Tillage homogenizes soil bacterial communities in microaggregate fractions by facilitating dispersal

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Abstract

2   Soil aggregation physically protects soil organic matter and promotes soil carbon persistence
3   through microaggregate formation and organo-mineral associations. Tillage is a ubiquitous
4   disturbance to arable soil that disrupts aggregation, thus affecting microbial resource availability,
5   soil microhabitat conditions, and microbial interactions. We investigated how tillage affects
6   bacterial community composition of soil microaggregate fractions (53–250 µm), specifically the
7   free microaggregate fraction in bulk soil, and the occluded microaggregate fraction from within
8   macroaggregates, using two long-term tillage vs. no-tillage experiments in southern WI, U.S.,
9   that represent two different silt loam soils (Alfisol and Mollisol). We applied 16S rRNA gene
10 amplicon sequencing to characterize the effects of tillage on microaggregate bacterial
11 communities by relating compositional changes and ecological community assembly patterns to
12 various tillage-driven changes in the soil environment, including aggregate size distribution and
13 carbon content. Tillage homogenized soil bacterial communities, as quantified by increased
14 compositional similarity at both within-plot and between-plot scales, and community assembly
15 was increasingly influenced by homogenizing dispersal with tillage. We did not identify major
16 distinctions between bacterial communities of the free and occluded microaggregate fractions,
17 thus highlighting how soil microaggregates readily shift between these operationally defined
18 fractions in temperate annual cropping systems, where the soil environment is subject to drastic
19 seasonal changes that are exacerbated by tillage. With this study, we improve our understanding
20 of the microbial response to soil disturbance, and thus the potential mechanisms through which
21 disturbances like tillage affect soil carbon persistence.

22

23 **Keywords:**
24 microaggregates; tillage; aggregate size fractionation; community assembly; soil microbial
25 communities, soil carbon
26
27 **Abbreviations:**
28 soil organic matter (SOM); soil organic carbon (SOC); particulate organic matter (POM)
29
30 **Highlights:**
31 • Tillage homogenized soil bacterial communities, within and between plots
32 • Homogenizing dispersal drove community assembly under tillage
33 • Free and occluded microaggregate fractions hosted similar communities

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1. **Introduction**

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35   Soil aggregation—the binding of soil particles and organic matter (Tisdall and Oades, 1982)—is

36   a primary mechanism of soil organic matter (SOM) protection that promotes microbially-

37   mediated soil carbon stability and persistence (Jastrow and Miller, 1998). Agricultural tillage,

38   employed on over 60% of U.S. farmland (Zulauf and Brown 2019), disrupts aggregation, thus

39   reducing physical protection of SOM and potentially increasing microbial mineralization of soil

40   organic carbon (SOC) (Elliott, 1986; Paustian et al., 1997; Six et al., 1998; Schimel and

41   Schaeffer, 2012). Improved understanding of the ecological factors that relate microbial

42   community composition to changes in aggregation and SOC persistence under mixing

43   disturbances will populate a key knowledge gap in soil microbial ecology (Wilpiszeski et al.,

44   2019). These relationships may be pertinent in the highly protected microenvironments of soil

45   microaggregates ($< 250\,\mu m$ in diameter), which are more stable and have lower turnover rates

46   than macroaggregates ($250–2000\,\mu m$ in diameter) (DeGryze et al., 2006; Davinic et al., 2012;

47   Totsche et al., 2018). Microaggregates are found both free in the bulk soil and occluded within

48   macroaggregate structures (Oades, 1984; Totsche et al., 2018).

49   Microaggregates are cemented and glued together by physicochemical interactions and

50   biomolecules that physically protect SOM, rendering it less accessible for microbial

51   decomposition (Edwards and Bremner, 1967; Jastrow and Miller, 1998; Six et al., 2004; Totsche

52   et al., 2018; Woolf and Lehmann, 2019). Microaggregate structures further inhibit microbial

53   activity due to nutrient and oxygen limitation (Sexstone et al., 1985; Ranjard and Richaume,

54   2001). These same microhabitats that protect SOM from microbial decomposition are also

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55   disproportionately high in microbial abundance; an estimated 70% of soil bacteria live within

56   microaggregates (Ranjard et al., 2000), despite the microaggregate fraction comprising perhaps

57   30–50% of arable soil by mass (Sheehy et al., 2015; Cates et al., 2016). Soil bacterial

58   communities moderate the soil carbon cycle through decomposition of SOM and mineralization

59   of SOC for food (Schimel and Schaeffer, 2012), while also contributing to persistent SOM

60   through the production of microbial necromass, which has been shown to accumulate in soil

61   (Simpson et al., 2004; Liang and Balser, 2008). Overall, the mechanisms that balance

62   microbially mediated SOC persistence with carbon-consuming microbial activity in

63   microaggregates (i.e., "microbial hotspots", sensu Kuzyakov and Blagodatskaya, 2015) are not

64   well-understood (Six et al., 2004; Wilpiszeski et al., 2019).

65   Agricultural tillage disrupts the fine roots and fungal hyphae that stabilize macroaggregates

66   (Tisdall and Oades, 1982; Elliott, 1986; Six et al., 1998), thus decreasing mean aggregate size

67   and/or proportion of aggregated soil (Frey et al., 1999; Six et al., 1999; Al-Kaisi et al., 2014;

68   Zheng et al., 2018). Because microaggregates can form within the protective environment of a

69   macroaggregate (Oades, 1984; Angers et al., 1997), tillage reduces the potential for occluded

70   microaggregate development within macroaggregates and, critically, the rate at which SOM is

71   potentially stabilized in microaggregates (Six et al., 2000a). Further, as macroaggregates

72   destabilize, their occluded microaggregates become more freely connected to the bulk soil

73   environment, increasing resource diffusion (e.g., oxygen and extracellular enzymes) and

74   decomposer pressure (Six et al., 1999; Garland et al., 2018; Piazza et al., 2020). Through these

75   mechanisms, tillage has been associated with decreases in total SOM content (Elliott, 1986),

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76   SOM residence time (Paustian et al., 2000), SOC content (Paustian et al., 1997; Al-Kaisi et al.,

77   2014; Zheng et al., 2018), aggregate-occluded particulate organic matter (POM) (Six et al.,

78   1999), microbial biomass (Zuber and Villamil, 2016), and microbial necromass accumulation

79   (Simpson et al., 2004). Though these effects are well-documented, they are typically only noted

80   in the top 5 or 10 cm of soil (Frey et al., 1999; Six et al., 1999; Simpson et al., 2004; Zheng et

81   al., 2018), and some work has suggested that tillage does not decrease total SOC across the full

82   soil profile (Powlson et al., 2014; Ogle et al., 2019). There is also evidence that minimum tillage

83   practices can be equally beneficial as no-tillage regarding SOC and microbial necromass

84   accumulation, by incorporating nutrients and alleviating compaction (Sae-Tun et al., 2022).

