

## **Impact of Microwave Disinfestation Treatments on the Bacterial Communities of No-till Agricultural Soils**

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**Running Title:** Microwave soil heating alters soil bacterial communities

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## Summary

Growing herbicide resistance has encouraged the development of new technologies for weed control. Pre-sowing microwave (MW) soil heating has been shown to reduce weed establishment in no-till farming systems, and substantially increases crop productivity. However, the effect of this technology on the soil microbial community in general, and on beneficial soil microbes such as ammonia oxidizers in particular, warrants further study. In order to check the effect of MW soil disinfestation treatment on the soil biota, indigenous soil microcosms were treated under a horn antenna of the MW prototype for three distinct durations. Immediately after heating (T0) and 28 days after heating (T28), the soil was collected at two penetration depths (0 – 5 and 5 – 10 cm) of MW energy to determine the bacterial community responses based on the 16S rRNA amplicon sequencing and the total abundance of bacteria and ammonia oxidisers with qPCR. While total bacteria and ammonia oxidizers exhibited no response to the MW treatments, bacterial community composition differed according to the treatment durations. Community responses clustered into two categories: no effect at low heating intensities (0 and 30 s, 17 – 45 °C); and strong effect at high heating intensities (60 and 90 s, 65 – 78 °C). For the latter group, community richness did not recover to its pre-heating levels within the four weeks studied. Immediately after high heating intensities treatments, the relative abundance of Firmicutes increased, and that of Proteobacteria decreased significantly regardless of penetration depth. The relative abundances of beneficial soil microbes (*Micromonosporaceae*, *Kaistobacter* and *Bacillus*) were significantly higher as soils recovered from high heating intensities compared with

untreated soils at T28. Our findings suggest that while pre-sowing MW treatments alter the soil microbial community, beneficial soil microbes exhibit faster recovery.

**Keywords:** microwave weed management, soil heating, soil bacteria, microbial recovery, heat resistant.

### **Highlights**

1. High heating intensities significantly reduced the bacterial community richness and community did not recover to its pre-heating condition within the studied time.
2. Heat resistant bacterial taxa survived the high heating intensities proposed for sterilizing weed seedbank, exhibited recovery and remained dominant in the community over 28 days after heating regardless of penetration depth.
3. High heating intensities did not induce a negative impact on the abundance of ammonia-oxidizing bacteria and archaea.
4. Overall, microwave soil heating altered the bacterial community and dormant heat-resistant taxa benefited and exhibited recovery.

### **Introduction**

Agricultural pests are enormously declining the sustainability of farmland (Fischer & Connor, 2018), and the traditional usage of chemicals for pest control has shifted the pests' adaptability to resist this widely adopted control measure. This adaptability of pest organisms (*i.e., pesticides and herbicides resistance*) can impose a significant challenge to sustainable food production. Therefore, modern agriculture systems require

sustainable pest control practices to ensure food security (Pretty & Bharucha, 2014). Among these practices, the development and adoption of non-chemical weed control techniques is one of the most pressing areas in need of development, due to the evolution of herbicide-resistant weeds (Heap, 2016), chemical residues in food chain (Fritschi *et al.*, 2015; Cozma *et al.*, 2017) and deterioration of the cropping environment (Fischer & Connor, 2018). Therefore, an alternative approach in farming system is needed to cope with pests' adaptability.

Microwave (MW) soil heating for weed management has been proposed as an alternative to chemicals in the agriculture industry (Nelson, 1996; Sartorato *et al.*, 2006; Brodie *et al.*, 2009; Khan *et al.*, 2017). MW is a part of electromagnetic spectrum within a frequency range of  $300 \text{ MHz} < f < 300 \text{ GHz}$  (Banik *et al.*, 2003). The heating process relies on the partial and/or complete dissipation of the MW electromagnetic field into the load, which ultimately induces polarization of dipolar molecules (*i.e.*, water), resulting in the rapid increase in the temperature of the dielectric material due to internal resistance (Metaxas & Meredith, 1983; Diprose *et al.*, 1984). In the case of a pre-emergence weed management, this rapidly increased temperature devitalises the weed seedbank by generating micro-steam explosions in the seeds, leading to their mortality in the top soil horizons and thus preventing the emergence of weeds (Khan *et al.*, 2017; Khan *et al.*, 2018). This characteristic, real time heating, of MW technology distinguishes it from the numerous classical thermal methodologies, for example: soil steaming and soil solarization, for weed and pathogen control (Brodie, 2018).

We have previously designed and developed a prototype to harness this property of MW energy by irradiating agricultural fields between crop rows for weed management (Brodie *et al.*, 2018). This prototype has been successfully employed to minimize the regeneration capacity of the soil weed seedbanks up to certain depths into soils. Recently, numerous field trials demonstrated that pre-sowing MW soil heating (75 – 80 °C; in top 0 – 8 cm) significantly reduced weed emergence (Khan *et al.*, 2017; Brodie *et al.*, 2018; Khan *et al.*, 2018). As this technology is in its pre-commercialization phase, the farming practitioners and policy makers are keen to know the response (*i.e.*, *recovery and/or reshaping after heat disturbance*) of the soil microbial community, which is important to soil health and plant productivity (Berendsen *et al.*, 2012), to MW energy along with the penetration depth into soil for disinfestation. Recently, Sabry *et al.* (2018) reported that a closed horn antenna, attached to a modified domestic MW (700W; 2.45GHz) system, reduced MW energy leakage and increased absorption. They found that penetration depth (up to 5 cm) did not change the temperature intensity below the soil surface during the soil disinfestation process. But this is not always the case, numerous factors can cause temperature fluctuations below the soil surface. Given that, soil temperature fluctuation is entirely depended on the MW system and soils conditions, and this fluctuation causes reductions in the lethality of MW soil heating. Therefore, the variation in temperature distribution along the penetration depth may change the response of the soil microbial community and this response is unknown in terms of soil disinfestation achieve through this MW prototype.

