Effects of biodegradable plastic film mulching on soil microbial communities in two agroecosystems

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Plastic mulch films are used globally in crop production but incur considerable disposal and environmental pollution issues. Biodegradable plastic mulch films (BDMs), an alternative to polyethylene (PE)-based films, are designed to be tilled into the soil where they are expected to be mineralized to carbon dioxide, water and microbial biomass. However, insufficient research regarding the impacts of repeated soil incorporation of BDMs on soil microbial communities has partly contributed to limited adoption of BDMs. In this study, we evaluated the effects of BDM incorporation on soil microbial community structure and function over two years in two geographical locations: Knoxville, TN, and in Mount Vernon, WA, USA. Treatments included four plastic BDMs (three commercially available and one experimental film), a biodegradable cellulose paper mulch, a non-biodegradable PE mulch and a no mulch plot. Bacterial community structure determined using 16S rRNA gene amplicon sequencing revealed significant differences by location and season. Differences in bacterial communities by mulch treatment were not significant for any season in either location, except for Fall 2015 in WA where differences were observed between BDMs and no-mulch plots. Extracellular enzyme rate assays were used to characterize communities functionally, revealing significant differences by location and sampling season in both TN and WA but minimal differences between BDMs and PE treatments. Overall, BDMs had comparable influences on soil microbial communities to PE mulch films.

1 **Effects of biodegradable plastic film mulching on soil**

2 **microbial communities in two agroecosystems**

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Abstract

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22 polyethylene (PE)-based films, are designed to be tilled into the soil where they are expected to

23 be mineralized to carbon dioxide, water and microbial biomass. However, insufficient research

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25 partly contributed to limited adoption of BDMs. In this study, we evaluated the effects of BDM

26 incorporation on soil microbial community structure and function over two years in two

27 geographical locations: Knoxville, TN, and in Mount Vernon, WA, USA. Treatments included

28 four plastic BDMs (three commercially available and one experimental film), a biodegradable

29 cellulose paper mulch, a non-biodegradable PE mulch and a no mulch plot. Bacterial community

30 structure determined using 16S rRNA gene amplicon sequencing revealed significant differences

31 by location and season. Differences in bacterial communities by mulch treatment were not

32 significant for any season in either location, except for Fall 2015 in WA where differences were

33 observed between BDMs and no-mulch plots. Extracellular enzyme rate assays were used to

34 characterize communities functionally, revealing significant differences by location and

35 sampling season in both TN and WA but minimal differences between BDMs and PE treatments.

36 Overall, BDMs had comparable influences on soil microbial communities to PE mulch films.

Introduction

38 Plastic mulch films are widely used in crop production systems to improve soil microclimate and

39 suppress weeds, translating into increased crop yields and/or improved fruit quality. Some of the

40 agronomic benefits of using plastic mulch films include reduction of weed pressure (Martín-

41 Closas et al. 2017), conservation of soil moisture (Kader et al. 2017; Shahi et al. 2017), and

42 moderation of soil temperature, among others. Low density polyethylene (PE) has been the

43 favored polymer for mulch films due to its many attractive properties such as low cost, easy

44 processability, high durability and flexibility (Bandopadhyay et al. 2018; Kasirajan & Ngouajio

45 2012). However, PE does not readily biodegrade, and thus must be disposed at the end of the

46 growing season, contributing to our global plastic waste problem (Brodhagen et al. 2015; Liu et

47 al. 2014). Even when removed from a field, fragments of film are left behind in the soil, which

48 can affect soil function and soil biota (Barnes et al. 2009; de Souza Machado et al. 2018b; Huerta

49 Lwanga et al. 2016; Rillig 2012; Sivan 2011; Teuten et al. 2009) or leach out into water systems

50 and pollute aquatic ecosystems (Fu & Du 2011; Kong et al. 2012; Magdouli et al. 2013; Van

51 Wezel et al. 2000; Wang et al. 2015; Wang et al. 2013). As these plastics break down in soil,

52 they form microplastics (de Souza Machado et al. 2018a), contributing to terrestrial microplastic

53 pollution (de Souza Machado et al. 2018a; de Souza Machado et al. 2018b).

54 Plastic mulch use is expected to increase to meet increasing global food demands; therefore, it is

55 imperative to find alternatives that will reduce the environmental footprint. Biodegradable mulch

56 films (BDMs) are a potential alternative: BDMs are made of polymers that can be degraded by

57 microbial action (Hayes et al. 2012; Kasirajan & Ngouajio 2012; Kyrikou & Briassoulis 2007;

58 Riggi et al. 2011). In the field, BDMs perform like other plastic films by altering the soil

59 microclimate and improving crop yields (DeVetter et al. 2017). However, unlike PE plastics,

60 which require removal and disposal, BDMs are designed to be tilled into the soil where resident

61 soil microbes are expected to degrade them over time. Under ideal circumstances, BDMs should

62 eventually be mineralized into carbon dioxide and water.

63   Despite being a promising sustainable alternative, adoption of BDMs has been limited in the US

64   (Goldberger et al. 2015). BDMs are currently more expensive than PE mulches, and breakdown

65   can be unpredictable. Growers and stakeholders have also cited insufficient knowledge regarding

66   the effects of BDMs on soil health as a barrier to adoption (Goldberger et al. 2015). Moreover,

67   the US National Organic Program (NOP) does not allow growers to use the currently available

68   BDM products in organic crop production because they are not 100% biobased (Miles et al.

69   2017). However, the source of the carbon does not dictate biodegradability of BDMs; a BDM

70   that is biodegradable and does not harm the soil, regardless of the source of feedstock, could

71   become a sustainable alternative to PE mulch (Ghimire et al. 2018a). Thus, evaluating the

72   impacts of incorporation of BDMs into soil on soil health is a critical part of adoption and policy

73   development surrounding BDMs (Brodhagen et al. 2017).

74   BDMs can impact soil health in two ways: indirectly, in a manner similar to PE films, by acting

75   as a surface barrier to soil and modifying the soil microclimate, and directly, by addition of

76   physical fragments and carbon into soil after tillage (Bandopadhyay et al. 2018). The body of

77   research on the impacts of polyethylene films on soil microbial communities and functions can

78   help us predict the indirect effect of BDMs on soil health. However, research on the direct effects

79   of BDMs on soil microbial community structure and function remains poorly answered due to a

80   dearth of research that directly compares BDMs and PE in the same study. Unless there is a

81   direct comparison of BDMs and PE, it is difficult to tease apart whether the observed changes

82   are above and beyond what you would expect from the application of PE mulch to the soil

83   surface (Bandopadhyay et al. 2018). These answers are critical if use of BDMs is to be advocated

84   as a sustainable alternative to PE. Previous studies have analyzed impacts of BDMs on soil

85   microbial communities using phospholipid fatty acid (PLFA) profiling (Li et al. 2014b) and

86   pyrosequencing (Moore-Kucera et al. 2014) methods. However, these studies did not use PE as a

87   negative control so direct effects of BDMs on soil microbial community structure and function

88   remain uncertain.

89   In this study, we compared the impacts of BDM and PE mulch on soil microbial communities

90   using two-year vegetable crop field trials in two diverse climates (Knoxville, TN, in the

91   southeastern USA and Mount Vernon, WA, in the northwestern USA). During this field trial,

92   measurements of soil health indices based on a suite of soil physical, chemical and biological

93   properties revealed that the overall effect of mulching on soil health indices was minimal and

94   that BDMs performed comparably to PE (Sintim et al. 2019). The study by Sintim et al. (2019)

95   included extracellular enzyme rates (expressed as C:N and C:P ratios), organic matter content

96   and soil respiration as biological indicators of soil health. To build on this finding, we extended

97   the study to focus on soil microbial communities, which are accepted as integral to soil

98   functioning, but not generally explicitly include in assessments of soil health. Here, we evaluated

99   the impacts of BDMs on 1) soil microbial community structure, characterized using 16S rRNA

100   gene amplicon sequencing 2) soil microbial abundances, estimated using qPCR and 3) soil

101   microbial community function, estimated by a suite of soil extracellular enzyme rates over the

102   two-year field trial experiment. We tested the hypothesis that plastic mulches would significantly

103   alter soil microbial community structure and function, but that there would be no significant

104   differences between PE and BDM mulches.

105

106   **Materials & Methods**

107   **Plastic Mulch Films**

108 Three commercially available biodegradable mulch films (BioAgri®, Naturecycle, and Organix

109 A.G. Film™) and one experimental film comprised of a blend of polylactic acid (PLA) and

110 polyhydroxyalkanoates (PHA) were tested alongside a polyethylene (PE) mulch (negative

111 control), and cellulose paper mulch (WeedGuard Plus®, positive control). The paper mulch used

112 in the experiment was a 100% biobased product and was chosen as the positive control because

113 its major constituent is cellulose which is known to rapidly disintegrate in the field (Li et al.

114 2014b). Physicochemical properties of mulches are reported in Table 1.

