for 30 minutes to perform FDA hydrolysis. Values were expressed as µg of fluorescein released per g of dried soil.

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

Soil (4 g) was shaken at 150 revolutions per minute (rpm) in 36 ml of NaCl 0.9% for 30 minutes at 28°C. Serial decimal dilutions were prepared in NaCl 0.9%, and 100-µl aliquots were spread-plated in four replicates on to R2A and King's B agar media to enumerate total bacteria and fluorescent Pseudomonad populations, respectively. Petri dishes were incubated at 28°C, and colonies were counted for one week. Fluorescent colonies on King's B medium were identified under UV light (366 nm). Results were expressed as $\log_{10}$ of colony-forming units (CFU) g^{-1} of dried soil.

Microbial biomass

Microbial biomass was assessed by the chloroform fumigation–extraction method (Brookes *et al.*, 1985) in accordance with the AFNOR standard NF ISO 14240-2. Measurements were performed by the Celesta Laboratory. Results were expressed as mg of organic carbon (OC) kg^{-1} dried soil.

Medicago truncatula *growth response*

Medicago truncatula seeds were pre-germinated, and 10 seedlings were transplanted per pot containing 100 g of microwave-treated or control soil (one pot per experimental soil bag), and then grown for 7 weeks under controlled conditions (14 hours day, 10 hours night, 22,15°C day, night). Soil moisture was adjusted with sterile distilled water. At harvest, root nodules were counted and chlorophyll content was evaluated with a SPAD52 (Minolta Soil-

Plant Analyses Development, Roissy, France). Fresh and dried (65°C for 2 weeks) shoot and root (nodules included) biomass values were evaluated.

Statistical analyses

We used the Rcmdr package in R Software (R Foundation for Statistical Computing, Vienna) for out statistical analyses of variances and XLSTAT (version 2018.1.49150) for multivariate analyses and graphs. The effects of microwave and control treatments were tested on soil physicochemical and biological characteristics first by one-way analysis of variance (ANOVA). The normality of the residuals and homoscedasticity of residual variances were checked by graphical analyses of the standardized residuals considering histograms and the quantile–quantile (Q–Q) plots. The significance of the difference between means was evaluated for ANOVA by Fisher's least significant difference (LSD), with $P < 0.05$ as the criterion of significance. When the residuals of a variable did not satisfy the assumptions of ANOVA, diverse data transformations were tested until the normality of new residuals was reached. When the assumptions of ANOVA could not be met, the non-parametric Kruskal–Wallis (KW) test was used.

The ISCP profiles were compared by principal component analyses (PCA): as no groupings congruent with some microwave treatments could be visualized (data not shown), ISCPs were not considered in the further statistical analyses below.

Effects of microwave treatments on properties of soil (except ISCP) and *Medicago* plants growing in the soils were then analysed separately by PCA (Pearson's correlation matrix) to (1) explore the sample relationships and possible groupings that could be related to some microwave treatments and (2) to identify major soil or plant variables contributing 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

Information). Inorganic phosphorus measured by Olsen's method (OIP) and dissolved organic carbon (DOC) values increased significantly after the two effective treatments (+18, +30% for OIP and +88, +38% for DOC values, respectively for the two treatments, Table 1). Intriguingly, these two effective treatments did not alter NO_3^- and water-soluble inorganic phosphorus (WIP), whereas the other microwave treatments did. The NO_3^- content was greatly and significantly increased by the two ineffective treatments (+154 and +127%), whereas the WIP content increased slightly following the 2 kW-4 minutes treatment (+14%).