Soil is a complex heterogeneous environment (Lu *et al.*, 2014), harboring a wealth of bacteria, fungi, protozoans, nematodes, and arthropods, which may be adversely affected by the MW soil treatment. Bacteria comprise the majority of the soil biota by biomass (Sánchez-Marañón *et al.*, 2017). In general, soil heating creates a transient disturbance for soil bacterial communities, and may affect their composition and function (O'Brien *et al.*, 2018). However, it is possible that the rapid growth and extreme diversity found in these communities results in a rapid recovery of both community structure and function, resulting in no long-term negative effects (Fierer, 2017). Nevertheless, it is likely that the long-term effects of heating depend on heating duration and intensity. For example, Nunes *et al.* (2018) evaluated the tolerance range of the soil bacterial community exposed to different MW heating durations and reported a 90% reduction in total bacterial communities with 6.8 minutes of MW soil treatment. Additionally, they found communities' tolerance to MW to be phylogenetically constrained: Actinobacteria were highly susceptible to heating, Proteobacteria showed intermediate tolerance, and Firmicutes exhibited the highest resistance. Therefore, the heating duration decides the extent of soil sterilization and the behavior of surviving species, as observed by Nunes *et al.* (2018). The majority of previous studies employed a domestic MW oven (closed cavity) for soil treatment to check the bacterial community responses; however, the MW prototype channels MW energy via an open-structured horn antenna. This could vary the temperature distribution in soils. Therefore, bacterial community responses to short term temperature exposure, achieved through

the MW prototype, is not well understood but is crucial for long-lasting productivity of soil systems after MW soil treatment for weed seedbank control.

In addition, the transient heat mediates the mineralization of organic nitrogen into ammonia ( $\text{HN}_3$ ) (Zagal, 1989; Glass *et al.*, 2008), and is subsequently converted to nitrate ( $\text{NO}_3^-$ ) via nitrification, which could enhance soil health for crop productivity. In soil, the conversion of ammonia to nitrite, an intermediate step in nitrification, is performed exclusively by two small, phylogenetically-conserved groups containing the ammonia monooxygenase (*amoA*) gene: ammonia oxidizing Archaea (AOA) and Bacteria (AOB; Banning *et al.*, 2015). The tolerance range and rate of recovery of ammonia oxidizers to MW treatment is unknown but may be crucial to the maintenance of soil fertility following MW weed disinfestation. Previous studies have reported ammonia oxidizing microbes to be a slow-growing and sensitive functional group (Leininger *et al.*, 2006; Banning *et al.*, 2015), suggesting that they could indeed be negatively affected by the MW treatment.

In order to fully understand the potential effects of the MW weed disinfestation treatment and its role in shaping the soil microbiota, we exposed soil microcosms to the MW weed disinfestation treatments at three MW durations and monitored the bacterial community composition (16S rRNA amplicon sequencing and 16S rRNA qPCR) as well as the total abundances of AOA's and AOB's (qPCR). We sampled the microcosms immediately after treatment (T0) and 28 days later (T28). Because the treatments were expected to be more extreme on the upper soil strata, we separately

sampled the soil at 5 cm and 10 cm to evaluate the effectiveness of the MW treatment in regard to penetration depth for disinfestation. We hypothesized that MW soil treatment would alter the bacterial community based on various taxa's temperature sensitivities and the damaged community would recover to its initial level within four weeks following the heat disturbance. Additionally, we hypothesized that the physiological adaptation of ammonia oxidisers to extreme events might help them to survive against this transient heat disturbance.

## **Materials and Methods**

### ***Soil Collection and Microwave Heating:***

Soil was collected from a wheat paddock on temperate agricultural land at the Dookie Campus (36.395°S; 145.703°E), of The University of Melbourne, Australia. This region is in the country's central grain growing belt, featuring a monocropping pattern with wheat-canola rotation, and is in the Goulburn Valley of North Victoria, Australia. The mean annual rainfall is 550 mm and mean annual temperature ranges from 9.4 – 20.9 °C (Australian Bureau of Meteorology). The average load of inorganic nitrogen fertilizer for crop production in this region is 100 – 120 kg ha<sup>-1</sup> and herbicide application is the most common weed management practices.

Soil at the sampling site was a brownish grey loam and was classified as Red Mesotrophic-Haplic Dermosol according to Australian Soil Classification (Isbell, 2016), with a field capacity of  $\approx 53\%$ . Soils were collected with cores (2 cm diameter; 0 – 10 cm depth), sieved (4-mm mesh size) and mixed thoroughly to produce a composite sample and shifted to the laboratory for microcosm establishment. Water content was

measured by oven drying the soil at 105 °C for 24 h and adjusted, with sterile distilled water, to 20% volumetric water content ( $\approx$ 45% field capacity). Soil was placed in polyethylene plastic, transparent to MW energy, microcosms (5×5×10 cm) and pre-incubated at 25 °C for three weeks in an incubator (Contherm Scientific Ltd.; Lower Hutt, New Zealand). To maintain the aerobic conditions, all the microcosms were aerated by opening lids every 3 days and moisture loss was replaced. Details of the soil properties are given in Table: S1 (Supplementary Material).

Microcosms were treated under one of the four open-structured horn antennas (5.5×11×36 cm) of a 2-kW MW weed treatment system. This experimental system was designed to apply MW energy for pre- and post-emergence weed control between crop rows, with an inter-antennae distance of 19.5 cm. It has four independently controlled 2-kW MW generators, which are powered through two on-board 7 kVA 3-phase electrical generators (Figure S1; Brodie *et al.*, 2017). Microcosms were subjected to three treatment durations (30, 60 and 90 s) as well as an untreated control conditions and sampled at two points following treatment (0 and 28 days), in quadruplicate.

Irrespective of cropping systems, previous field trials have demonstrated that MW soil treatment for 60 and 90 s was enough to achieve the necessary temperature for killing weed seedbanks (75 – 80 °C; Brodie *et al.*, 2018), and these durations were thus selected for further study. A 30 s duration was selected as an intermediate treatment. Thermal images were taken immediately (2 – 5 s) after soil heating by using an infrared thermal camera (C2; FLIR Systems Inc.; Wilsonville, Oregon, USA). The raw images of each MW treatment were post-processed in the MATLAB<sup>®</sup> (The Mathworks Inc.,

Natick Massachusetts, USA) to evaluate the temperature distribution in the microcosm. At each sampling point, a small core (1.5 cm in diameter) was collected at 0 – 5 cm and 5 – 10 cm depth from each replicate. For soil collection at T0, a small corer (1.5 cm in diameter) was used to evacuate the soil column at each depth. Immediately after sampling, to avoid any disturbance in soil profile, the corer was positioned into the microcosm exactly where the sampling occurred until the final sampling happened at T28. This final sampling was done with a separate, but same sized, corer from both depths. The samples were immediately transferred to the laboratory and stored at – 20 °C.