115 **Field trial description**

116 Field experiments were set up in two locations: East Tennessee Research and Education Center

117 (ETREC), University of Tennessee, Knoxville, TN and the Northwestern Washington Research

118 & Extension Center (NWREC), Washington State University, Mount Vernon, WA. The soil at

119 Knoxville is a sandy loam (59.9% sand, 23.5% silt, and 16.6% clay), classified as a fine

120 kaolinitic thermic Typic Paleudults. The soil at Mount Vernon is a silt loam (14.2% sand, 69.8%

121 silt, and 16% clay), classified as a fine-silty mixed nonacid mesic Typic Fluvaquents. Henceforth

122 in the paper, Knoxville, TN will be referred to as TN and Mount Vernon, WA will be referred to

123 as WA. The mulches were tested in the field over two years (2015 to 2016) under pie pumpkin

124 (*Cucurbita pepo*) as a test crop, with full experimental details described in H. Y. Sintim et al.

125 (2019) and S. Ghimire et al. (2018b). Briefly, before mulch application began in TN and WA,

126 the plots were under winter wheat (*Triticum aestivum*) cover crop in TN and clover (*Trifolium*

127 *spp.)* at WA. Each field site was arranged as a randomized complete block design with four

128 replications of seven main plot treatments (six mulch treatments described above and one no

129 mulch control). Mulches were machine-laid on raised beds at the end of May to early June, and

130 harvest was completed in September-October. PE mulch was removed soon after harvest, while

131 BDMs were tilled in; all beds were tilled within two weeks of harvest. The two sites were

132 planted with a winter wheat cover crop following harvest in the fall. Pie pumpkin (*Cucurbita*

133 *pepo*) was used as the test crop because it met requirements of a large-scale, multi-location field

134 experiment; namely, pie pumpkin is economically important, commonly grown in both our field

135 experimental locations, and has sufficient season length to maximize treatment exposure.

136 Additionally, pie pumpkins, like other cucurbits such as cucumbers, melons and squash, are

137 commonly grown on plastic mulch films throughout the United States (Inglis et al. 2015).

138 Soil water content and temperature were monitored as described in Sintim et al. (2019). Briefly,

139 sensors (5TM, Decagon Devices Inc., Pullman, WA) installed in the center of each mulch

140 treatment at 10-cm and 20-cm soil depths for one field block were connected to data loggers

141 (EM50G, Decagon Devices Inc., Pullman, WA) that recorded the soil water and temperature data

142 hourly. Soil water content and temperature data is reported in Sintim et al. (2019). Air

143 temperature, precipitation, relative humidity, wind, and solar radiation were collected from a

144 meteorological station located at the field site at TN (Decagon Devices Inc. Weather Station,

145 Pullman, WA), and about 100 m away from the field site at WA (WSU AgWeatherNet Station,

146 Mount Vernon, WA). Weather data for the two locations were continuously collected from 2015-

147 2017.

148 Soil physical, chemical, and biological properties were assessed over the two-year study for this

149 site, in order to assess changes in soil health. Detailed protocols for these measurements and raw

150 data is provided in Sintim et al. (2019).

151 **Soil sampling**

152   Soil samples were collected from each of the 28 plots (seven treatments, replicated four times) at

153   both locations in the Spring (May) and Fall (September) of 2015 and 2016. Spring samples were

154   collected approximately 2 weeks prior to mulch application. Fall soil samples were collected

155   while the mulches were still in the field, approximately 2 to 3 weeks before the mulch was tilled

156   in (BDMs) or removed (PE). In our studies, BDMs did not full degrade over the winter, so the

157   2016 samples represent communities that have been exposed to (and are presumably still

158   degrading) tilled-in mulch from the previous season; Spring 2016 soil had been exposed to tilled-

159   in mulches from the previous season, and Fall 2016 soil had been exposed to both tilled-in

160   mulches from the previous season and new mulch laid for the 2016 season. Soil was collected

161   from the top 10 cm using a 2 cm diameter stainless steel auger. Thirty 10-cm soil cores were

162   taken about 20 cm apart and composited for each of the plots. All sampling equipment was

163   cleaned with 70% ethanol between plots to limit cross contamination. Roots and pebbles were

164   removed by hand, and soils homogenized and stored in plastic bags for transport back to the lab.

165   Soils were stored at -80°C until DNA extraction and extracellular enzyme assays.

166   **Soil DNA extraction and quantification**

167   Extraction of DNA from soil samples was completed using the MoBio™ PowerLyzer™ Power

168   Soil DNA isolation kit (now branded under Qiagen™) with inhibitor removal technology, as per

169   manufacturer's instructions. 0.25 grams of soil were used for the extractions, and the DNA

170   obtained after the final elution step was stored at -20°C until further analyses.

171   Quantification of the DNA extracted from soil was completed using the Quant-It™ PicoGreen™

172   dsDNA Quantification Kit (ThermoFisher Scientific) per manufacturer's instructions and quality

173   of DNA was measured by 260/280 ratios in a NanoDrop™ Spectrophotometer (Table S1).

174 **Quantitative PCR for bacterial and fungal abundances**

175 As a proxy for bacterial and fungal abundances, 16S rRNA (bacteria) and ITS (fungi) gene copy

176 abundances were quantified from soil DNA samples using Femto™ Bacterial DNA

177 quantification kit (Zymo Research) and Femto™ Fungal DNA quantification kit (Zymo

178 Research) following the manufacturer's protocol. DNA extracts were diluted 1:10 prior to

179 quantification and 1 µl of the diluted samples was used for each qPCR reaction. All samples

180 were analyzed in triplicate. No template negative controls were included in each run. Bacterial

181 and fungal DNA standards were provided in the kit and the ng DNA standard per well was

182 converted to copy numbers which were used for final calculations. qPCR reactions were

183 performed in a CFX Connect Real-Time PCR Detection System (BioRad). qPCR efficiencies

184 averaged around 85% and 90% for bacterial and fungal assays, respectively.

185 **DNA amplification and sequencing**

186 16S rRNA amplicon sequencing of DNA extracts was conducted by the Genomic Services

187 Laboratory (GSL) at Hudson Alpha, Huntsville, AL, following their standard operating

188 procedures. Extracted DNA samples were shipped frozen in 96 well plates. The V4 region of the

189 16S rRNA gene was amplified using primers 515F (GTGCCAAGCAGCCGCGGTAA) and

190 806R (GGACTACHVGGGTWTCTAAT) (Caporaso et al. 2012). The first PCR used V4

191 amplicon primers, Kapa HiFi master mix, and 20 cycles of PCR. All aliquots and dilutions of the

192 samples were completed using the Biomek liquid handler. PCR products were purified and

193 stored at -20^0C until further processing was completed. PCR indexing was completed for 16S

194 (V4) amplicons using GSL3.7/PE1 primers, Kapa HiFi master mix, and 12 cycles of PCR.

195 Products were purified using magnetic beads using the Biomek liquid handler. Final libraries

196 were quantified using Pico Green. V4 amplicon size obtained was 425 bp for the soil samples.

197   The amplified 16S rRNA genes were sequenced using 250 paired-end reads on an Illumina

198   MiSeq platform. Sequence reads were deposited in the NCBI sequence read archive (Accession

199   PRJNA564156).

200   Raw sequence data was processed using mothur v.1.39.5 following the MiSeq SOP (Schloss et

201   al. 2009). Before aligning to the reference database (SILVA release 102), unique sequences were

202   identified, and a count table generated. After alignment to SILVA database, sequences were

203   filtered to remove overhangs at both ends, and sequences de-noised by pre-clustering sequences

204   with up to two nucleotide differences. Chimeras were removed using the VSEARCH algorithm.

205   Sequences were classified using the Bayesian classifier (Wang et al. 2007) against the mothur-

206   formatted version of the RDP PDS training set (v.9) with a bootstrap value of > 80% (Wang et

207   al. 2007). Following this step, untargeted (i.e. non-bacterial) sequences classified as *Eukaryota*

208   and *Arachaeota* were removed. Sequences were binned into phylotypes according to their

209   taxonomic classification at the genus level. A consensus taxonomy for each OTU was generated

210   by comparison to the RDP training set. The resulting OTU count table and taxonomy

211   assignments were imported into R (v. 3.4.0) (R Core Team 2018) for further downstream

212   statistical analyses. Mothur code, R code and associated input files are available at:

213   https://github.com/jdebruyn/BDM-Microbiology.

214   **Extracellular enzyme assays**

215   Fluorescence microplate enzyme assays were conducted using fluorescently labelled substrates

216   to assess enzyme activities in soil (Bell et al. 2013). Seven enzymes were assayed using their

217   respective fluorescent substrates and standards (Table S2).