85   A recent meta-analysis demonstrated that the occluded microaggregate fraction preferentially

86   accumulates SOC at a higher rate than the free microaggregate and other soil fractions (King et

87   al., 2019). One study found over 90% of the increase in SOC content in no-tillage as compared to

88   conventional tillage systems was attributable to the occluded microaggregate fraction, across

89   soils of various clay mineralogies (Denef et al., 2004), while another study found that the

90   occluded microaggregate fraction contributed 49–112% of the increase in SOC following a shift

91   to no-tillage across the U.S. (Six and Paustian, 2014). Together, these results indicate a higher

92   capacity for SOC persistence in the occluded versus the free microaggregate fraction.

93   The effects of tillage on soil microenvironments (e.g., aggregate size and porosity), and the

94   resulting redistribution of resources (e.g., oxygen, water, biomass), suggests that tillage also

95   alters soil microbial community composition and function (Bhattacharyya et al., 2021). Tillage-

96   driven decreases in aggregate size may select for more oligotrophic communities due to lower

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97   substrate and oxygen availability (Trivedi et al., 2017), though some have found fast growing,

98   copiotrophic competitors to dominate soil communities under tillage or disturbance (Srour et al.,

99   2020; West and Whitman, 2022). Tillage also shifts communities towards bacterial dominance

100   and away from fungal dominance, which is thought to contribute to SOC loss (Six et al., 2006).

101   Understanding changes to microbial communities under a given management practice, such as

102   tillage, is essential for improving predictions of SOC persistence and storage.

103   In the limited number of studies that have applied high-throughput sequencing to aggregate

104   fractions, distinct and more diverse bacterial communities are supported by the free

105   microaggregate fraction than the macroaggregate fraction (Trivedi et al., 2017; Bach et al., 2018;

106   Upton et al., 2019). One study that separately assayed free and occluded microaggregate

107   fractions found similar community compositions in the free and occluded microaggregate

108   fractions (53–250 µm), yet suggested that copiotrophic bacteria live in association with free

109   microaggregates whereas oligotrophic bacteria are characteristic of occluded microaggregates

110   (Biesgen et al., 2020). This assessment is consistent with the idea that free microaggregates have

111   higher resource availability, notably C and oxygen, that may support copiotrophic

112   microorganisms, whereas occluded microaggregates may be more insulated from perturbation,

113   resource fluxes, and decomposers, as evidenced by increased SOC persistence (King et al.,

114   2019). Another study found that microbial necromass concentration was greater in the occluded

115   microaggregate fraction under no-tillage as compared to tillage, whereas there was no difference

116   in the free microaggregate fraction under tillage versus no-tillage (Simpson et al., 2004). These

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117 results suggest that tillage-related impacts on macroaggregate formation and turnover (and, thus,

118 occluded microaggregates) extend to microbial community composition (Six et al., 2004).

119 In this study, we assess differences in community composition of the free and occluded

120 microaggregate fractions to tease out the ecological processes governing community assembly,

121 in order to better predict how management-driven changes to soil structure affect soil

122 communities. The community assembly processes (Vellend, 2010) of interest are as follows:

123 Dispersal describes the generally stochastic movement and establishment of organisms in space,

124 and may occur in soil via physical disturbance or mass flow of pore water (Zhou and Ning,

125 2017). Homogenizing dispersal increases compositional similarity between communities,

126 whereas dispersal limitation increases compositional differences between communities, and may

127 allow for stochastic demographic changes to community composition — termed 'drift' (Stegen et

128 al., 2013). Selection refers to deterministic or niche-based processes dictated by biotic factors,

129 such as inter-taxa fitness differences, and abiotic factors, such as environmental filters

130 (Hutchinson, 1957). Homogeneous selection decreases phylogenetic differences between

131 communities due to community assembly under similar conditions or filters (Dini-Andreote et

132 al., 2015). Variable selection increases phylogenetic differences between communities due to

133 variable conditions (Stegen et al., 2015). To statistically infer the relative influences of these

134 community assembly processes in soil microbial communities, Stegen et al. (2012, 2013, 2015)

135 developed a null modeling approach that compares observed phylogenetic distances and

136 dissimilarity metrics between communities to null models of stochastically assembled

137 communities. A more recent approach separately assesses community assembly processes within

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138   phylogenetically related 'bins' of OTUs, thus enabling representation of various assembly

139   processes that may influence subsets of community members (Ning et al., 2020). These

140   approaches have not yet been used to directly compare the effects of tillage on community

141   assembly, let alone at the microaggregate fraction scale.

142   We sought to better understand how bacterial communities are affected by tillage, as modulated

143   through soil aggregation. We collected soil samples in no-tillage and chisel-plowed tillage plots

144   from two long-term studies in southern Wisconsin, U.S., and related soil properties to tillage-

145   driven differences in bacterial community composition, diversity, and community assembly

146   processes of the bulk soil, free microaggregate, and occluded microaggregate fractions, using

147   16S rRNA gene amplicon sequencing. In addition to expecting standard responses to tillage

148   including decreased SOC and aggregation, we hypothesized that the free microaggregate and

149   occluded microaggregate fractions would support distinct bacterial communities, and

150   demonstrate differences due to tillage treatments. Specific hypotheses included: H1) With tillage,

151   community assembly would be driven by homogenizing dispersal and homogeneous selection,

152   whereas in the no-tillage system, community assembly would be driven by dispersal limitation

153   and variable selection. H2) The occluded microaggregate fraction would demonstrate stronger

154   evidence for dispersal limitation, whereas the free microaggregate fraction would demonstrate

155   stronger evidence for homogeneous selection. H3) Tillage would increase sample-to-sample

156   similarity in community composition (i.e., lower beta diversity). Better understanding microbial

157   community composition and assembly in microaggregate environments will improve efforts to

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158 understand mechanisms of SOC persistence, thus contributing to climate change resilience

159 (Paustian et al., 2000), ecosystem services, and crop productivity (Janzen, 2006).

2. Methods

161 *2.1 Soil collection*

162 Soil was sampled from two separate long-term tillage studies located at 1) the University of

163 Wisconsin (UW) Arlington Agricultural Research Station in Arlington, WI, U.S., (43°17'56"N,

164 89°21'11"W, 314 m a.s.l.) on a Plano silt loam soil (fine-silty, mixed, superactive, mesic Typic

165 Argiudoll), under a corn (*Zea mays* L.) – soybean (*Glycine max* L.) rotation; and, 2) the UW

166 Lancaster Agricultural Research Station in Lancaster, WI, U.S., (42°49'53"N, 90°47'35"W, 313

167 m a.s.l.) on a Fayette silt loam soil (Fine-silty, mixed, superactive, mesic Typic Hapludalfs),

168 under a continuous corn rotation. The tillage study at Arlington, WI was established in 1987 with

169 a no-tillage treatment, in which crops are planted directly into the undisturbed residue of the

170 previous crop, and a tillage treatment, which consists of fall chisel plow followed by two spring

171 field cultivator passes prior to planting. Further details regarding management practices and

172 agronomic findings have been reported (Pedersen and Lauer, 2003; Chamberlain et al., 2021).