***DNA Extraction and qPCR:***

DNA was extracted from 0.25 g of soil with the Power Soil™ DNA Isolation Kit according to the manufacturer's instructions (MoBio Laboratories Inc., Carlsbad, CA, USA). DNA extracts were quantified using a Nanodrop™ ND2000c spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and their concentrations ranged between 20 – 80 ng g<sup>-1</sup> soil.

qPCR was used to quantify the absolute abundances of archaeal and bacterial *amoA* genes, as well as the total bacterial abundances. All qPCR reactions were performed in a CFX384™ optical qPCR detection system (Bio-Rad, Laboratories Inc.; Hercules, CA, USA), and each sample was measured in triplicate. The reaction for quantification of the archaeal-*amoA* gene contained 10 µL of SensiFAST (Bio-Rad Laboratories, USA), 1 µL of each primer set in accordance with Francis *et al.* (2005; 10 µM; Arch-amoAF and Arch-amoAR), 2 µL of 10-fold diluted DNA template and 6 µL of water. Each

bacterial-*amoA* qPCR reaction contained 10  $\mu\text{L}$  iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 1  $\mu\text{L}$  of each primer set according to Rotthauwe *et al.* (1997; 10  $\mu\text{M}$ ; *amoA*-1F and *amoA*-2R), 2  $\mu\text{L}$  of 10-fold diluted DNA template and 6  $\mu\text{L}$  of water. The thermal cycling conditions for both archaeal and bacterial *amoA* gene quantifications were as follows: 95  $^{\circ}\text{C}$  for 3 min then 40 cycles of 95  $^{\circ}\text{C}$  for 5 s, 60  $^{\circ}\text{C}$  for 30 s and 72  $^{\circ}\text{C}$  for 45 s. Standard curves, generated in each qPCR reaction by using plasmids, were linear over six orders of magnitude ( $10^4 - 10^9$  gene copies  $\mu\text{L}^{-1}$ ) with  $r^2$  values of 0.98 – 0.99; efficiency was 85 – 100%.

For the quantification of total bacteria, we quantified copies of 16S rRNA. Each 20  $\mu\text{L}$  qPCR reaction consisted of 10  $\mu\text{L}$  iTaq Universal SYBR GREEN Supermix (Bio-Rad Laboratories, USA), 1  $\mu\text{L}$  of each primer set according to Suzuki *et al.* (2000; 10  $\mu\text{M}$ ; 1369F and 1492R), 2  $\mu\text{L}$  of 10-fold diluted DNA template and 6  $\mu\text{L}$  of water. PCR thermal cycling conditions for 16S rRNA gene amplification were as follows: 95  $^{\circ}\text{C}$  for 10 min; followed by 40 cycles of denaturation at 95  $^{\circ}\text{C}$  for 15 s, annealing at 54  $^{\circ}\text{C}$  for 30 s, and extension at 72  $^{\circ}\text{C}$  for 30 s. Standard curves were generated for each qPCR reaction, which were linear over six orders of magnitude ( $10^4 - 10^9$  gene copies  $\mu\text{L}^{-1}$ ), with  $r^2$  of 0.97 – 0.99 and efficiency of 80 – 100%.

#### ***16S rRNA Sequencing:***

16S rRNA amplicon sequences were used to estimate the relative abundances within the soil bacterial community. Two samples per condition were sequenced. The V3-V4 regions of the 16S rRNA gene was amplified using primers 341F and 806R, as previously described (Yu *et al.*, 2005). Paired-end sequencing was performed in an

Illumina MiSeq sequencer (San Diego, CA, USA). Paired-end reads were merged with PEAR (v. 0.9.5; Zhang *et al.*, 2013) and primers were trimmed. Reads were quality-filtered, full-length duplicates were removed, and chimeras were filtered against the “rdp\_gold” database using USEARCH (v. 8.0.1623; Edgar, 2010; Edgar *et al.*, 2011) and UPARSE (Edgar *et al.*, 2011). Singletons were removed. Reads were clustered at 97% identity and assigned with the Greengenes database (Version 13\_8, Aug 2013; DeSantis *et al.*, 2006) in QIIME (v.1.8; Caporaso *et al.*, 2010)

***Statistical Analyses:***

All statistical analyses were performed in the R 3.4.0 software (Team, 2014) using the *phyloseq* (McMurdie & Holmes, 2013) and *vegan* (Oksanen *et al.*, 2008) packages.

Prior to the analysis of amplicon sequencing data, reads were randomly subsampled to 30,745 reads for sample. The final dataset contained 6,154 prokaryotic OTUs. Alpha diversity was evaluated using Shannon's diversity index, and differences between MW durations and sampling time (days after heating) were tested with a two-way ANOVA, and Tukey post-hoc test was performed to determine changes in response to soil heating intensities. Beta diversity was evaluated with a principal coordinate analysis (PCoA) of Bray-Curtis distances. Clustering patterns of the resulting ordination were assessed using *adonis* and *betadisper* from the *vegan* package. For genus-level analyses, taxa were agglomerated by genus, and individual ANOVAs for the effect of time and MW duration were performed for each genus. qPCR data was transformed to  $\log_{10}$  prior to analyses. Differences between gene copy numbers for AOA, AOB, and total 16S data were compared between treatments using ANOVA. Specific measurements are presented as *mean  $\pm$  standard deviation*.

## Results

### *Temperature Distribution in Soil:*

The mean temperature of the untreated control soil was  $20\pm 2$  °C (Figure 1A). Immediately after heating for 30, 60 and 90 s, soils exhibited sharp increases in temperature to  $45.1\pm 2.1$ ,  $64.3\pm 2.8$  and  $78.3\pm 2.1$  °C, respectively, confirmed by infrared thermal images of the microcosm and liquid-in-glass thermometers (Figure 1B-D). The peak temperature for treatment duration was retained for less than 20 s in the soils following heat disturbance. A cross-sectional infrared thermal image of the microcosms indicated that the heating intensity gradually decreased along the penetration depth (Figure S4), and we categorized 0 – 5 cm depth as a high heating zone and 5 – 10 cm depth as a moderate heating zone based on temperature. Despite this, we detected a slightly higher reflection of MW energy in the moderate heating zone than in the high heating zone.

### *Bacterial Community Composition:*

We performed 16S rRNA amplicon sequencing in order to observe the effect of MW heating on the bacterial community. A visual inspection of the PCoA of Bray-Curtis distances between samples, revealed clustering of samples exposed to 0 – 30 s MW, and samples exposed to 60 – 90 s MW (Figure 2). The first axis represented 53.6% of the variation in community composition, and the second axis represented 13.8% of the variation. At T0, the soils exposed to 30 s MW heating clustered closely to control samples, regardless of penetration depths. A similar pattern was found at T28. In contrast, microcosms which were exposed to 60 – 90 s MW, clustered separately from

the untreated controls soils and 30 s MW soil treatment. Therefore, we categorized these heating durations into two groups: low-intensity (0 – 30 s) and high intensity (60 – 90 s) treatments. Community composition significantly clustered according to these MW heating intensity categories (PERMANOVA, pseudo-F = 26.3,  $R^2 = 0.28$ ,  $p < 0.001$ ), days after heating (PERMANOVA, pseudo-F = 17.7,  $R^2 = 0.18$ ,  $p < 0.001$ ), and the interaction of MW intensities  $\times$  days after heating (PERMANOVA, pseudo-F = 14.56,  $R^2 = 0.15$ ,  $p < 0.001$ ) on the bacterial community composition (Figure 2). Community composition varied along the penetration depth of MW energy (PERMANOVA, pseudo-F = 3.22,  $R^2 = 0.03$ ,  $p = 0.034$ , Figure 2).