218   Soil slurries were prepared in a sodium acetate trihydrate buffer whose pH was matched closely

219   with the soil pH. 800 µl of soil slurry was pipetted into deep well 96 well plates. Separate plates

220   were prepared for 4-methylumbelliferone (MUB) and 7-amino-4-methylcoumarin (MUC)

221   standard curves for each sample. 200 µl of appropriate standards and substrates were added to

222   the soil slurries. The plates were sealed and inverted to mix the contents. Incubation was done for

223   3 hours at room temperature, after which the substrate and standard plates were centrifuged at

224   1500 rpm (~327 x g) for 3 min. The supernatants were pipetted into black 96 well plates and

225   fluorescence measured at 365 nm excitation wavelength and 450 nm emission wavelength in a

226   BioTek® Synergy plate reader.

227   **Statistical analyses**

228   Beta diversity was computed using Bray-Curtis distances of microbial community composition

229   using the vegan package (v 2.4-3) in R version 3.4.0 (R Core Team 2018) based on OTU tables,

230   and were then visualized using non-metric multidimensional scaling (NMDS) using phyloseq

231   package v.1.21.0 in R (McMurdie & Holmes 2013). To determine whether significant

232   differences existed in bacterial community composition between bacterial communities across

233   different locations, seasons, and mulch treatments, a permutational multivariate analysis of

234   variance (PERMANOVA) was performed using the ADONIS function implemented in R, based

235   on the Bray-Curtis dissimilarity matrix. All libraries were subsampled to even depth (minimum

236   sample read count, i.e. smallest library size, of 34,266) before analysis was performed. Similarity

237   percentage analyses (SIMPER) was completed in R to reveal the most influential OTUs driving

238   differences between soil bacterial communities in different locations, and across different

239   seasons. Difference in relative abundances of taxa between locations and seasons were

240   determined using Kruskal-Wallis rank sum non-parametric test. A post-hoc test was completed

241   using pairwise Wilcoxon rank sum test if significant differences were reported using Kruskal-

242   Wallis test. P values were adjusted using the method of Benjamini & Hochberg (Benjamini &

243   Hochberg 1995) to control the false discovery rates (p < 0.05). Canonical analysis of principal

244   coordinates (CAP) was done to relate environmental variables reported in H. Y. Sintim et al.

245   (2019) to changes in bacterial community composition. Our *a priori* hypothesis for conducting

246   this statistical test was that we would see changes in microbial communities across the two

247   locations driven by specific environmental variables. The ordination axes were constrained to

248   linear combinations of environmental variables, then the environmental scores were plotted onto

249   the ordination. A PERMANOVA was performed on the CAP axes. These analyses were

250   completed in R following the online tutorial by M. Berry (Berry 2016).

251   Libraries were subsampled with replacement to equal size prior to computing alpha diversity

252   metrics. The estimate_richness function was used in R phyloseq package to calculate observed

253   richness and inverse Simpson indices (for diversity). A mixed model analysis of variance was

254   completed using the generalized linear mixed model (GLIMMIX) procedure in SAS V. 9.3 to

255   assess changes in richness and inverse Simpson over time. The fixed effects were location (TN

256   and WA), mulch treatments (seven treatments total) and time/season of soil sampling (four time

257   points), while the random effect was block (total three blocks to serve as replicates). Repeated

258   measures were incorporated in the model as sampling was done over time. The model was a

259   completely randomized design (CRD) split-split-plot with repeated measures in the sub-sub plot.

260   Normality of data was checked using Shapiro-Wilk test (W > 0.9) and equal variance using

261   Levene's test ($\alpha = 0.05$). All data were normal and hence no transformations were performed.

262   Raw experimental values and standard errors are reported in the figures.

263   To visualize differences in the functional profile of the communities; i.e. all seven enzyme rates,

264   NMDS ordination of Bray-Curtis similarities was done in Primer 7 v. 7.0.13 (PRIMER-E). A

265   mixed model analysis of variance with repeated measures was completed using the generalized

266   linear mixed model (GLIMMIX) procedure in SAS V. 9.3 to assess changes in enzyme activities

267   over time. Fixed and random effects were same as specified above. Boxplots for equal variance

268   and outliers, reported in SAS, were used to remove outliers in the dataset. Normality was

269   checked using Shapiro-Wilk test (W > 0.9) and probability plots for residuals, and equal variance

270   using Levene's test ($\alpha = 0.05$). Data were log transformed as necessary when these conditions

271   were not met. Type III tests of fixed effects and interaction effects are reported.

272   To assess for potential enrichment of bacteria and fungi, a paired t-test was conducted using

273   initial and final 16S rRNA and ITS gene copy abundances (determined by qPCR) from Spring

274   2015 and Fall 2016 to see if there was a significant change over the course of the experiment.

275   Initial 16S and ITS gene copy abundances from Spring 2015 were also subtracted from final

276   abundances in Fall 2016 to get change in abundance over time. To determine if the enrichment or

277   depletion of bacterial and fungal abundances was significantly different between treatments, a

278   mixed model analysis of variance in SAS v. 9.3 using the GLIMMIX procedure was conducted

279   on the differences. Significance level of all analyses were assessed at $\alpha = 0.05$. All data were

280   checked for normality using Shapiro-Wilk test (W > 0.9).

281   **Results**

282   **Environmental and soil physicochemical data**

283   Environmental data collected during the experiment is reported in Sintim et al. (2019) and in

284   Table S3. The mean daily air temperature in 2015 to 2016 was about 4ºC higher in Knoxville,

285   TN than in Mount Vernon, WA (Table S3). The total annual precipitation during the

286   experimental years was higher in Knoxville, TN than in Mount Vernon, WA.

287   In all plots with plastic mulching, fragments of the mulches (i.e. remnants from the previous

288   season's mulches) were visible in the soils throughout the experiment. Soil temperature, moisture

289   and physicochemical properties were measured and reported previously by Sintim et al. (2019).

290   In summary, significantly increased soil temperature was observed in the early growing seasons

291   in the plastic mulch plots compared to the cellulose and no-mulch plots. On average, the monthly

292   soil temperature was greater in TN than in WA. Overall, mulched plots had higher water content

293   than the no mulch plots, with PE mulch having the highest soil water content for the greatest

294   time. The soil health analysis revealed some effects of mulching on certain properties (namely

295   aggregate stability, infiltration, soil pH, electrical conductivity, nitrate, and exchangeable

296   potassium), but these were not consistent among BDMs, nor across sampling times and

297   locations.

298   **Soil bacterial community diversity and structure**

299   For the 16S rRNA gene sequences, the percentage of bases with a Phred quality (Q) score ≥ 30 was

300   78% (24,090,356 total reads with 94% reads identified), and 90% (21,712,542 total reads with

301   93% reads identified) for the two flow cells used. The NMDS ordination revealed a clear difference

302   in community structure between TN and WA when combining data from all four sampling seasons

303   (Spring 2015 to Fall 2016) (Fig. 1a). Permutational ANOVA (PERMANOVA) tests confirmed

304   significant differences between TN and WA soil microbial communities (Table 2, Table S4). The

305   mean relative abundances of the most abundant classes of bacteria are shown in Fig. 1b. Similarity

306   percentage tests (SIMPER) revealed the most influential OTUs contributing to the variation seen

307   between location (Fig. 1b). The most influential OTUs belonged to several classes of microbes

308   such as *Acidobacteria_Gp7, Acidobacteria_Gp16, Acidobacteria_Gp4, Planctomycetacia* and

309   *Spartobacteria*. CAP analysis revealed that the differences in soil communities between TN and

310   WA were most related soil moisture and organic matter content (Fig. S1).

311   In addition to locational differences, bacterial communities also differed significantly between

312   the different seasons (Table 2, Table S4). For both locations, Spring communities were more

313   similar to each other than Fall communities (Fig. 2a, b). SIMPER tests revealed that several

314   genera of *Acidobacteria. Planctomycetaceae, Spartobacteria* and *Actinobacteria* (such as

315   *Streptomyces*) were cumulatively responsible for 60% of the seasonal variance in bacterial

316   communities (Fig. S2 and S3). Interestingly, relative abundance of *Streptomyces* increased over

317   time from Spring 2015 to Fall 2016 in both TN and WA (Fig. S2).

318   Unlike location and season, the mulch treatments did not have a significant effect on bacterial

319   community structure. Because of the locational and seasonal differences, we additionally

320   analyzed each time-location set separately, and did not detect any significant effects of treatment

321   on community structure except for Fall 2015 in WA (Fig. S4, Table 2, Table S4). We further

322   used a pairwise.adonis function in R (Salazar 2019) to determine pairwise differences for Fall

323   2015 in WA, but no significant patterns emerged.

324   Alpha diversity of the soil bacterial communities was estimated using observed species richness

325   and inverse Simpson index of diversity (Table S5). The observed species richness estimator

326   measures count of unique OTUs in each sample. There were significant differences between TN

327   and WA ($p < 0.05$) in richness estimates (Table 3, Fig. 3a). TN had greater richness than WA

328   throughout the experiment, ranging from 260 to 300 unique OTUs. WA richness estimates

329   ranged from 250 to 280 OTUs over the two years. The locational differences in richness were

330   due to a lower richness in Fall 2015, Spring 2016 and Fall 2016 in WA (Fig. 3a). The Inverse

331   Simpson diversity index ranges were similar between TN and WA, ranging from 7 to 11 (Fig

332   3b).

333   For both TN and WA, there was a significant difference between seasons in both richness and

334   inverse Simpson index (Table 3). Post hoc testing showed that the richness estimates in TN

335   significantly differed between 2015 and 2016. In WA, Fall 2015 differed in richness from the

336   other time points. In TN, Fall 2016 diversity was significantly higher than other seasons.