173 The tillage plots at Lancaster, WI were established in 1993, consisting of no-tillage and tillage

174 treatments, the latter of which consists of fall chisel plow and a spring field cultivator pass prior

175 to corn planting. The Lancaster plots have been used for various research projects over the years

176 (e.g., Gupta et al., 2004; Dolliver and Gupta, 2008), which sometimes included manure

177 application treatments (1993–1997, 2003–2005, 2014) or corn fungicide treatments (2008–2010).

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178   Soil was sampled once in each location (23 October 2021 at Arlington, WI and 6 November

179   2021 at Lancaster, WI), following corn grain harvest and prior to fall tillage. At Arlington, three

180   plots were sampled for each treatment, collecting five intact cores per plot for a total of 15 cores

181   per treatment. Due to our interest in discerning dispersal processes, our sampling design focused

182   on ensuring relatively high spatial proximity of individual cores within a given plot. Soil cores

183   were 7.9 cm dia, evenly spaced just within the perimeter of a 48 cm dia circle; distance between

184   adjacent cores was approximately 15 cm. As detailed below, the top 5 cm was analyzed to target

185   soil under the greatest intensity of tillage disturbance. Lancaster was sampled in the same

186   fashion, but only two plots per treatment were used for analysis (see section 2.7), for a total of

187   ten cores per treatment. Intact cores were temporarily kept in a cooler, and then held at 4 °C for

188   up to ten days until sample processing.

189   *2.2 Aggregate size fractionation and sample processing*

190   To assess variability in community composition and community assembly at a relatively small

191   spatial scale, each core was processed separately. The top 5 cm of each field-moist soil core were

192   gently passed through a 2 mm sieve (henceforth referred to as "bulk" soil). Then, 80 g of this

193   field-moist bulk soil was subjected to aggregate size fractionation via wet sieving (Elliott, 1986)

194   to isolate the macroaggregate fraction (250 µm–2000 µm), free microaggregate fraction (53 µm–

195   250 µm), and the silt + clay-sized fraction (< 53 µm). Then, 20 g of the moist macroaggregate

196   fraction was separated into occluded fractions via rapid shaking with glass beads to break up the

197   macroaggregates under water flow to encourage liberated microaggregates and other material to

198   pass through a 250 µm sieve, as previously described (Six et al., 2000a, 2002); macroaggregate-

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199   occluded fractions included the occluded microaggregate fraction (53 µm–250 µm), occluded silt

200   + clay-sized fraction (< 53 µm), and occluded coarse POM + coarse sand-sized fraction (250 µm

201   – 2000 µm). Other modifications to the wet sieving method included a slaking for two minutes

202   prior to the first wet sieving step (Arlington samples only) and draining each wet sieved fraction

203   for two minutes prior to subsampling as described below. The largely unaggregated Lancaster

204   soil samples did not undergo slaking, and required wet sieving of an additional 80 g of bulk soil

205   to obtain enough macroaggregate soil for the occluded fraction separation step. The primary

206   objective of fractionation was to isolate the free and occluded microaggregate fractions, but the

207   relative dry mass of each size fraction was also determined. Correction for sand content of

208   aggregate fractions was not performed and thus all size fractions also include the primary

209   mineral particles of that size.

210   Moisture content was estimated for bulk soil, the wet-sieved macroaggregate fraction, the free

211   microaggregate fraction, and the occluded microaggregate fraction by drying subsamples in a 60

212   °C oven for 24 hours. Bulk, free microaggregate, and occluded microaggregate soil was

213   subsampled for DNA extraction (see section 2.4), and bulk soil was also subsampled to measure

214   soil respiration (see section 2.3). The remaining wet-sieved soil was washed from each sieve (or

215   washbasin) into aluminum pans to determine the dry mass of each fraction. Overall recovery

216   (macroaggregate + free microaggregate + [silt + clay] fractions) was 99% for both treatments at

217   both sites, and macroaggregate recovery (occluded microaggregate + [occluded silt + clay] +

218   occluded coarse POM) was 101% for tillage treatments, and 96–97% for no-tillage treatments.

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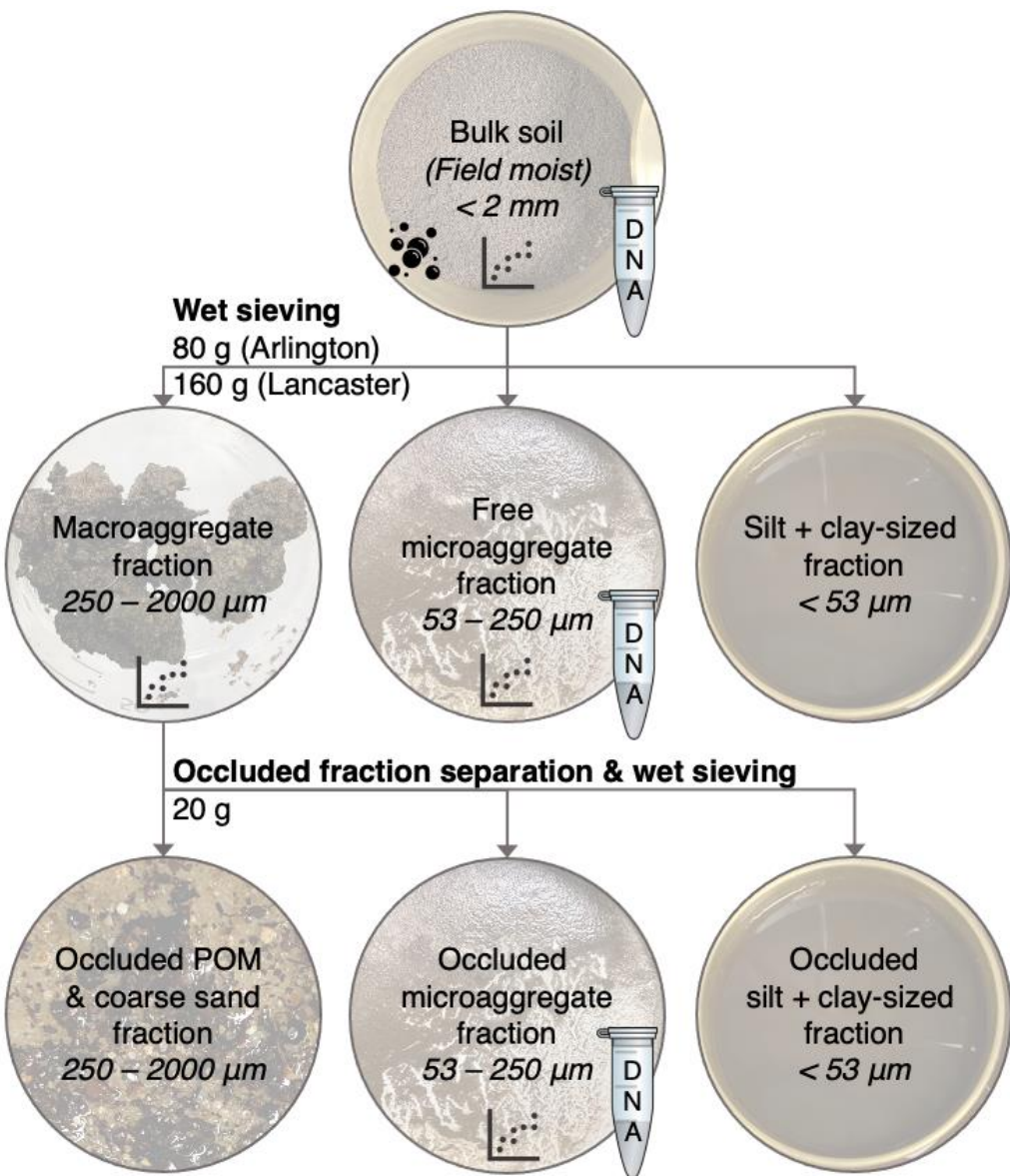
Figure 1. Aggregate size fractionation schematic. Bulk soil (80 g or 160 g) was subjected to wet sieving to separate macroaggregate (250–2000 µm), free microaggregate (53–250 µm), and silt + clay-sized (< 53 µm) fractions. A 20 g (wet) subsample of the macroaggregate fraction was then further separated into occluded microaggregate (53–250 µm), occluded silt + clay-sized (< 50 µm), and occluded POM + coarse sand fractions (250–2000 µm). The "DNA" tube indicates that subsamples were retained for 16S rRNA gene amplicon sequencing. The graph icon indicates that subsamples were collected to measure total carbon and total nitrogen. The bubble icon indicates that soil respiration was measured on bulk soil.