***Bacterial Community Richness:***

High MW soil heating intensities significantly reduced bacterial community diversity relative to the low intensity treatments, evidenced by decreasing Shannon richness index (ANOVA;  $p < 0.001$ , Figure 3) at T0. This reduction in richness was still detected at T28 (Tukey post-hoc test;  $p < 0.0001$  for comparison among low and high intensities at T28 for either penetration depths). Additionally, the combination of MW heating intensity  $\times$  days after heating had a significant impact on the community richness (ANOVA;  $p < 0.001$ ; Figure 3). Immediately after heating (T0), high intensity treatment induced a 58% reduction in richness, on average, regardless of penetration depths.

***Phylum- and Genus-Level Responses:***

Bacterial community responses to the combined effect of MW heating intensities and penetration depth were assessed at the phylum and genus levels. Across all samples, Actinobacteria (37.3%) was the predominant phylum followed by Proteobacteria

(26.7%), Gemmatimonadetes (7.9%), Acidobacteria (7.2%) and Firmicutes (6.1%). At 0 – 5 cm depth, the relative abundance of Proteobacteria significantly increased and Firmicutes decreased with high heating intensities at T0 (ANOVA;  $p < 0.001$ ; Figure 4) while Proteobacteria remained dominant, and Firmicutes exhibited recovery at T28. At 5 – 10 cm depth, Proteobacteria significantly decreased and did not recover to control levels by T28, and Firmicutes increased according to high heating intensities at T0 (ANOVA;  $p < 0.001$ ; Figure 4) and remained higher in the community by T28. Actinobacteria showed significantly higher relative abundance by T28 than at T0 (ANOVA;  $p < 0.001$ ; Figure 4).

Of the 10 most abundant genera across all samples, at 0 – 5 cm depth, the relative abundance of the *Bacillus* and an unclassified *Gaiellaceae* significantly increased with high heating intensity (ANOVA;  $p < 0.001$ ; Figure 4) at T0 and they remained relatively higher in the community by T28. While the relative abundance of *Kaistobacter*, *Micromonosporaceae*, *TM7-1*, *Xanthomonadaceae* was significantly higher at T28 than at T0. At 5 – 10 cm depth, the relative abundance of the *Bacillus*, *Gaiellaceae* and *Kaistobacter* significantly increased with high heating intensities while the relative abundance of the *Micromonosporaceae*, *Rhodospirillaceae*, *Myxococcales* and *Solirubrobacterales* significantly decreased at T0 (ANOVA;  $p < 0.001$ ; Figure 4). *Micromonosporaceae*, *TM7-1* and *Bacillus* exhibited recovery at T28 (Figure 4, top right panel).

#### ***Taxonomic and Functional Gene Abundances:***

The average abundance of 16S rRNA bacterial gene for the untreated control soil was  $10^{10} \pm 0.42$  gene copies  $g^{-1}$  soil. Overall, during the experiment, no significant difference was detected in total 16S rRNA abundance in any of the studied factors (Tukey post-hoc test;  $p > 0.05$ ; Figure 5D). Similarly, MW soil treatments had no detectable effect on *amoA* gene abundances at any time point (Figure 5). The total AOB *amoA* abundance ranged between  $10^{7.7} \pm 0.28$  –  $10^{7.9} \pm 0.38$  gene copies  $g^{-1}$  soil (Figure 5A), while the total AOA *amoA* abundance ranged between  $10^{6.4} \pm 0.43$  –  $10^7 \pm 0.39$  *amoA* gene copies  $g^{-1}$  soil (Figure 5B). Overall, the AOB were more abundant than AOA (Figure 5C). Also, the AOB:AOA did not significantly change ( $p > 0.05$ ) in response to MW soil heating intensities (Figure 5C).

## Discussion

The primary purpose of the MW weed management technology is to reduce the viability of the soil weed seedbank of the herbicide-resistant weeds as an alternative to chemicals in the no-till agricultural system. A MW prototype has designed to deactivate the germination capacity of soil weed seedbank before crop sowing under field conditions. In this case, MW energy penetrates soil up to 8 cm for weed seedbank control, and after every centimeter downward the intensity of MW energy declines leading to changes in temperature distribution along the penetration depth. This depends on the soil characteristics, dielectric properties of the soil and the soil moisture content. They potentially regulate the MW-soil interactions (Nelson, 1996). The heating intensities and penetration depth of microwave energy into top soil layer decide the fate of the seedbank. We previously observed that the temperature requirement for this task ranged between 75 – 80 °C for effective weed seedbank depletion (Brodie *et al.*, 2018). Likewise, soil microorganisms also affect by MW energy along the penetration depth. Understanding the response of soil bacterial communities to this changing temperature intensities is crucial for the maintenance of ecosystem services provided by the soil biota and this information will help in calibrating the heating intensities for different soil conditions and farming practices.

Therefore, a major aim of this study was to evaluate the effect of MW soil heating on the soil bacterial communities and on the total abundance of AOB and AOA at two penetration depths. The temperature distribution, achieved through MW energy

application in the top soil layer has been simulated elsewhere (Brodie, 2016), where it was reported that most of the MW energy attenuation (*i.e., conversion of electric field into heat energy*) occurred between 0 – 8 cm of soil. A similar temperature distribution pattern was found in the microcosm of this study (see, Figure S4 and S5). Generally, when the temperature equilibrium of soil is altered by MW heating, both MW energy diffusion and heat transfer happen, and the majority of MW energy attenuation occurs in the top few centimeters for disinfestation. The temperature distribution of deeper sites (for example, 5 – 10 cm in this study) depends on heat conduction, and during conduction, heat lost to the atmosphere causes cooler and hotter spots (*i.e., inconsistency in soil heating*) in the lower penetration depth, as detected in the microcosm of the present study (Figure S4). Relevant to this, Casu *et al.* (2018) presented a 1-D transmission-line model to predict the feasibility of MW soil treatment for disinfestation purposes in raised-bed agricultural systems. Their simulation represented the time required to attain the lethal temperature and its retention into the soil against various penetration depths to eradicate pathogens. They computed that moist soil requires lesser time to achieve the target temperature for up to 15 cm depth on the raised bed than dry soil, and the temperature differed considerably along with penetration depth. Therefore, the temperature variability along the penetration depth would potentially alter the disinfestation process, as evident by the present study.