337   Diversity estimates were significantly lower in Spring than in the Fall seasons for WA.

338   In TN in 2016, PE had the lowest richness and BioAgri had the highest. However, treatment

339   differences in richness estimates were not significant (Table 3) when analyzing data using a

340   mixed model. Inverse Simpson diversity indices were also not significantly different between

341   treatments (Table 3). Looking at the final time point in TN, diversity estimates were highest for

342   Weedguard, and lowest for PE, and in WA, the estimates were highest for Weedguard, followed

343   by PE with BDMs having lower diversity than PE or Weedguard, however these differences

344   were not significant (Fig. 3b). It is important to note, however, that Weedguard completely

345   disintegrated in TN after the field season (Ghimire et al. 2018b), which could explain some of

346   the treatment differences observed in the alpha diversity metrics.

347   **Microbial community abundances**

348   As a proxy for bacterial and fungal abundances, bacterial (16S) and fungal (ITS) rRNA gene

349   copies were quantified using qPCR assays for soil samples from all seasons. In general, bacterial

350   (16S) gene copy numbers averaged to 2.5 x 10^9 gene copies per gram dry weight soil in Spring

351   2015 and 4.2 x 10^9 gene copies g^{-1} in Fall 2016 in TN; 1.8 x 10^9 gene copies g^{-1} in Spring 2015

352   and 6.9 x 10^9 gene copies g^{-1} in Fall 2016 in WA. For fungal (ITS) abundances, values ranged

353   from 2.9 x 10^8 gene copies g^{-1} in Spring 2015 to 3.8 x 10^8 gene copies g^{-1} in Fall 2016 in TN, and

354   4.5 x 10^8 gene copies g^{-1} in Spring 2015 to 8.4 x 10^8 gene copies g^{-1} in Fall 2016 in WA. In order

355   to assess if gene abundances had significantly changed over the course of the experiment (Spring

356   2015 to Fall 2016) for each mulch treatment, a paired t-test was used to identify changes that are

357   significantly different from zero (Table S6). There was a significant increase in bacterial gene

358   copies under BDM and Weedguard treatments in WA, but no significant change for no mulch

359   and PE treatments (Table S6). There was also a significant enrichment in fungal gene copies over

360   time for two of the BDMs (PLA/PHA and Naturecycle) in WA. In TN, significant enrichment in

361   bacterial gene copies was seen under Organix, PLA/PHA and PE treatments (Table S6) but no

362   enrichment was seen in fungal gene copies. In order to determine if these changes were

363   significantly different between treatments, the differences between the final (Fall 2016) and the

364   initial (Spring 2015) abundances were analyzed using a mixed model analysis of variance and

365   Tukey post hoc tests. In both locations, mulch treatments did not have a significant effect on the

366   changes in either 16S or ITS gene copies over the course of the experiment (Fig. 4 a, b).

367   **Microbial community functions**

368   To assess potential functional responses of the soil microbial communities to the mulching

369   treatments, extracellular enzyme potential rate assays were conducted for common carbon,

370   nitrogen, and phosphorus cycling enzymes in soil (Table S2). The data were combined over the

371   two years to visualize Bray Curtis similarities of the enzyme rate profiles (Fig. 5). Locational

372   differences in the enzyme profile were significant ($p < 0.05$), as were seasonal differences in

373   both TN ($p < 0.05$) and WA ($p < 0.05$) evaluated using PERMANOVA (Fig. 5). However, mulch

374   treatment did not have a significant effect on the enzyme profile for any of the seasons at either

375   location ($p < 0.05$). NMDS ordination for the final sampling time point Spring 2017 is shown in

376   Fig. S5, showing no clear treatment differences. In general, the enzyme activity rates oscillated

377   between higher activities in the Spring and lower activities in the Fall. When analyzed separately

378   for each enzyme, the data over the two years revealed a significant effect of sampling time in TN

379   for all seven enzymes assayed. In WA, enzyme activities of β-xylosidase, β-glucosidase, α-

380   glucosidase, N-acetyl β-glucosaminidase and phosphatase were significantly different between

381   sampling times (Fig. 6). In WA, cellobiosidase and leucine amino peptidase activities remained

382   unchanged across the seasons (10-22 nmol activity g^{-1} dry soil h^{-1} for cellobiosidase and 200-375

383   nmol activity g^{-1} dry soil h^{-1} for leucine amino peptidase) (Fig. 6).

384   When averaged across seasons, mulch treatment differences were not significant for any soil

385   enzymes in WA (Table 4). However, in TN, an effect of mulch treatment was observed for N-

386   acetyl β-glucosaminidase activities (Table 4). N-acetyl β-glucosaminidase activity was reduced

387   under BDMs and PE compared to no mulch plots. Interaction effects of mulch treatment and

388   time of sampling were not detectable for any of the enzymes assayed in TN or WA (Table 4).

389   **Discussion**

390   In this study, soil microbial community composition was not significantly altered by mulch type.

391   This is in contrast to other studies that have reported altered bacterial communities in soils under

392   BDMs (Koitabashi et al. 2012; Li et al. 2014b; Muroi et al. 2016), and under non-biodegradable

393   plastic mulches (Farmer et al. 2017; Munoz et al. 2015). Such opposite findings could be due to

394   differences in methodology: for example, the studies by Koitabashi et al. (2012) and Muroi et al.

395   (2016) were shorter laboratory incubation studies in controlled conditions (28°C to 30°C), used

396   pure polymer feedstock rather than commercial film formulations which include plasticizers and

397   other additives, and relied on different detection methods such as polymerase chain reaction-

398   denaturing gradient gel electrophoresis (PCR-DGGE). Laboratory studies under controlled

399   conditions often result in more rapid microbial responses to treatments compared to field studies

400   where variable environments introduce more noise. Our lack of observed difference may also be

401   because we used a realistic, but low, plastic to soil ratio: For example, in the study by Muroi et

402   al. (2016), 1.8 g PBAT films was used in 300 g soil. In the field, tilled-in BDMs are a very small

403   input of carbon when taking into account the volume of soil into which they are incorporated

404   (Bandopadhyay et al. 2018). For comparison, the input of mulch carbon added to the soil in this

405   study was a significantly smaller amount (6 to 25 g C m^{-2}) (Hayes et al. 2017) compared to the

406   amount typically added from cover crop residues (142 g C m^{-2}) (Al-Kaisi & Lal 2017). However,

407   several studies have demonstrated responses by soil microbes to these small inputs

408   (Bandopadhyay et al. 2018), suggesting that even if they are not a major carbon source, they do

409   influence microbial activities by some other mechanism, and may result in a difference between

410   BDMs and PE after multiple seasons of BDM incorporation. Finally, because our aim was to

411   characterize responses in bulk soil communities to understand the overall system level response

412   to plastic films, we likely missed changes happening on smaller spatial and temporal scales. For

413   example, Li et al. (Li et al. 2014b) reported changes in microbial communities in soils that were

414   sampled in close proximity to buried mulch films, indicating that microbial communities in the

415   immediate vicinity of the films may be affected. Here we show that any local effects of mulch

416   films are not detectable at a field scale, at least over a two-year period.

417   We did note significant differences in soil bacterial composition by location and season, which

418   has been observed in other studies (Li et al. 2014b; Moore-Kucera et al. 2014). In our study,

419   mulch effects were minimal compared to other drivers of community structure variation. It is

420   well accepted that local soil conditions such as temperature, moisture and pH play a pivotal role

421   in shaping microbial communities (Fierer & Jackson 2006; Moore-Kucera et al. 2014; Rousk et

422   al. 2010). In this study, the location differences in communities were attributed to higher relative

423   abundances of *Acidobacteria, Actinobacteria* and *Planctomycetes* in TN and higher abundances

424   of *β-* and *γ-Proteobacteria* in WA. This corresponds with higher pH and saturated K in TN and

425   higher soil organic matter and soil moisture in WA. Both pH and water content are major

426   edaphic factors that influence temporal and spatial variation in soil microbial communities

427   (Docherty et al. 2015; Rousk et al. 2010). Changes in soil physicochemical properties and

428   different climates and soil types between TN and WA could explain such locational differences.

429   Seasonal differences in communities were driven by significantly increased percent relative

430   abundance of *Acidobacte*r *Gp6, Gp4* and *Gp7* in Spring in TN as compared to Fall. Additionally,

431   significantly greater abundances of *Planctomycetaceae* and *Streptomyces* were seen in Fall

432   compared to Spring in TN. In WA, *Acidobacteria_Gp6* and *Spartobacteria* showed significantly

433   greater percent abundances in Spring compared to Fall whereas *Streptomyces* showed

434   significantly higher percent abundance in Fall compared to Spring (Fig. S2). Seasonal tillage

435   operations often reset many of the soil properties, which can explain why the abundances of

436   some taxa oscillated between Spring and Fall. Actinobacteria such as *Streptomyces* have

437   demonstrated polymer degrading capabilities (Pathak & Navneet 2017). However, because we

438   did not observe differences in the relative abundance of this taxa between BDMs, PE or no

439   mulch control, this increase is likely attributable to the agronomic management of the plots (e.g.