Effects of microwaves on soil microbial properties

All soil microbial properties were negatively affected by microwave radiation to different extents (Table 2), except the diversity of the ISCP of the soil microbial community (data not shown). The two effective microwave treatments systematically triggered similar significant and major declines in all properties, whereas the two ineffective treatments had no such effect. A decrease of more than 60% in microbial biomass was induced by the two effective treatments. In addition, smaller but significant reductions of microbial biomass were also observed following the ineffective treatments (–21 and –35%). The greatest reductions in the soil microbial biomass were mirrored by significant decreases in the culturable densities of heterotrophic bacteria by almost 10 times of CFU. For fluorescent Pseudomonads, the corresponding density losses even exceeded 100 times of CFU. The decrease in bacterial densities was correlated to the increase in soil temperature measured immediately after microwave radiation (Pearson' coefficient r = –0.52 (total bacteria) and –0.48 (Pseudomonads), Table S2 ($P < 0.05$), Supporting Information) to the increase in the soil temperature.

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

group III (untreated soil) (Figure 1a). The last two groups were further resolved along the second principal component F2. On the first axis, group II is closed to group III containing the soil samples of the control which is also ineffective. The 4 kW-2 and 2 kW-4 minutes treatments were not fully resolved within group II, whereas the 2 kW-8 and 4 kW-4 minutes treatments were fully resolved within group I. All microbial variables (microbial biomass, bacterial densities and hydrolase activity) were strongly correlated with one another according to the Pearson correlations (Table S2, Supporting information). The eigenvectors show that these microbial variables together with the total N, OIP and DOC variables contribute strongly to the definition of the first component in the correlation circle (F1=45.3%, Figure 1b). Thus, soil replicates in Group I could be distinguished from those of Group II and III, mostly on the basis of that subset of variables (e.g. effective treatments caused soil OIP and DOC contents to increase while diminishing the total N content and microbial properties, in particular the microbial biomass, Pseudomonads density and enzyme FDA hydrolysis (see also Tables 1 and 2)). Soil NO_3^-, TOC and NH_4^+ variables were preponderant in the definition of the second principal component (Figure 1b). Yet, only NO_3^- was useful to distinguish soil replicates from Groups II and III as confirmed in Table 1.

In contrast, the PCA ordination of *Medicago* seedling properties (Figure 2) did not show a similar pattern congruent with the treatment groups previously defined with soil properties in Figure 1. Indeed, soil replicates of the 2 kW-4 treatment were the only ones that could be unambiguously distinguished from the other soil samples and treatments along the first principal plan (Figure 2a). All *Medicago* plant properties, except the nodule number, contributed strongly to the definition of the first principal component (F1=53.9% Figure 2b). Thus the relative distribution of the 2 kW-4 minutes treatment in this plane (Figure 2a) seemed to be explained mainly by diminished shoot and root biomasses, and lower SPAD unit values (Figure 2b) compared with all the other microwave treatments and the control.

Correlations between soil and *Medicago* plant properties were further analysed by CCA (Figure 3, Table 4 and Table S3 Supporting Information). The first two canonical correlations calculated between the two sets of variables were almost maximal (0.96 and 0.91, Table 4) revealing that soil and plant properties datasets were highly correlated according to the first two canonical components and legitimating the interpretation of their relationships in this plane. This analysis essentially revealed that plant properties (except nodules) were mostly positively correlated with soil DOC and to a lesser extent with soil OIP (see also Table S2, Supporting Information). By contrast, plant SPAD, shoot and root biomass were negatively correlated with nodule number and most of the other soil variables, especially microbial densities and enzyme FDA hydrolysis.

Discussion

The effects of 915-MHz microwave treatment of a soil to eradicate the seed bank of invasive species were investigated under four radiation conditions that combined power level and exposure time. The effectiveness of the four conditions chosen according to De Wilde *et al.* (2017), related to the rates of seed germination of three invasive plant species, was confirmed here on *Festuca rubra* seed germination. More precisely, the 2 kW-8 and 4 kW-4 minute conditions caused soil temperature to reach at least 80°C leading to complete inhibition of *Festuca* seed germination and were designated effective treatments. By contrast, the 2 kW-4 and 4 kW-2 minute treatments failed to affect the germination rate of *Festuca* seeds significantly (50–60°C soil temperature) and were considered ineffective treatments. The effectiveness of our microwave-based seed sterilization thus appeared to be positively related to the final soil temperature, which has been reported previously by several authors (Sartorato *et al.*, 2006; Brodie *et al.*, 2007; Sahin, 2014). In addition to temperature, we measured chemical (total nitrogen, NH_4^+, NO_3^-, inorganic phosphorus and organic carbon