Based on the PCoA of Bray-Curtis distance, we categorised different MW heating durations into two groups: the low heating intensities, where soil exposed to 30 s did not induce a discernible effect on the bacterial communities and clustered closely to untreated control soil; and the high heating intensities, where 60 and 90 s treatments induced a significant divergence in bacterial communities, and clustered closely overtime. The observed patterns likely depended on temperature distribution, peak temperature retention into soil and bacterial differential recolonization patterns following heating (Neary *et al.*, 1999).

Microbial mortality is caused by the hyperthermic conditions induced by MW energy application into soil (Vela *et al.*, 1976; Vela & Wu, 1979), and, during recovery, community change occurs, which can then decide the fate of survival taxa and their role in shaping the overall bacterial communities' structure (Voort *et al.*, 2016). In this study, the high heating intensities (65 – 78 °C) resulted in a divergence in the bacterial communities' composition (Figure 2). This is in agreement with findings of Russell (2003), where high temperature treatment (50 – 60 °C) dramatically altered the structure and physiological properties of the bacterial communities, resulting in a changed community structure. In contrast, Pietik inen *et al.* (2000) reported that heating soils to 45 – 160 °C did not reduce the bacterial abundances in soils.

Therefore, based on this, it can be inferred that complete soil sterilization is somehow hard to occur.

Based on the bacterial richness, the communities disturbed with high intensity MW treatment did not recover to their pre-heating richness within the period studied (28

days; Figure 3). This suggests that the four weeks for bacterial recolonization following heat disturbance of 75 – 80 °C, appeared to be an insufficient duration for bacterial recovery. A similar pattern was observed in a recent study where the soil bacterial community declined with a 90 s MW treatment (800 W; 2.45 GHz), and subsequently underwent a successional shift, approaching community recovery by 29 days after soil heating (Jurburg *et al.*, 2017). In contrast, Voort *et al.* (2016) found higher community richness with 80 °C soil heating treatment for 1 h and argued that this was in agreement with the *intermediate disturbance hypothesis* (Connell, 1978). According to this hypothesis, species exhibit a trade-off between their ability to compete for survival/regrowth on available resources and their capacity to withstand a certain disturbance (Connell, 1978). In this study, the relative abundance of heat-resistant Firmicutes increased and heat-sensitive Proteobacteria decreased with high intensity MW soil treatment at T28 (see Figure 4, right below panel), which potentially exhibits a trade-off between species in response to MW soil heating. Our results show that bacterial diversity declined significantly at lower penetration depth of MW energy into the microcosms (Figure 3). While the lower soil depth should be classified as a moderate heating zone (Figure S4), based on temperature distribution, because MW energy loses its intensity with increasing penetration depth (Nelson, 1996). Therefore, the reduction in community diversity at lower depth (5 – 10 cm) is probably linked to the reflection of MW energy from the working surface beneath the samples. Thus, the reabsorption of this reflected energy might have changed the soil temperature distribution at lower depths resulting in higher bacterial

communities' mortality. This phenomenon is highly complex and needs sound simulation modelling for assessment but can be visualized in the cross-sectional thermal image of the microcosm (Figure S3).

Figure 4 depicts that, at T0 the relative abundance of Proteobacteria decreased and Firmicutes increased significantly according to high MW heating intensities while at T28 the TM-7, Firmicutes and Actinobacteria exhibited recovery in response to high heating intensities. In general, the immediate negative impact of soil heating on bacterial communities is mortality (O'Brien *et al.*, 2018), which is evident with a relative abundance of heat-sensitive (e.g., Proteobacteria) to heat-tolerant (e.g., Firmicutes) bacterial taxa in this study. A similar community shift trend has been detected in a recent microcosm study in response to soil heating at up to 65°C (Jurburg *et al.*, 2017). In our study, the relative abundance of Actinobacteria, which have previously been identified as being moisture-dependent in another microcosm experiment (Jacquiod *et al.*, 2013), increased with respect to days after heating. We did not observe a decrease in a relative abundance of this phylum with the high intensity MW soil treatments at T0, indicating that they survived the transient heating disturbance.

In our soils, Proteobacteria was the second most abundant phylum. Within this phylum, Beta-proteobacteria have been reported to resist MW heating durations of over 180 s (Nunes *et al.*, 2018), which is in accordance with our findings.

In this study, the relative abundance of the gram-positive spore-forming genus *Micromonosporaceae* increased with the high heating intensities of MW soil treatments at lower depths in the microcosm. The heat-induced activation of this genus at 70 °C has been reported previously (Hoskisson *et al.*, 2000). In addition, Suarez *et al.* (1980) reported that the dormant spores of *Micromonospora* were resistant to high temperature exposures (75 – 90 °C). *Micromonospora* species have also been reported to mediate crop protection by producing anti-fungal and anti-microbial compounds (Hirsch & Valdés, 2010), and help in promoting nitrogen-fixing symbioses, root nodulation and phytohormones formation (Solans *et al.*, 2006) and carbon decomposition (Yeager *et al.*, 2017). The favorable response of this genus to heating treatments may explain the reason for a good growth of crops after MW soil treatment for up to two cropping seasons, as reported by Khan *et al.* (2019).

At T28, the relative abundance of *Kaistobacter* increased significantly. This genus has been known to play an important role in the carbon and nitrogen cycles (Rampelotto *et al.*, 2013). Overall, the high heating intensities (65 – 78 °C) appeared to reshape the bacterial communities, and the bacterial community's richness did not recover to its pre-heating conditions within the time studied (*i.e.*, *four weeks*). Further, a less diverse community may be functionally altered (Boynton & Greig, 2016), and its ability to regulate key soil nutrient processes may be impacted, as soil functionality depends on the soil bacterial community composition (Wagg *et al.*, 2014).

We did not detect a negative impact of MW soil heating on the ammonia oxidisers over the period studied (28 days; Figure 5). These results supported our hypothesis that ammonia oxidisers are resistant to MW energy, which was initially argued by Vela *et al.* (1976). They reported that nitrifying bacteria are resistant to at least 40,000 J cm<sup>-2</sup> of MW energy applied to the soil surface. In this study, the maximum applied energy was 2,500 J cm<sup>-2</sup> associated to high intensities MW treatment, as measured by a simulation modelling according to Brodie (2016). Moreover, Ferriss (1984) found that prokaryotes were resistant to MW soil treatment and concluded that soil moisture and MW soil treatment time, which directly relates to the temperature increase, decide the fate of soil microorganisms.