440   plant species, irrigation or fertilizer regimes etc.), rather than a response to mulch type.

441   Mulch materials did not have a consistent impact on bacterial richness or diversity. A previous

442   study evaluating microbial diversity using PCR-DGGE showed no difference in ammonia

443   oxidizer diversity under biodegradable and non-biodegradable mulching materials one year after

444   tilling plastics into soil (Kapanen et al. 2008).The higher richness estimates under BDMs

445   compared to PE treatments, which was significant in Fall 2015 in WA, suggested that tilled

446   BDMs may help promote richness in the soil environment.

447   Using gene copy abundances as a proxy for bacterial and fungal abundances, we observed some

448   evidence of a BDM-induced enrichment. In WA, both bacteria and fungi increased in abundance

449   under BDM and Weedguard treatments over the course of the two-year experiment. Because we

450   did not see an increase under PE, this suggests that this is in response to the incorporation of

451   BDMs into the soil (as opposed to an indirect effect of microclimate modification, such as soil

452   warming). In TN, we observed bacterial, but no fungal, enrichment in two of the four BDM plots

453   and PE plot. Previous studies have also demonstrated increased fungal abundances in soil

454   because of BDM incorporation (Li et al. 2014b; Ma et al. 2016; Muroi et al. 2016; Rychter et al.

455   2006). Fungi are important colonizers and degraders of BDMs; several plant pathogenic fungal

456   species such as *Alternaria brassicicola Aspergillus fumigates, Humicola insolens,* and

457   *Aspergillus oryzae* are known to produce cutinases which can accelerate degradation of

458   biodegradable mulch films (Koitabashi et al. 2012; Moore-Kucera et al. 2014; Muroi et al. 2016).

459   There is precedent for the differential responses in microbial enrichment we observed between

460   the two locations, with both fungal and bacterial enrichment in WA, but only bacterial

461   enrichment in TN. In a similar study comparing BDM effects in three locations, it was found that

462   BDMs resulted in soil fungal enrichment in Lubbock, TX, and bacterial enrichment in Knoxville,

463   TN (Li et al. 2014b). From other soil systems, we know that soil pH can be the best predictor of

464   bacterial community composition, while fungal communities were more closely associated with

465   changes in soil nutrient status (Lauber et al. 2008). Both TN and WA soils had comparable

466   fungal gene abundances initially (Spring 2015). However, since the microbial communities in

467   WA were related to organic matter (Fig. S1) and WA soils had higher C:N ratios than TN soils

468   this could explain the fungal enrichment in WA but not in TN.

469   Enzyme assays were conducted to assess potential activity rates for common carbon, nitrogen

470   and phosphorus cycling enzymes in soil. As with bacterial community structure, enzyme activity

471   profiles showed the greatest differences by location and season (Fig. 5, Table 4). The seasonal

472   oscillation in enzyme activities seen for almost all the enzymes could be attributed to seasonal

473   tillage operations which tend to offset many of the soil biological functions (Alam et al. 2014;

474   Busari et al. 2015; Zuber et al. 2015) (Fig. 6). This was also observed for many of the soil

475   physicochemical properties (Sintim et al. 2019). Mulch treatments had significant effects on N-

476   acetyl-β -glucosaminidase (NAG) in TN. NAG was decreased under mulches compared to no

477   mulch treatments, with the greatest decrease observed under PE. NAG catalyzes the hydrolysis

478   of chitin oligomers to form amino sugars which are major sources of mineralizable nitrogen in

479   soils and thus is important in carbon and nitrogen cycling in soils. Xylosidase activity was also

480   reduced under mulch treatments compared to no mulch plots in TN though not significant.

481   Because we saw decreases under all mulch treatments for NAG in TN, this is likely an indirect

482   effect of the mulches via microclimate modification, rather than a direct effect of mulch

483   fragments tilled into the soil. All mulches warm the soil, with PE often having a greater soil

484   warming potential compared to BDMs (Kader et al. 2017; Moreno & Moreno 2008). Mulches

485   also increase soil moisture levels (Qin et al. 2015). Consequently, changes in soil temperature

486   and moisture will affect enzyme pool sizes (Steinweg et al. 2013). The reduction in activity

487   under plastic mulches may be because TN has a warmer climate where plastic mulches can push

488   temperatures above optima limiting soil microbial activity (Moreno & Moreno 2008). Mean soil

489   temperatures in summer under mulched plots were 24.7ºC at 10 cm depth in TN, whereas in WA

490   it was 18.7°C. Un-mulched plots had mean summer soil temperatures of 23.8 °C for TN and

491   17.0°C for WA (Sintim et al. 2019). In the month of June in both years, soil temperatures

492   exceeded 30 °C under mulched plots in TN, but were less than 30 °C for no mulch plots. It has

493   been reported that fungal and bacterial growth rates have optimal temperatures around 25 to

494   30°C in agricultural and forest humus soils, while at higher temperatures lower growth rates are

495   found (Pietikainen et al. 2005). This decrease in growth rate was shown to be more drastic for

496   fungi than for bacteria, resulting in an increase in the ratio of bacterial to fungal growth rate at

497   higher temperatures. Thus, the high temperatures under mulches in the summer in TN were

498   above optimum growth conditions for soil microbes and may have reduced soil enzyme

499   activities. Cold-adapted microorganisms, which are expected to be more prevalent at the WA

500   site, tend to respond more efficiently to increased temperature than warm-adapted microbes

501   (Brzostek & Finzi 2011). The greatest relative temperature sensitivity of decomposition

502   processes has been observed at low temperatures (Kirschbaum 1995). Warming experiments

503   have revealed reduced xylosidase activity in soils under medium-warmed plots compared to

504   unwarmed plots (Steinweg et al. 2013). It has also been reported that warming induces decreases

505   in the temperature sensitivity of β-xylosidase activity in the H horizon (Souza et al. 2017). One

506   study reported greater increase of the relative temperature sensitivity of XYL and NAG

507   (important for C cycling) at lower temperatures, compared to amino peptidase enzymes

508   suggesting that temperature plays a pivotal role in regulating the use of substrates. Thus, the

509   turnover of easily degradable C substrates (like glucose) is more sensitive to temperature than

510   higher molecular compounds, at least for cold soils (Koch et al. 2007).

511   Looking specifically at studies which assessed soil enzyme activities after treatment with

512   biodegradable plastic film, one field study reported that soil microbial biomass and β-glucosidase

513 activity were most responsive to mulch incorporation; however that study did not have PE as a

514 control, so it is unclear if this response was specific to BDMs or just related to plastic mulching

515 generally (Li et al. 2014a). The cited study also focused on soils in close proximity to plastic,

516 rather than bulk soil responses. Laboratory studies have shown increased esterase activity in soils

517 during the degradation of poly(butylene succinate-co-adipate) (PBSA) (Yamamoto-Tamura et al.

518 2015), and increased microbial activity as per a fluorescein diacetate hydrolysis test during the

519 degradation of a variety of biodegradable polymers (Barragán et al. 2016). These studies provide

520 insight into the potential of these enzymes in the degradation process of BDMs. Other studies

521 that have looked at more general activity responses by microbes under plastic mulches (i.e.

522 respiration) have reported mixed results: some have observed increases in activity under plastic

523 mulches (Chen et al. 2017; Mu et al. 2016; Mu et al. 2014; Zhang et al. 2015), while others

524 report decreased activities (Moreno & Moreno 2008).

525 **Conclusions**

526 Two years of biodegradable and PE mulch treatments in a vegetable agroecosystem in two

527 locations revealed only minor effects on soil microbial communities and their functions. We

528 previously showed that biodegradable mulches did not have a significant impact on a suite of soil

529 quality parameters at these sites (Sintim et al. 2019). The investigation of the microbial

530 communities from the same experiment corroborate these results showing that locational and

531 seasonal variations are more important drivers of changes in soil health under BDM tillage

532 operations than the type of mulch treatment at these field sites.

533 It should be noted that marginal but significant location-dependent effects of mulches were

534 observed. For example, in WA, BDM incorporation caused a significant enrichment in both soil

535 bacterial and fungal abundances, suggesting a direct response to BDM incorporation into soils;

536 while only bacterial enrichment was observed in TN. We additionally observed decreases in

537 specific enzyme activities (NAG) under mulch treatments in TN but not WA, which may be

538 attributable to increased temperatures under the plastics (i.e. microclimate modification) in the

539 warmer climate. Together, this shows that plastic mulches had minor impacts on soil microbial

540 communities and their functions. BDMs may have effects different from PE plastic mulches, and

541 these responses may be location-specific. As microbes are the drivers of soil carbon and nutrient

542 cycling, changes in bacterial and fungal abundances and/or activity can have repercussions for

543 soil organic matter dynamics and nutrient availabilities. Longer term studies of repeated BDM

544 incorporation are needed to determine if these microbial responses will significantly affect soil

545 functioning and health. In addition, the fact that we saw different responses by the communities

546 in two locations under identical management may mean that the ultimate impact of plastic

547 mulching on soil functioning may be dependent on local climate and soil conditions.