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228   *2.3 Soil analysis*

229   The bulk soil (sieved to < 2 mm), macroaggregate, free microaggregate, and occluded

230   microaggregate fraction subsamples that were retained for dry mass conversion were ground to a

231   powder and used to quantify total soil carbon and nitrogen by flash combustion with a Flash EA

232   1112 CHN Automatic Elemental Analyzer (Thermo Finnigan, Milan, Italy) and soil pH

233   (excluding the macroaggregate fraction). Soil pH was determined from the supernatant of a 1:1

234   soil:$CaCl_2$ (0.1 M) slurry using a micro pH electrode (modified from Braus and Whitman, 2021).

235   For routine soil analysis, a composite soil sample representing each treatment was comprised of

236   an equal mass of bulk soil from each plot. Samples were submitted to the UW Soil and Forage

237   Analysis Lab (Madison, WI, U.S.) to determine soil texture, organic matter content, pH, and

238   plant-available P, K, Ca, and Mg. Routine soil properties can be found in Table S1.

239   Soil respiration (CO_2 evolution) from fresh sieved soil was measured using the MicroResp

240   system (James Hutton Ltd., Aberdeen, Scotland), following general instructions for use

241   (Campbell et al., 2003) without added substrate. At the time of aggregate fractionation, 300 mg

242   of freshly sieved (< 2 mm), field-moist soil from each soil core was placed into each of six wells

243   of a deep-well plate. Wells were covered and stored at 4 °C for up to six hours. Each deep-well

244   plate, containing soil from up to ten different cores, was covered in parafilm and firmly tapped

245   on the benchtop 20 times to repack soil and minimize large air pockets. The deep-well plate was

246   then incubated at 25 °C in a CO_2-free environment overnight (approximately 16 hours), to help

247   deplete CO_2 from the well headspace and soil air. The following day, a colorimetric detection

248   plate was read at absorbance wavelength 570 nm using a BioTek Synergy 2 spectrophotometer

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249   microplate reader (time = 0). After confirming that all wells had highly similar readings (< 5 %

250   coefficient of variance), the detector plate was inverted over the deep-well plate, connected by

251   the 96-well seal, and clamped together. After six hours of incubation at 25 °C, the clamp set-up

252   was dismantled, and the colorimetric plate was immediately re-read to determine CO_2 evolution.

253   The colorimetric agar in the detection plates was made per the MicroResp manual instructions

254   (version 4), and CO_2 evolution was calculated following the manual's instructions, with a minor

255   modification to subtract CO_2 values for a set of empty deep wells in each plate (blanks).

256   *2.4 DNA extraction and 16S rRNA gene sequencing*

257   Total genomic DNA was extracted from bulk soil, free microaggregate, and occluded

258   microaggregate soil fractions using the DNeasy PowerLyzer PowerSoil Kit (Catalog No. 12855,

259   Qiagen, Germantown, MD), following manufacturer's instructions. We used 250 mg samples of

260   field-moist bulk soil for DNA extraction, but, due to the wetness of the microaggregate fractions

261   following wet sieving, we used 450 mg samples of these fractions to capture the same dry-mass

262   equivalent of 250 mg of field-moist bulk soil, based on preliminary testing. The microaggregate

263   samples were transferred directly from the drained sieves into the DNA extraction tubes, which

264   were immediately frozen at −20 °C, and stored at −80 °C for up to three months prior to DNA

265   extraction. Complete library preparation details can be found in the Supplementary Information.

266   Briefly, the 16S rRNA genes of extracted DNA were amplified in triplicate using PCR. Variable

267   region V4 of the 16S rRNA gene was targeted using forward primer 515f and reverse primer

268   806r (Walters et al., 2016). Primers also contained barcodes and Illumina sequencing adapters

269   (Kozich et al., 2013). The following reagents comprised each 25 μL PCR reaction: 12.5 μL Q5

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270   Hot Start High-Fidelity 2X Master mix (Catalog No. M0494, New England BioLabs, Ipswich,

271   MA), 1.25 μL 515f forward primer (10 mM), 1.25 μL 806r reverse primer (10 mM), 1 μL DNA

272   extract, 1.25 μL Bovine Serum Albumin (20 mg/mL; Catalog No. 97064-342, VWR

273   International, Radnor, PA), and 7.75 μL PCR-grade water. The plate was sealed and briefly

274   centrifuged prior to 30 PCR cycles on an Eppendorf Mastercycler nexus gradient thermal cycler

275   (Hamburg, Germany) using the following parameters: 98 °C for 2 min + 30 × (98 °C for 10

276   seconds + 58 °C for 15 seconds + 72 °C for 10 seconds) + 72 °C for 2 min and 4 °C hold.