In contrast to our heating method, soil steaming with the temperature gradient of about 80 – 100 °C for 10 minutes induced a 10-fold reduction in the population of AOB in an organic farming soil (Roux-Michollet *et al.*, 2008). In the present study the peak temperature exposure was less than 2 minutes. The difference in response of ammonia oxidizers to MW and steam treatment could be explained through changes in the heat transfer mechanism associated with both soil disinfestation strategies. A similar range of ammonia oxidisers (10<sup>4</sup> – 10<sup>5</sup> amoA gene copies g<sup>-1</sup> soil) as those found in our study has been reported in the Southern Australian soils regardless of the management practices (Jenkins *et al.*, 2016).

In this study, the survival of ammonia oxidisers could be explained by a number of factors: (1) variation in microbial distribution into the soil matrix (soil type, soil moisture, soil pH and agricultural strategies); (2) specific mass and volume of seeds

(much higher than microbes); and (3) uneven MW energy distribution into soil causes hot and cold spots. Spatial distribution of ammonia oxidisers into soil particles, and preferential localization, can help protect them from extreme conditions. The resistance of soil bacteria and fungi to MW based soil heating is reported in numerous classical studies (Vela & Wyss, 1965; Vela *et al.*, 1976; Vela & Wu, 1979; Van Wambeke *et al.*, 1983), and they concluded that both soil heterogeneity and inconsistency of MW heating may have supported the survival of microorganisms.

Overall, this study highlights that high heating intensities (65 – 78 °C), which are proposed for weed seedbanks depletion, have a strong impact on the bacterial community composition. Four weeks appeared to an insufficient duration for community recovery, following soil heating to 75 – 80 °C, and a longer monitoring of microbial community recovery following MW weed disinfestation is needed in order to develop appropriate management practices that preserve the long-term functionality of soil biota. We also did not observe the recovery of community richness during the four weeks of study, and further research must also tease apart whether recovery would eventually happen from the remaining microbial seedbank or via immigration of microbes from the surrounding area. At present, the impact of MW soil heating on soil fertility has been monitored for three cropping seasons (Khan *et al.*, 2019). Further long-term monitoring will reveal of whether there are long-term consequences to its application on the soil biota.

## **Conclusions**

Our study shows that high heating MW intensities, which are proposed for weed management, have a strong effect on the bacterial community composition. The heat-resistant bacterial group survived those high heating intensities and remained dominated in the community until T28. Four weeks appeared to be an insufficient duration for community recovery following soil heating at 75 – 80 °C. This suggests that a longer temporal investigation after MW soil heating is needed to standardize the bacterial community recovery of different cropping systems. In addition, we did not detect any discernible effect of high temperature on the total abundance of ammonia oxidizers. Overall, our study highlights that MW soil treatment can potentially be applied for weed control without significantly damaging the biologically important taxa, but further research is needed to determine how often it may be reapplied, and whether there are long-term consequences to its application on the soil biota.

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### **Data Availability Statement**

The data will not be shared in order to maintain ethical standards.

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## FIGURE CAPTIONS

**Figure 1** Infrared thermal images of microcosm treated through microwave energy under horn antenna of microwave weed killer depicting the gradual increase in soil temperature. Note: the untreated control soil (0 s; A), 30 s exposure (B), 60 s exposure (C) and 90 s exposure (D). Image was captured within 2 – 3 s after microwave soil heating and temperature loss during this time was negligible. The raw images were post-processed in MATLAB for temperature forecasting.

**Figure 2** Principle coordinate analyses of Bray-Curtis dissimilarity index-based variation in the community composition with microwave soil heating treatment over two-time points.

**Figure 3** Variation in bacterial diversity against microwave soil heating. Shannon richness calculated from the 16S rRNA gene sequence data for the different treatment combinations at two-time points.

**Figure 4** Relative abundance of the predominated bacterial taxa and genera that significantly changed over time in response to microwave soil heating intensities.

**Figure 5** Influence of microwave soil heating on the total abundance of the AOB (A), AOA (B), AOA:AOB (C) and 16S rRNA bacterial gene (D). The log-transformed gene copies number per gram soil are plotted against the various doses of soil microwave treatment monitored at two times points.