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564 **References**

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Manufacturers, major constituents, and physicochemical properties of the mulches used in the study.

Bio-based content was provided by the manufacturers. Data reported from (Hayes et al. 2017) .

1 **Table 1: Manufacturers, major constituents, and physicochemical properties of the mulches used in the study.** Bio-based
2 content data was provided by the manufacturers. Data adapted from Hayes et al. (2017).

Mulches	Manufacturer	Major constituents[a]	Weight (g m^{-2})	Thickness (μm)	Elongation[b] (%)	Contact angle[c] (°)	Total carbon (%)	Biobased content (%)
BioAgri®	BioBag Americas, Inc., Dunedin, FL	Mater-Bi® grade EF04P (blend of starch and PBAT)	18.0	26	260	87.6	57.6	20-25
Naturecycle	Custom Bioplastics, Burlington, WA	Blend of starch and polyesters	25.4	48	213	69.2	54.8	~20
Organix A.G. Film™	Organix Solutions, Maple Grove, MN	BASF®ecovio® grade M2351(blend of PLA and PBAT)	17.8	20	273	86.2	51.4	10-20
Experimental PLA/PHA	Metabolix Inc., Cambridge, MA	88.4% MD05-1501 (56% Ingeo PLA, 24% Mirel™ amorphous PHA, 15% CaCO$_3$ and 5% additives), 10.0% Techmer PLA M91432 (20% carbon black in PLA 3052) and 1.6% PLA	25.0	33	247	67.8	47.5	86
WeedGuardPlus ®	Sunshine Paper Co., Aurora, CO	Cellulose	240	479	6.4	<10	46.0	100
Polyethylene	Filmtech, Allentown, PA	Linear low-density polyethylene	25.4	47	578	79.3	82.9	< 1

4 [a]PBAT: Polybutylene co-adipate co-terephthalate; PLA: Polylactic acid; PHA: Poly(hydroxyalkanoate); [b]Measured in machine
5 direction; [c]Measured at 22°C.

PERMANOVA results (F values) for differences in bacterial community composition by location, time and mulch treatment.

Significant differences are in bold; *p < 0.05; **p < 0.01; ***p < 0.001.

1 **Table 2:** Results (F values) of PERMANOVA tests for differences in bacterial community

2 composition by location (Knoxville (TN) and Mount Vernon (WA)), season (Time) and mulch

3 treatment. Significant differences are in bold; *p < 0.05; **p < 0.01; ***p < 0.001.

Factor/treatment	Levels	TN (F)	WA (F)
Location	TN, WA	**117.34*****	
Time	Spring 2015, Fall 2015, Spring 2016, Fall 2016	**17.83*****	**32.84*****
Mulch treatments (Spring 2015 – initial sampling)	7 treatments: 5 BDMs[a] (BioAgri, Organix, PLA/PHA, Naturecycle, Weedguard), PE[b], no mulch control	0.61	0.81
Mulch treatments (Fall 2015)		0.87	**1.96****
Mulch treatments (Spring 2016)		0.84	0.81
Mulch treatments (Fall 2016)		1.15	1.26

4 [a]BDMs = Biodegradable mulches; [b]PE = polyethylene

F values obtained from a mixed model analysis of variance of the alpha diversity metrics richness (number of observed OTUs) and diversity index (inverse Simpson).

Significant values are in bold, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

1 **Table 3: F values of fixed effects and interaction effects obtained from a mixed model**

2 **analysis of variance of the alpha diversity metrics richness (number of observed OTUs) and**

3 **diversity index (inverse Simpson) from Spring 2015 to Fall 2016 in Knoxville, TN and**

4 **Mount Vernon, WA.** Significant values are in bold, *p < 0.05; **p < 0.01; ***p < 0.001.

Factor/treatment	Levels	Richness F	Diversity F
Location	TN, WA	**24.42*****	2.98
Treatment	7 treatments: 5 BDMs^a (BioAgri, Organix, PLA/PHA, Naturecycle, Weedguard), PE^b, no mulch control	1.93	1.20
Location*Treatment		1.22	1.58
Time	Spring 2015, Fall 2015, Spring 2016, Fall 2016	**19.28*****	**122.23*****
Location*Time		**6.06*****	**3.84****
Treatment*Time		**2.4****	1.63
Location*Treatment*Time		0.55	1.09

5 ^{a}BDMs = Biodegradable mulches; ^{b}PE = polyethylene

F values obtained from a mixed model analysis of variance of the soil enzyme activities.

Significant values are in bold, *p < 0.05; **p < 0.01; ***p < 0.001.

1   **Table 4: F values of fixed effects and interaction effects obtained from a mixed model analysis of variance of the soil enzyme**

2   **activities from Spring 2015 to Spring 2017 in Knoxville, TN and Mount Vernon, WA.** Significant values are in bold, *p < 0.05;

3   **p < 0.01; ***p < 0.001.

Location	Factor	β-xylosidase	β-glucosidase	α-glucosidase	N-acetyl β glucosaminidase	β-D cellubiosidase	Phosphatase	Leucine amino peptidase
TN	Treatment	2.21	1.49	2.62	2.53*	1.03	1.37	1.71
	Time	46.48***	29.56***	40.16***	34.60***	32.82***	68.23***	28.83***
	Treatment*Time	1.55	0.92	1.52	1.26	0.88	1.04	0.96
WA	Treatment	0.89	0.84	1.12	0.64	0.75	1.13	0.34
	Time	5.12***	3.44*	13.31***	6.06***	0.27	4.10**	0.65
	Treatment*Time	0.88	0.91	0.65	0.78	0.72	0.96	0.77

Figure 1

Bacterial community composition differences between the two field locations, showing communities from all four sampling times.

(A) Non-metric multidimensional scaling (NMDS) ordination of Bray-Curtis dissimilarities of OTU relative abundances, highlighting differences between location (PERMANOVA p = 0.001). Each point corresponds to the microbial community of one plot in the field (4 sampleing times * 3 replicate plots, resulting in 12 points for each treatment). Ellipses denote clustering at 95% confidence. NMDS stress value: 0.14. (B) Bar plot showing differences in mean relative abundance of the most abundant classes of bacteria in TN and WA, aggregating all treatments and all four sampling times. Asterices denote significant differences between locations, determined by ANOVA (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001). Red stars indicate taxa which cumulatively contributed up to 46% of the variance in microbial communities between TN and WA, determined using SIMPER.
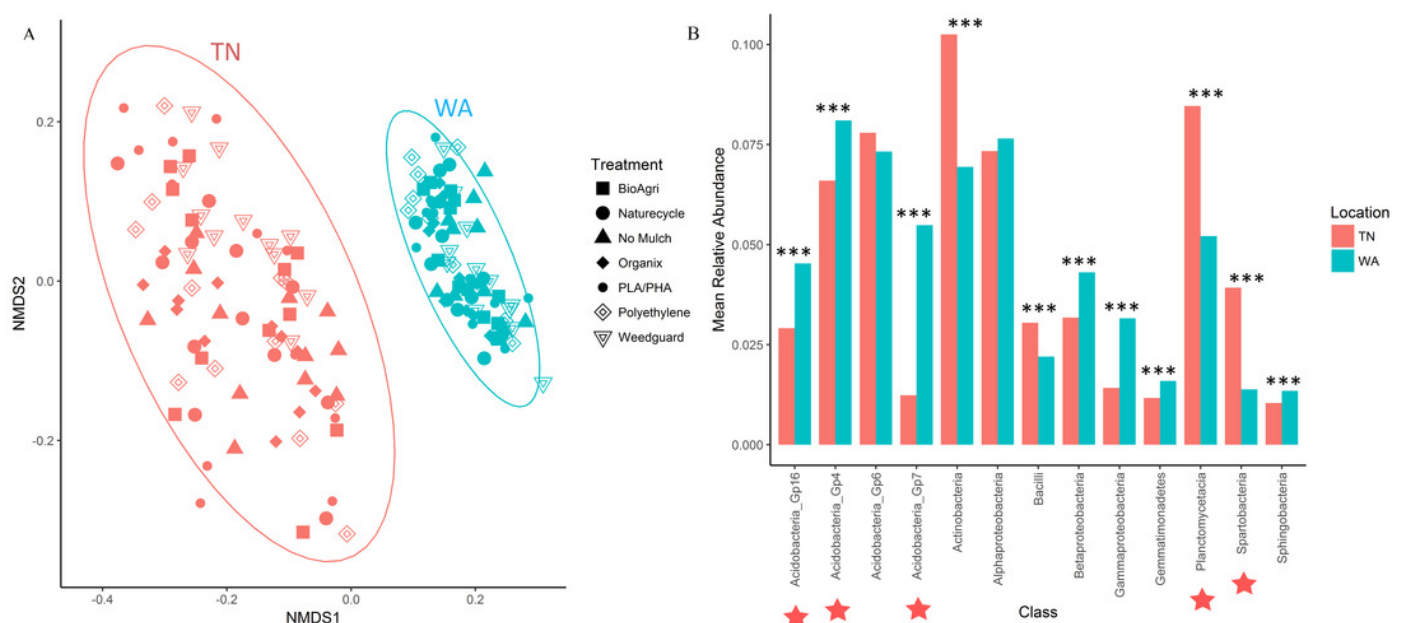
Figure 2

NMDS ordination of Bray Curtis dissimilarities of soil bacterial communities showing significant differences between season.