277   Amplified DNA was confirmed via gel electrophoresis, then normalized and purified, prior to

278   paired-end 250 base pair sequencing on an Illumina MiSeq sequencer at the UW–Madison

279   Biotech Center. To obtain high coverage, the same library was sequenced twice under identical

280   conditions, and total reads were pooled for each sample after processing as described next.

281   Sequencing data were processed using a QIIME2 (Bolyen et al., 2019) pipeline, with DADA2

282   (Callahan et al., 2016) as the operational taxonomic unit (OTU, or amplicon sequence variant)-

283   picking algorithm, and taxonomy assignment using the SILVA 132 reference database (Quast et

284   al., 2013; Yilmaz et al., 2013). This yielded 10,102,355 demultiplexed sequences, which was

285   reduced to 6,307,452 after denoising, with a mean length of 227 base pairs (SD = 2.2). Excluding

286   extraction blanks, a total of 18,180 OTUs were identified. Amplicon sequences are available in

287   the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under

288   accession [TBD]. Our primers targeted both bacteria and archaea, but because our communities

289   were dominated by bacteria (94.5% of total reads), for simplicity, we will refer to bacteria when

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290   discussing communities in this manuscript. Over 99% of archaeal reads represented the phylum

291   *Crenarchaeota*.

292   *2.5 Data analysis*

293   Data analysis was performed in R (R-Core-Team, 2018), using *ggplot2* (Wickham, 2016) for

294   data visualization. The R code used to perform these analyses and to create the following figures

295   is available at https://github.com/jaimiewest/Soil-Disturbance-Tillage. To test for a significant

296   effect of tillage treatment on proportion of soil in each fraction, C content of each fraction, and

297   respiration, we used ANOVA followed by Tukey's HSD *post-hoc* comparison for significant

298   results ($p < 0.05$). To test for a significant effect of tillage treatment, soil fraction, or interaction

299   of these factors on soil C content, soil N content, soil C:N ratio, and soil pH, we performed

300   ANOVA as described above. Unless otherwise noted, reported *p* values refer to ANOVA tests.

301   Community composition was visualized using principal coordinates analysis (PCoA) created

302   with the *ordinate* function in the *phyloseq* package for R (*phyloseq::ordinate*) (McMurdie and

303   Holmes, 2013) using Bray-Curtis dissimilarities (Bray and Curtis, 1957) of Hellinger-

304   transformed relative abundance data (Legendre and Gallagher, 2001). To test for a significant

305   effect of tillage treatment, soil fraction, or interaction of these factors on community

306   composition, we used permutational multivariate analysis of variance (PERMANOVA) to

307   partition Bray-Curtis dissimilarity matrices among sources of variation using *vegan::adonis2*

308   (Anderson, 2001). A significant result ($p < 0.05$) was subjected to *post-hoc* pairwise factor

309   comparisons, adjusting p-values using the Benjamini-Hochberg method (Benjamini and

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310   Hochberg, 1995) to identify significant differences. To compare differences in community

311   composition due to tillage treatment or soil fractions, we tested for homogeneity of multivariate

312   dispersions (PERMDISP; *vegan::betadisper*) (Anderson, 2006), using ANOVA to test the

313   distances to group spatial median. Further, we also evaluated the effect of tillage treatment on

314   dispersion of free and occluded microaggregate fraction communities within each soil core. To

315   describe richness, we used the weighted linear regression model of OTU richness estimates,

316   which weights observations based on variance, using *breakaway::betta* (Willis et al., 2017). We

317   also calculated Faith's phylogenetic diversity (PD) (Faith, 1992; Pérez-Valera et al., 2015) to

318   assess differences in phylogenetic distance (i.e., sample branch length) using *picante::pd*

319   (Kembel et al., 2010).

320   To further understand changes in community composition, we calculated the weighted mean

321   predicted 16S rRNA gene copy number (Nemergut et al., 2016), which has been shown to

322   correlate with potential growth rate (Klappenbach et al., 2000) and disturbance (Whitman et al.,

323   2019; West and Whitman, 2022), and compared tillage treatments and soil fractions using

324   ANOVA and *post-hoc* testing as described above. 16S rRNA gene copy numbers were predicted

325   using the ribosomal RNA operon database (rrnDB) (Stoddard et al., 2015).

326   After evaluating our key questions, we used differential abundance to identify significant

327   treatment-driven shifts in relative abundances of individual taxa as well as phyla. For this

328   analysis, we compared the tillage treatments to each other (excluding taxa with mean relative

329   abundance < 0.00001) and subjected those data to a beta-binomial regression model and "Wald"

330   hypothesis test in *corncob∷differentialTest* (Martin et al., 2021), which controls for the effect of

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331 the treatment on dispersion. We report the µ value, which is the coefficient used to estimate

332 relative abundance in the *corncob* model and is proportional to the fold-change in relative

333 abundance between the treatment and control. We also assessed differential abundances of taxa

334 in the microaggregate fractions as compared to the bulk soil communities.

335 *2.6 Community assembly process assignment*

336 In order to determine the influential community assembly processes within each treatment and

337 fraction, we first applied the method developed by Stegen et al. (2012, 2013, 2015), which

338 compared sample pairs of interest (each possible pair of samples from the same site, tillage

339 treatment, and fraction) to null models that represent stochastic assembly in order to determine

340 the relative influence of selection (based on phylogenetic distances), or dispersal (based on

341 taxonomic dissimilarities). Briefly, the influence of selection was first tested using the

342 abundance-weighted beta-mean nearest taxon distance (βMNTD; the mean phylogenetic distance

343 between each OTU in one community and its closest relative in another community)

344 (*picante::comdistnt*) (Kembel et al., 2010). Homogeneous selection was identified in

345 comparisons for which βMNTD was more than 2 standard deviations below the mean of the null

346 distribution, indicating lower mean phylogenetic distance between pairwise communities than

347 observed in the null. Variable selection was identified in comparisons for which βMNTD was

348 more than 2 standard deviations above the mean of the null distribution, indicating higher mean

349 phylogenetic distance between pairwise communities than observed in the null. Comparisons that

350 fell within 2 standard deviations of the null mean were considered to lack a dominant influence

351 of selection, and were subsequently tested for the influence of dispersal using the modified

18

352   Raup-Crick metric based on Bray–Curtis dissimilarities (RC_{Bray}) (*phyloseq::distance*) (Chase et

353   al., 2011). Homogenizing dispersal was identified in comparisons for which RC_{Bray} was

354   significantly lower than the mean of the null distribution, indicating a higher level of similarity

355   between community compositions than was observed in the null condition; and dispersal

356   limitation was identified in comparisons for which RC_{Bray} was significantly higher than the null

357   mean, indicating lower similarity. Comparisons that were similar to the null mean for both

358   metrics were considered undominated by any particular community assembly process. This

359   analysis, including null model construction, was first performed for each site, and then separately

360   for sets of samples representing each site $\times$ treatment $\times$ fraction combination.