## Figures

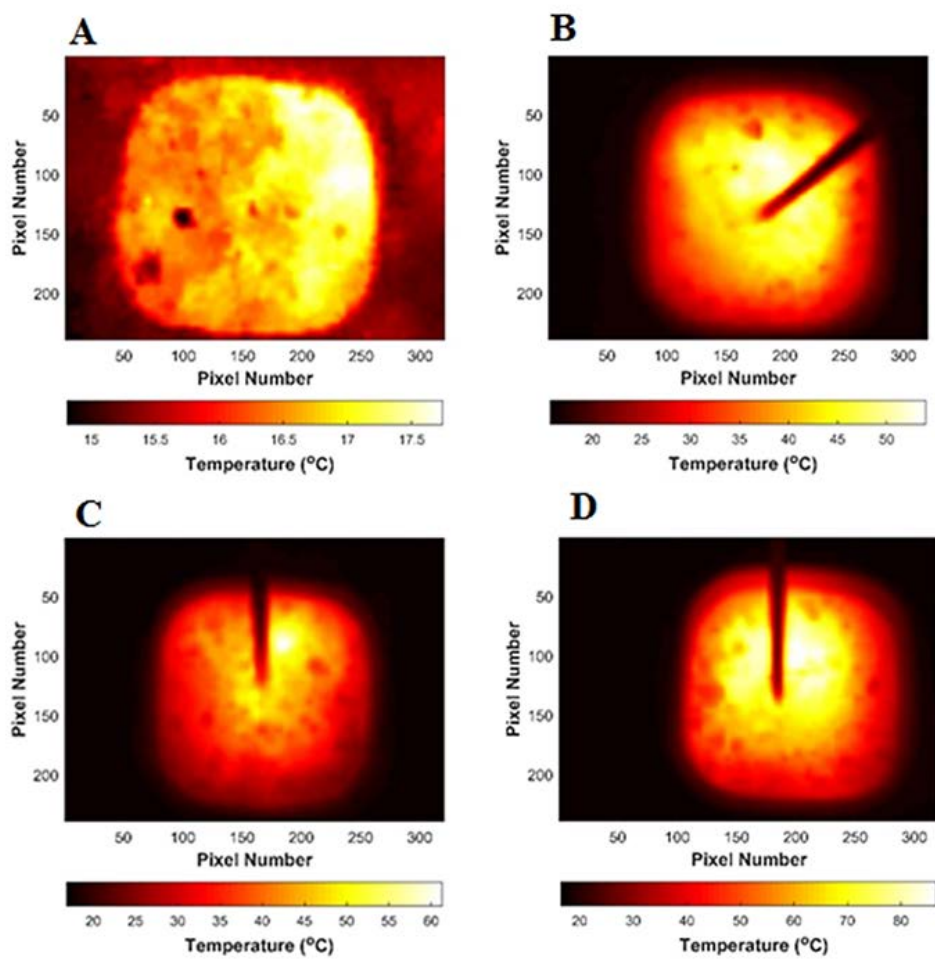


Figure 1

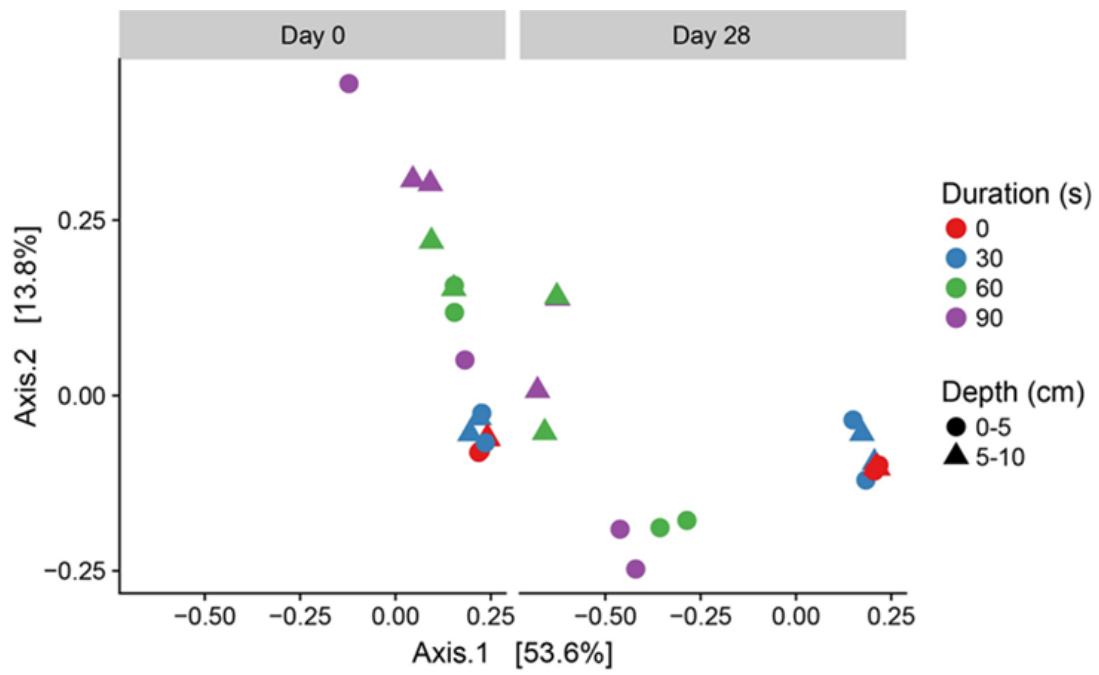


Figure 2

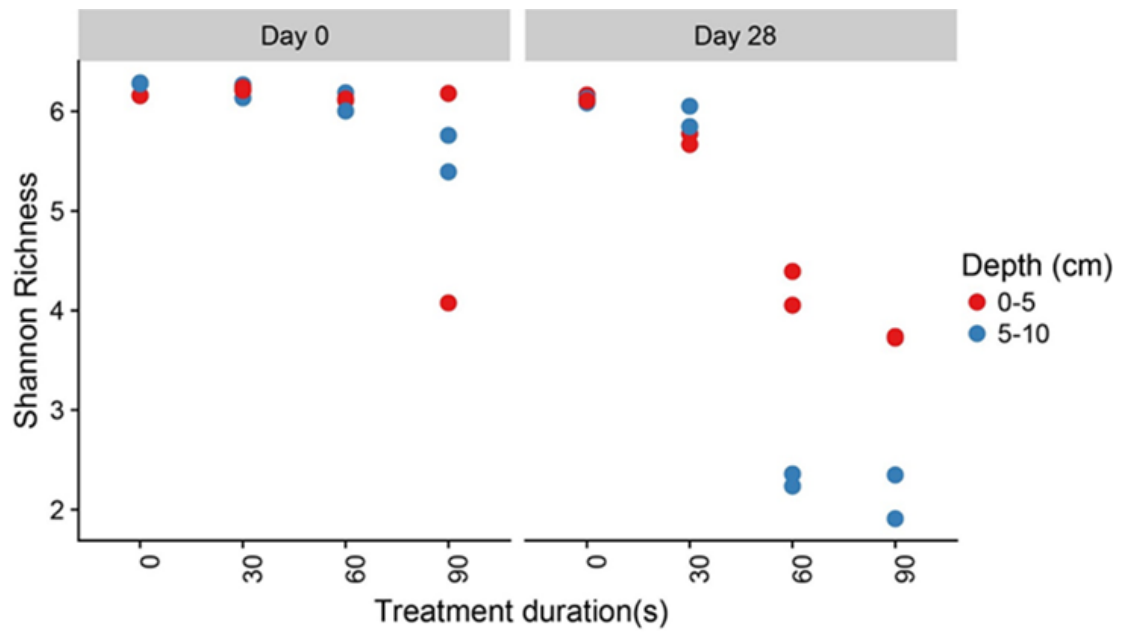
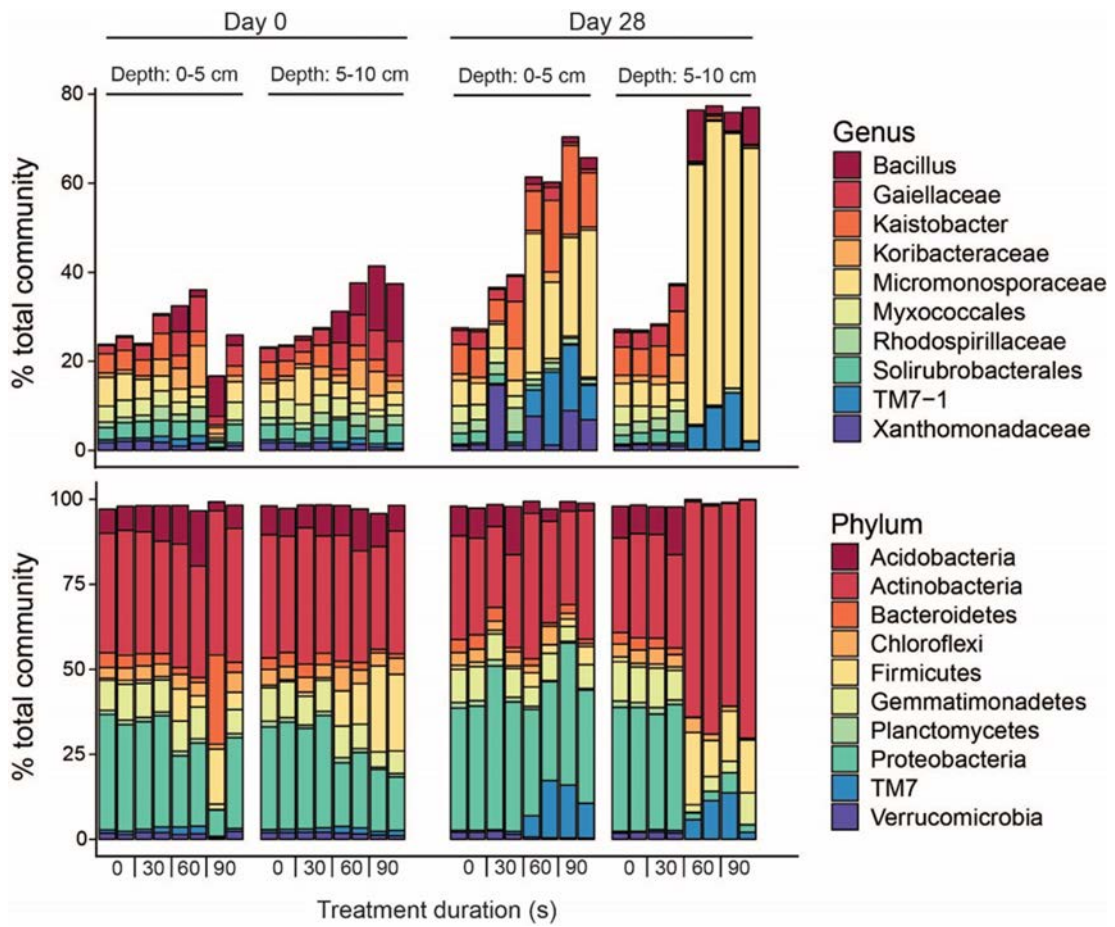