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

362 Soil biological properties were the most responsive to microwave treatments. Microbial
363 biomass, has been used traditionally as a bioindicator of soil quality (Andrews *et al.*, 2004).
364 In this study it was affected considerably by microwaves, especially under the effective
365 treatments (–60%). The high temperatures recorded (80°C) probably weakened biological
366 membranes, e.g. by denaturing their proteins, leading to their poration and disruption. Local
367 temperatures in some soil micro-niches might even have reached >100°C leading to boiling
368 of the cellular contents. Banning & Murphy, 2008) reported that the direct heating of field
369 soil microcosms in an oven at 120°C for 20 minutes resulted in a similar loss of microbial
370 biomass (–50%). Our soil density of total heterotrophic culturable bacteria and especially
371 fluorescent Pseudomonads were also affected by effective treatments (about 10-fold and
372 100-fold less CFU, respectively). It can be speculated that Pseudomonads could be more
373 easily lysed because they are Gram-negative bacteria and that Gram-negative bacteria show
374 lower resistance towards microwaves than Gram-positive bacteria, as previously published
375 (Woo *et al.*, 2000, Gedikli *et al.*, 2008, Tahir *et al.*, 2009). Moreover, certain Gram-positive
376 bacteria can tolerate heat shocks better because of their sporulation ability, which could have
377 contributed to the smaller decrease we observed in our total bacteria counts. In addition to
378 loss in cell vitality, microwaves might also have decreased bacterial culturability and our
379 subsequent CFU counts. Against this background, it would be interesting to refine the
380 analysis by monitoring the proportion of culturable sporulating bacteria and evaluating the
381 density of viable but non-culturable bacteria from microscopic counts combined with vital
382 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.

The microwave treatments we applied, had less effect on soil chemical properties than microbiological traits, as already observed by Cooper & Brodie (2009). These authors reported that microwave treatment (750 W heating power, 2.45 GHz) had no effect on soil pH or nitrate, phosphorus, potassium and sulphate availability: they found that only the size of the bacterial community was reduced by 78% in the top layer of the soil. Nevertheless in our experiment, soil DOC and OIP contents increased significantly following the two effective treatments. The increase in DOC we recorded (+38-88%) might have been fueled largely by the decrease in microbial biomass (r = –0.82, Table S2) probably resulting from disruption of the plasma membrane and leading to the release of cytosolic soluble organic compounds into the soil. Furthermore, some soil organic compounds might have undergone abiotic hydrolysis with increased temperatures and thus might have taken part in the increase in DOC. The release or accumulation of DOC had also been reported before with the temperature increase induced by microwave treatment (625 W heating power, 2.45 GHz) (Ferriss 1984). The dissolved organic carbon DOC is traditionally described as a tool for monitoring adverse and rapid effects of management on soil quality (Silveira, 2005) and is therefore useful for assessing unusual soil management techniques such as microwave treatments. In our assay, the soil OIP content evolved in the same way as DOC in response to the increased soil temperature. Again, this extra supply of OIP might have originated from microbial biomass lysis and from hydrolysis of insoluble phosphorus forms. Applying microwave treatment (1000 W heating power, 2.45 GHz for 5 minutes) has already been shown to induce the release of soluble phosphorus into sewage sludge solution (+76% under all temperature settings from 60 to 110°C) (Liao *et al.*, 2005). While our soil DOC and OIP contents were solely reactive to effective treatments, there was a significant increase in the NO_3^- content under the two ineffective treatments only. Both ammonium and nitrate are 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

unaltered. However, the smaller nodulation of *M. truncatula* raises concern about the future establishment of plant–microbe symbioses and calls for a thorough interpretation before engineering new plant covers in such microwave-treated soils.