361   In addition to determining the dominant community assembly processes based on comparison of

362   the full communities represented by each sample, we also assigned community assembly

363   processes at a finer taxonomic scale, by separately assessing phylogenetically-related bins of

364   OTUs (*iCAMP::pdist.big* and *iCAMP::icamp.big*), as detailed by (Ning et al., 2020). This

365   approach is analogous to the Stegen method, but captures the various ecological mechanisms

366   governing community assembly of the microbial subcommunities within each soil sample. Like

367   the above-detailed full community assessment, here we also compared βMNTD and RC_{Bray}

368   metrics to null models to determine the influences of selection and dispersal, respectively,

369   though sample-to-sample comparisons were made within phylogenetic bins, and the dominant

370   process was weighted by that bin's relative abundance. We selected bins and ran the analysis

371   using the default parameters as detailed in Ning et al. (2020) and the R documentation (i.e.,

372   minimum of 24 OTUs per bin, confirmed by phylogenetic signal testing using *iCAMP::dniche*

19

373   and *iCAMP::ps.bin*; phylogenetic null model randomization within bins; taxonomic null model

374   randomization across all bins), with the exception of the phylogenetic distance metric, for which

375   we used βMNTD so that results would be more comparable to the full-community scale

376   assessment based on the method by Stegen et al. (2012, 2013, 2015). To test for a significant

377   effect of tillage treatment on the influence of community assembly processes that had $> 5\%$

378   influence, we performed ANOVA as described above. Community relative abundance data was

379   Hellinger-transformed for all community assembly assessments (Legendre and Gallagher, 2001).

380   *2.7 Exclusion of plots from analysis*

381   At the Lancaster site, one no-tillage plot and one tillage plot were excluded from analysis.

382   Though this field has been under long-term tillage treatments since the early 1990's, there are

383   strong indicators in the dataset that the two plots in question were subjected to treatments or

384   conditions that differentiate them from the other plots, likely due to the split-plot use of manure

385   and/or corn fungicide treatments over the years, or the disruptive installation of large pan

386   lysimeters. Though we made every attempt to avoid areas where manure was applied or

387   lysimeters were installed, it was challenging to confirm the precise boundaries of historic split-

388   plots, and the history of fungicide application (unpublished) was unbeknownst to us prior to

389   sampling. The microbial communities of the excluded plots are clearly differentiated in the

390   PCoA (Fig. S1).

20

3. Results

3.1 Tillage generally decreased aggregation, with different site responses

At Arlington, over 60% of soil (dry mass basis) was in water-stable aggregate fractions (Fig. 2A), with over 50% of soil in the macroaggregate fraction. There were no significant differences in proportion of soil in macroaggregate, free microaggregate, or silt + clay fractions due to tillage treatment, but within the macroaggregate fraction (Fig. 2C) there was a significant decrease in proportion of soil in the occluded microaggregate fraction ($p < 0.001$; Fig. 2C) from 28% in no-tillage to 22% with tillage, with a complementary increase in the occluded silt + clay fraction ($p < 0.001$).

At Lancaster, soil was largely unaggregated, with 25% and 14% of soil (dry mass basis) in water-stable aggregate fractions in the no-tillage and tillage treatments, respectively (Fig. 2B). In particular, the tillage treatment had a significantly lower proportion of soil in the macroaggregate fraction, with 6% as compared to 14% in the no-tillage treatment ($p < 0.001$). This was complemented by a significantly higher proportion in the silt + clay fraction ($p < 0.001$). Within the macroaggregate fraction at Lancaster (Fig. 2D), the proportions of the occluded microaggregate fraction and occluded silt + clay fractions were both significantly lower with tillage as compared to no-tillage ($p < 0.001$ and $p < 0.05$, respectively), with 17% of macroaggregate soil in the occluded microaggregate fraction in no-tillage, down to 11% with tillage. There was also a significant increase in occluded POM ($p < 0.001$), from 9% in the no-tillage treatment to 21% with tillage.
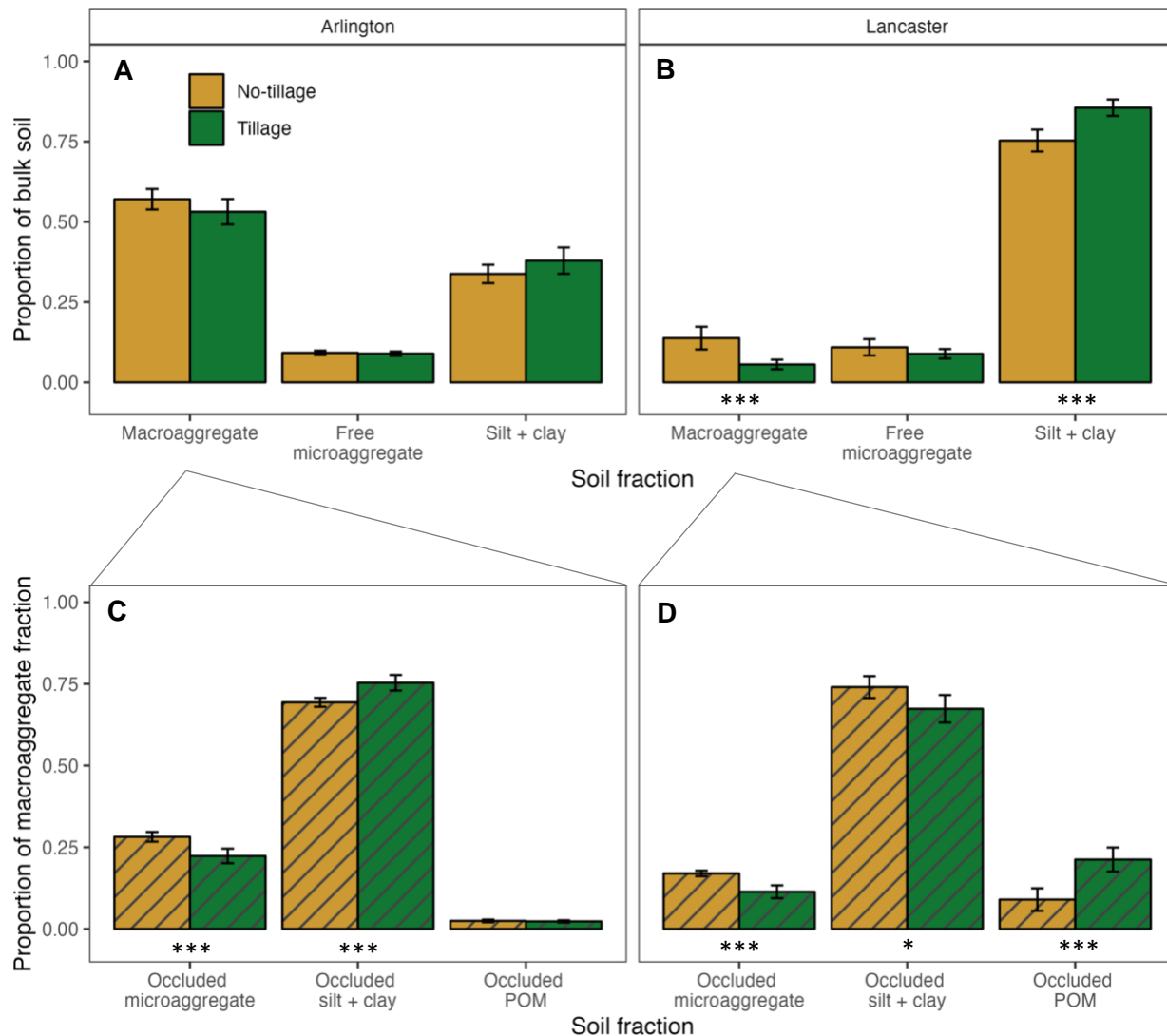
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Figure 2. Distribution of bulk soil in various fractions at Arlington, WI (**A**) and Lancaster, WI (**B**), on a dry soil basis. Lower panels show distribution of macroaggregate soil in the occluded fractions (**C** and **D**). Macroaggregate = macroaggregate fraction, 250–2000 µm; Free microaggregate = microaggregate fraction from bulk soil, 53–250 µm; Silt + clay = silt and clay-sized fraction from bulk soil, <53 µm; Occluded microaggregate = microaggregate fraction occluded within macroaggregate fraction, 53–250 µm; Occluded silt + clay = silt and clay-sized fraction occluded within the macroaggregate fraction, <53 µm; Occluded POM = particulate organic matter and sand occluded within the macroaggregate fraction, 250–2000 µm. Error bars represent ± 1.96 SE. Asterisks indicate significant tillage treatment differences within soil fraction: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. Striped bars represent occluded fractions.