(A) TN (PERMANOVA p = 0.001; stress: 0.17) and (B) WA (p = 0.001; stress: 0.16). Ellipses denote clustering at 95% confidence. Spring 2015 samples represent initial soils prior to mulch application. Fall 2015 represents soils exposed to a season of surface applied mulches. Spring 2016 and Fall 2016 represent soils exposed to BDM mulch fragments tilled into soil from the previous season.
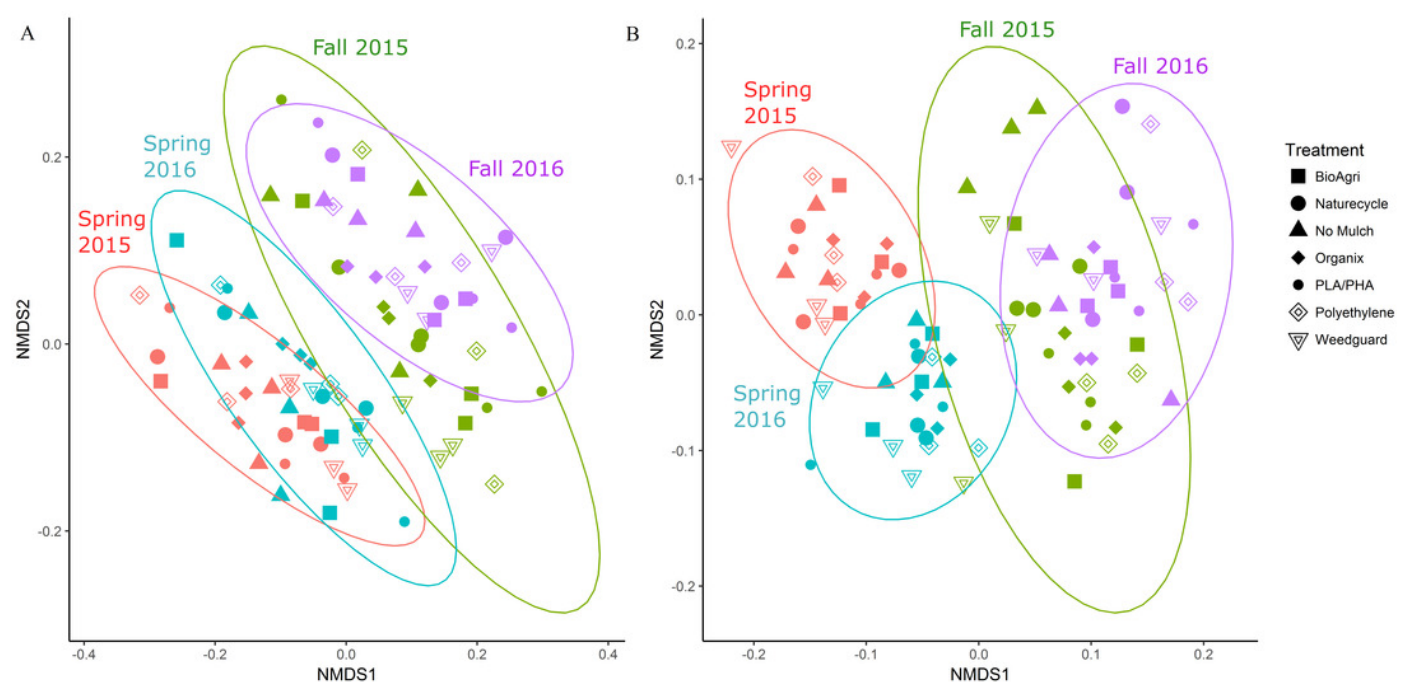
Figure 3

Changes in alpha diversity of soil microbial communities over time in TN and WA.

(A) Richness (number of unique OTUs) and (B) Inverse Simpson estimates. Spring 2015 samples represent inital soils prior to mulch application. Error bars indicate SEM of three replicate samples.
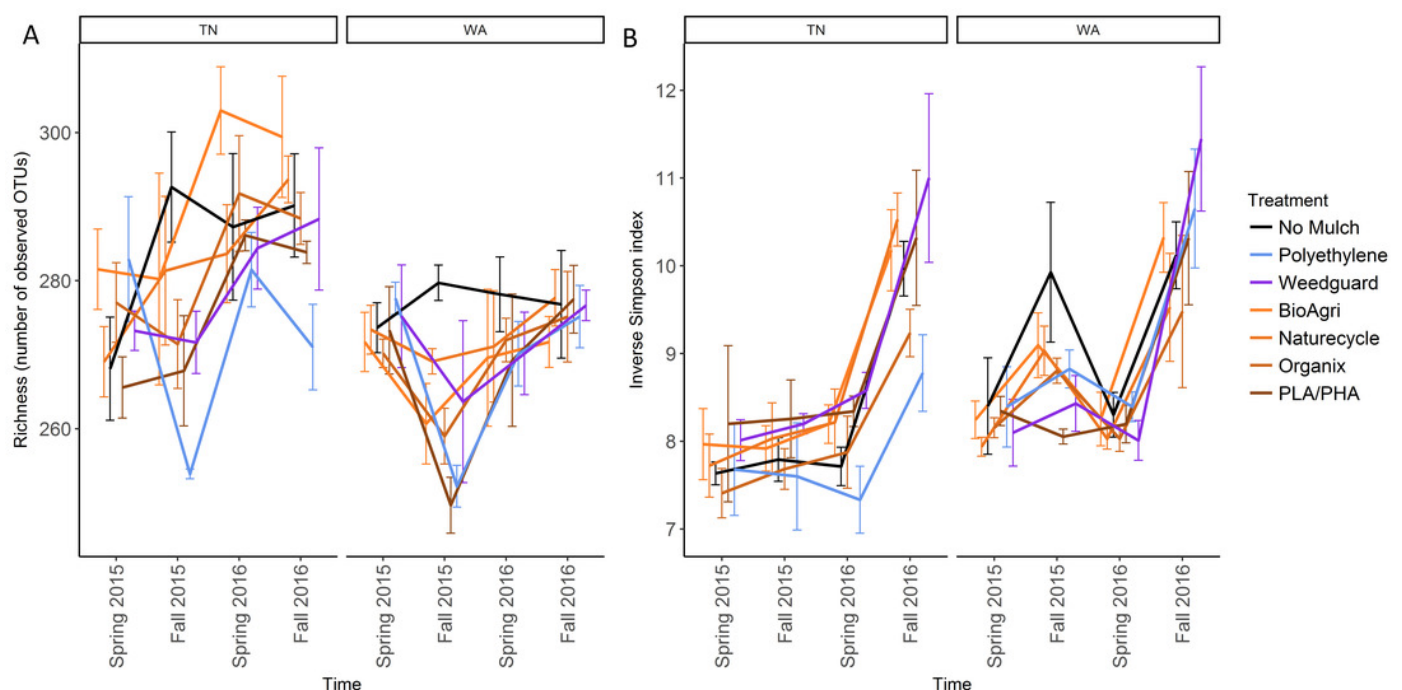
Figure 4

Change in bacterial and fungal abundances over the experiment in TN and WA.

Net change in (A) bacterial 16S rRNA and (B) fungal ITS gene copy abundances per gram dry weight soil over the two year experiment in TN and WA. Net changes were calculated by subtracting starting abundances (Spring 2015) from final abundances (Fall 2016). Error bars are SE of four replicate samples. Lowercase letters denote significant groupings between treatments (p ≤ 0.05, Tukey's HSD). Asterices indicate treatments which showed significant enrichment (i.e. significant difference from 0) using a paired t-test (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001).
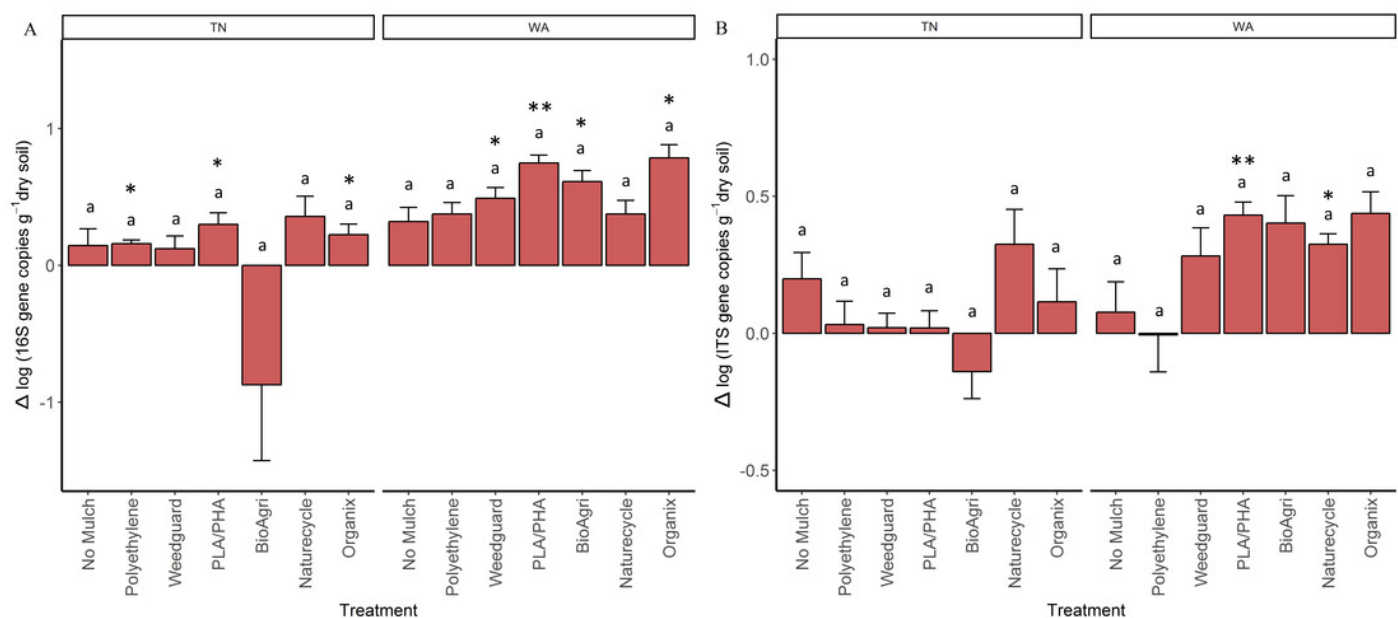
Figure 5