Supporting Information

The following supporting information is available in the online version of this article:

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

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.

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.

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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 /g kg^{-1} DS	TOC /g kg^{-1} DS	OIP /μg^{-1} g DS	N-NH_4^+ /$\mu g\ g^{-1}$ DS	N-NO_3^- /$\mu g\ g^{-1}$ DS	DOC /mg g^{-1} DS	WIP /$\mu g\ g^{-1}$ DS	Moisture content /% 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 /mg OC kg^{-1} DS	Density of total heterotrophic bacteria /log_{10} CFU g^{-1} DS	Density of fluorescent Pseudomonads /log_{10} CFU g^{-1} DS	FDA hydrolysis /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

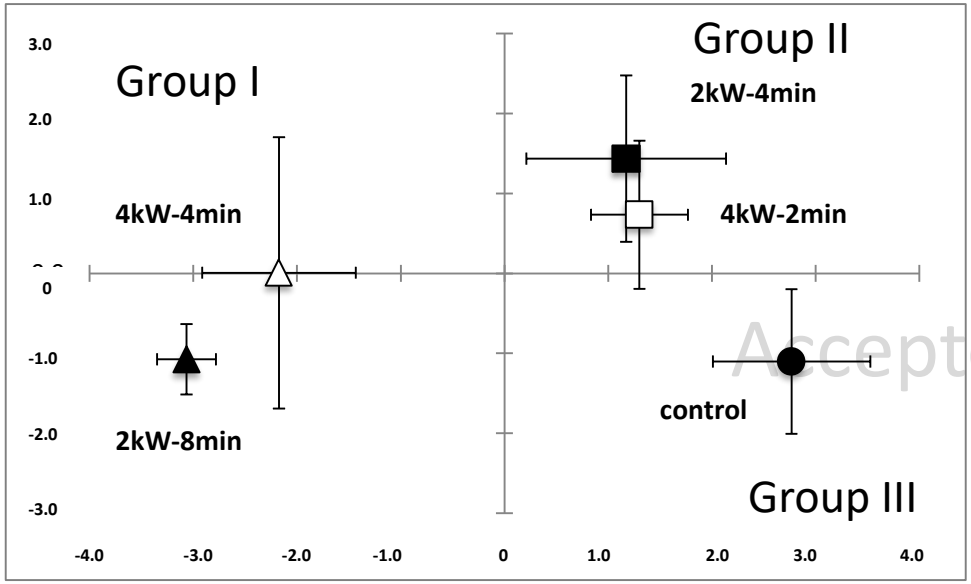
650 **Table 4** Eigenvalue, variance accounted for and cumulative variance from principal component
651 analysis (PCA) or canonical correlation analysis (CCA).