22

3.2 Tillage reduced total soil carbon

Tillage decreased total carbon content in all measured fractions at both sites, reported here on a per unit of bulk soil basis (Fig. 3; $p < 0.001$ for each fraction at Arlington, $p < 0.05$ for each fraction at Lancaster).
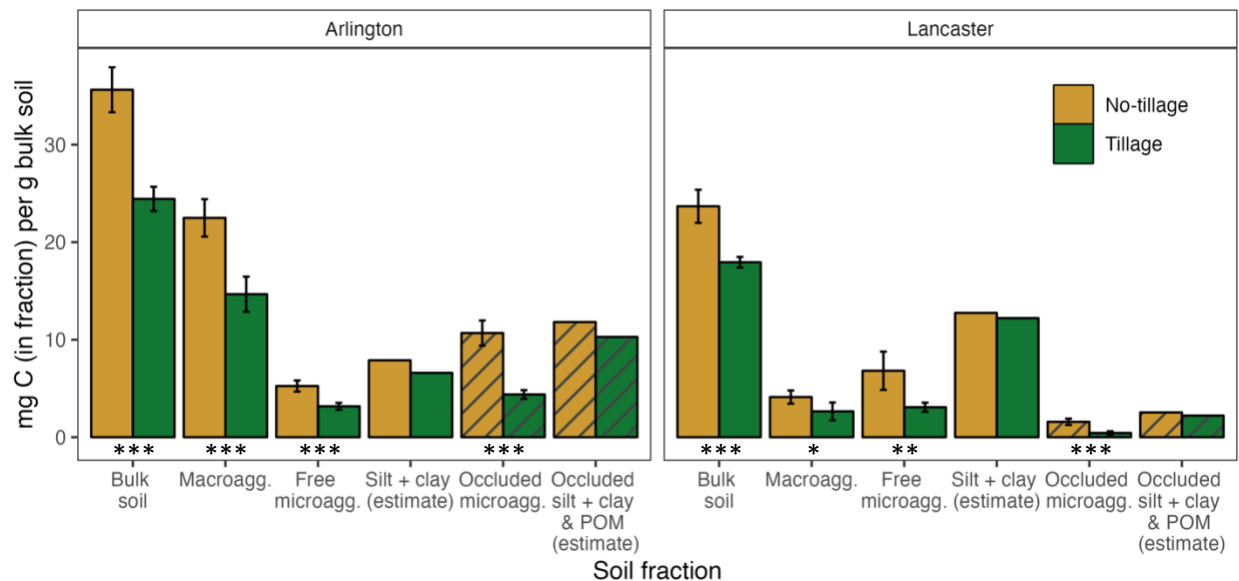

Figure 3. Carbon content of each soil fraction, on a per unit bulk soil basis. Bulk soil = whole soil; Macroagg. = macroaggregate fraction, 250–2000 µm; Free microagg. = microaggregate fraction from bulk soil, 53–250 µm; Silt + clay (estimate) = Carbon content in the < 53 µm fraction, estimated as Bulk soil – (Macroagg. + Free microagg.); Occluded microagg. = microaggregate fraction occluded within macroaggregate fraction, 53–250 µm; Occluded silt + clay & POM (estimate) = Carbon content in the < 53 µm fraction occluded within the macroaggregate fraction, estimated as Macroagg. – Occluded microagg. Error bars represent ± 1.96 SE. Asterisks indicate significant treatment differences within soil fraction: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$. The estimated silt + clay carbon contents do not have associated error bars or statistics. Striped bars represent occluded fractions.

Soil C concentration was affected by tillage treatment (Arlington only, $p < 0.001$), soil fraction (both sites, $p < 0.001$), and had a significant interaction effect (both sites, $p < 0.001$) (Table 1 and

23

443   Fig. S3). At Arlington, C concentration decreased with tillage, overall and within each fraction (p

444   < 0.001, Tukey's HSD). Comparing aggregate fractions, C concentrations of both free and

445   occluded microaggregate fractions were greater than those of the bulk soil and macroaggregate

446   fraction ($p < 0.05$, Tukey's HSD) in both treatments, and C concentration of the occluded

447   microaggregate fraction was greater than the free microaggregate fraction in no-tillage only ($p <$

448   0.01, Tukey's HSD). At Lancaster, C concentration decreased from occluded microaggregate >

449   free microaggregate > macroaggregate > bulk soil ($p < 0.01$, 0.05, and 0.001, respectively,

450   Tukey's HSD). Comparing aggregate fractions, the C concentrations of both free and occluded

451   microaggregate fractions were greater than C concentrations in the macroaggregate fraction and

452   the bulk soil in the no-tillage treatment only ($p < 0.001$, each comparison, Tukey's HSD), and C

453   concentration of the free microaggregate fraction was greater in no-tillage as compared to tillage

454   ($p < 0.001$, Tukey's HSD).