NMDS ordination of Bray-Curtis similarity of the functional profile of soil microbial communities (based on 7 soil enzyme activities).

Points are differentiated by (A) location and (B) sample time. Spring 2015 samples represent initial soils prior to mulch application.
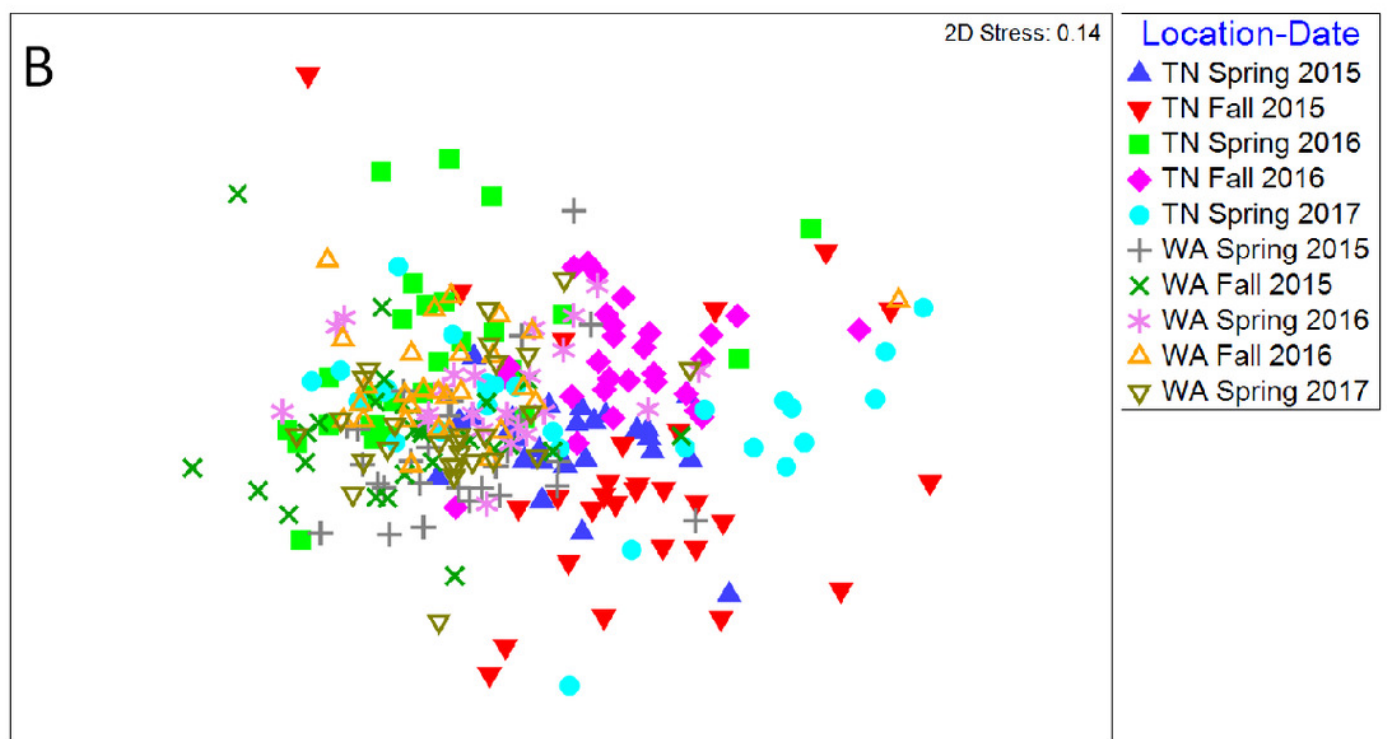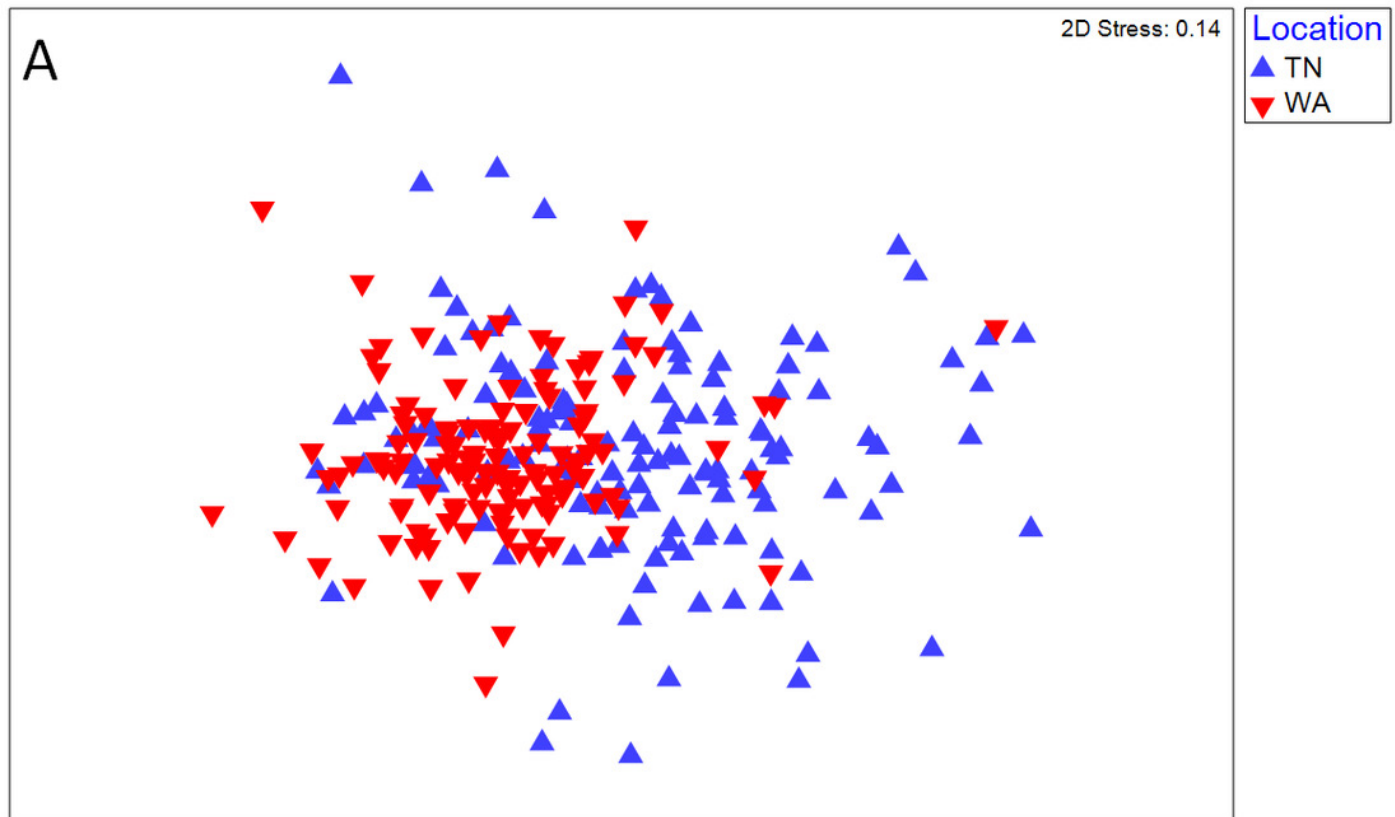
Figure 6

Soil enzyme activity over time in TN and WA under different mulching treatments.

P values arereported in Table 4. Spring 2015 samples represent initial soils prior to mulch application. Error bars indicate SEM of four replicate samples.