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

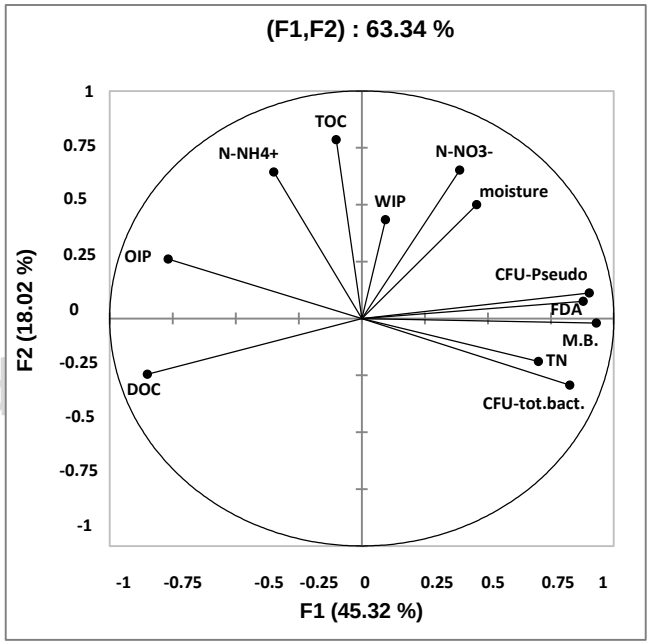
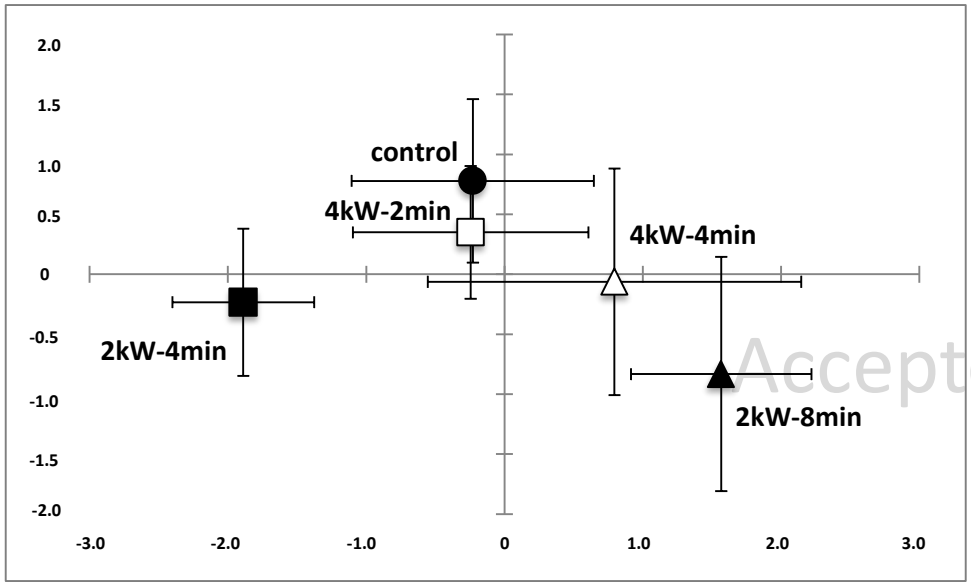


Fig. 2

a



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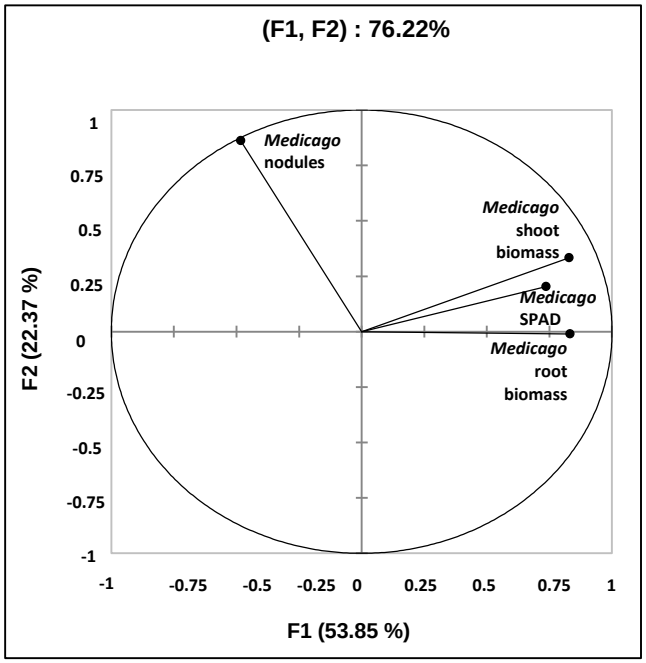

Fig. 3