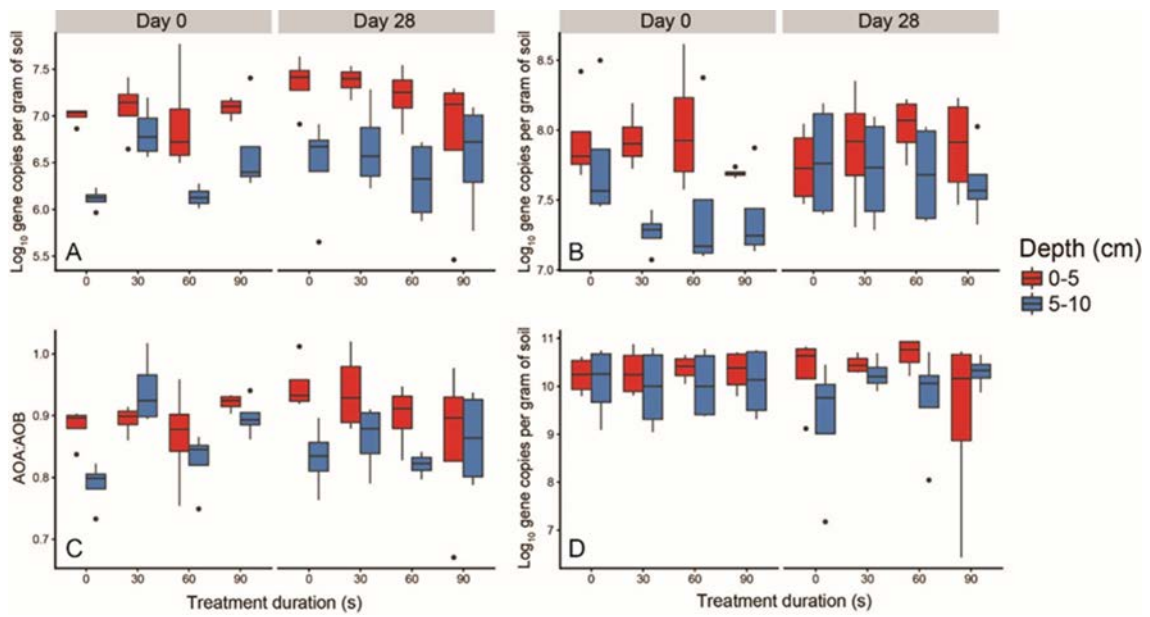


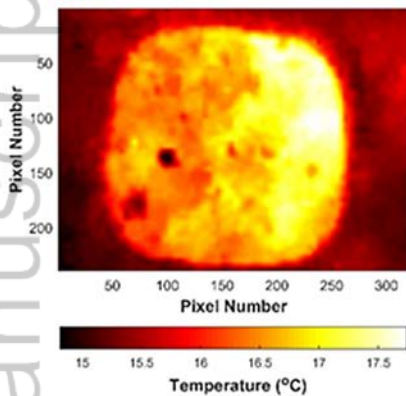
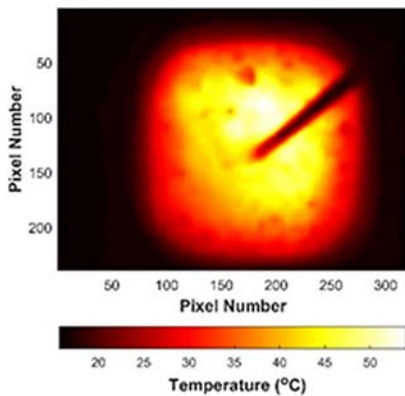
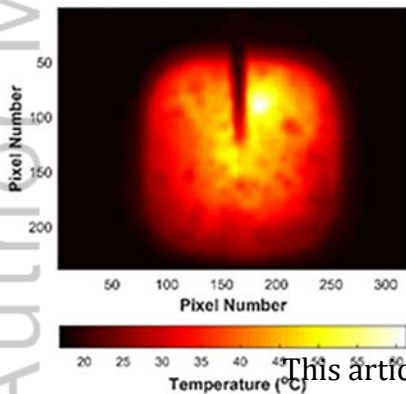
Figure 3



**Figure 4**



**Figure 5**

**A****B****C****D**