455   Soil N concentration trends were very similar to those of total C (Table 1), where N

456   concentration at Arlington decreased with tillage for all soil fractions ($p < 0.001$, all fractions,

457   Tukey's HSD), and N concentrations of the free and occluded microaggregate fractions were

458   greater than the macroaggregate fraction or bulk soil concentrations ($p < 0.001$, all comparisons,

459   Tukey's HSD). At Lancaster, N concentrations in the microaggregate fractions were greater than

460   the macroaggregate fraction and bulk soil concentrations for the no-tillage treatment ($p < 0.001$,

461   all comparisons, Tukey's HSD). The N concentration of the free microaggregate fraction

462   decreased by almost half with tillage ($p < 0.001$, Tukey's HSD).

24

463   The soil C:N ratio demonstrated significant effects of tillage treatment ($p < 0.001$) and soil

464   fraction ($p < 0.001$) at both sites (Table 1 and Fig. S4). At Arlington, C:N ratio was greater in no-

465   tillage compared to tillage ($p < 0.001$, Tukey's HSD), and greater in free and occluded

466   microaggregate fractions compared to the macroaggregate fraction or bulk soil ($p < 0.001$,

467   Tukey's HSD). At Lancaster, there was a significant interaction effect of tillage and soil fraction

468   ($p < 0.001$), with a significantly higher C:N ratio in the macroaggregate fraction with tillage.

469   Tillage decreased respiration (CO_2 evolution from sieved, field-moist bulk soil) by 50% at

470   Arlington ($p < 0.01$; Fig. S5) on a *per unit soil* basis, was though this difference was not

471   significant on a *per unit soil C* basis ($p = 0.106$). Tillage did not have a significant effect on

472   respiration at Lancaster. No-tillage plot samples averaged 23% soil moisture at both sites,

473   whereas tillage plots averaged 19–20% soil moisture; no adjustments to soil moisture were made

474   prior to respiration measurements.

475   Soil pH varied significantly by fraction ($p < 0.001$ for both sites; Fig. S6), with bulk soil >

476   occluded microaggregate ≥ free microaggregate fraction, and mean pH of 7.1, 6.8, 6.5

477   (Arlington) and 7.2, 6.9, and 6.9 (Lancaster), respectively. Lancaster pH data also demonstrated

478   a significant interaction between tillage treatment and soil fraction ($p < 0.001$); bulk soil pH in

479   no-tillage was higher than with tillage (7.4 vs. 7.1), whereas in the occluded microaggregate

480   fraction, soil pH in no-tillage was lower than with tillage (6.5 vs. 7.3), with no significant

481   difference in pH between treatments within the free microaggregate fraction (Fig. S6).

25

482 **Table 1**. Soil carbon and nitrogen concentrations and C:N ratio in soil fractions, by tillage
483 treatment, presented as mean (and standard error) with ANOVA *p* values. Means followed by the
484 same letter (within site) are not statistically different. Bulk soil = whole soil; Macroaggregate =
485 250–2000 µm; Free microaggregate = microaggregate fraction from bulk soil, 53–250 µm;
486 Occluded microagg. = microaggregate fraction occluded within macroaggregate fraction, 53–250
487 µm.

		Arlington		Lancaster	
	Soil fraction	No-tillage	Tillage	No-tillage	Tillage
Soil total carbon concentration (% C)	Bulk soil	3.56 (0.12) c	2.44 (0.06) d	2.37 (0.09) DE	1.79 (0.03) E
	Macroaggregate	3.95 (0.14) c	2.73 (0.09) d	3.14 (0.16) CDE	4.90 (0.58) BC
	Free microaggregate	5.72 (0.23) b	3.55 (0.15) c	6.38 (0.61) AB	3.67 (0.45) CD
	Occluded microagg.	6.67 (0.32) a	3.75 (0.09) c	6.94 (0.32) A	6.35 (0.55) AB
	Source of variation	*p* value		*p* value	
	Tillage treatment	< 0.001 ***		0.0713	
	Soil fraction	< 0.001 ***		< 0.001 ***	
	Tillage × fraction	< 0.001 ***		< 0.001 ***	
Soil total nitrogen concentration (% N)	Bulk soil	0.288 (0.007) c	0.212 (0.004) d	0.240 (0.010) BC	0.186 (0.003) C
	Macroaggregate	0.323 (0.009) c	0.237 (0.006) d	0.305 (0.015) B	0.313 (0.026) B
	Free microaggregate	0.420 (0.014) b	0.284 (0.009) c	0.478 (0.039) A	0.266 (0.032) BC
	Occluded microagg.	0.498 (0.018) a	0.307 (0.006) c	0.578 (0.022) A	0.476 (0.036) A
	Source of variation	*p* value		*p* value	
	Tillage treatment	< 0.001 ***		< 0.001 ***	
	Soil fraction	< 0.001 ***		< 0.001 ***	
	Tillage × fraction	< 0.001 ***		< 0.001 ***	
C:N ratio	Bulk soil	12.4 (0.15) b	11.5 (0.12) c	9.9 (0.32) D	9.7 (0.22) D
	Macroaggregate	12.2 (0.16) b	11.5 (0.13) c	10.5 (0.68) CD	15.3 (0.62) A
	Free microaggregate	13.6 (0.18) a	12.5 (0.16) b	13.3 (0.34) B	13.7 (0.25) AB
	Occluded microagg.	13.3 (0.19) a	12.2 (0.13) b	12.0 (0.21) BC	13.3 (0.33) B
	Source of variation	*p* value		*p* value	
	Tillage treatment	< 0.001 ***		< 0.001 ***	
	Soil fraction	< 0.001 ***		< 0.001 ***	
	Tillage × fraction	0.428		< 0.001 ***	

26

488   *3.3 Tillage affected bacterial community composition, but not richness*

489   Bacterial community composition was significantly affected by tillage treatment at both sites

490   (Fig. 4; $R^2 = 0.30$ and $p < 0.001$ at Arlington, $R^2 = 0.22$ and $p < 0.001$ at Lancaster;

491   PERMANOVA). The homogeneity of variance test (BETADISPER) was also significant for

492   tillage treatment at Arlington and Lancaster ($p < 0.001$, $p < 0.05$, respectively), which indicates

493   that the assumptions of the PERMANOVA were not met due to differences in sample dispersion.

494   Tillage decreased dispersion of community composition by 14% and 6% relative to the no-tillage

495   treatment at Arlington and Lancaster, respectively, as quantified by between-plot mean distance

496   to spatial median (Fig. 5A and D; $p < 0.001$ for Arlington and $p < 0.01$ for Lancaster). This

497   trend, which indicates higher dissimilarity of samples within the no-tillage treatment, was also

498   apparent at the plot scale, where tillage decreased sample dispersion within plots by 13% and 5%

499   relative to no-tillage at Arlington and Lancaster, respectively (Fig. 5B and E; $p < 0.001$ for

500   Arlington and $p < 0.05$ for Lancaster). The dispersion of the free microaggregate and occluded

501   microaggregate communities within each soil core did not significantly differ between tillage

502   treatments, though there was a trend towards decreased dispersion with tillage at Lancaster ($p <$

503   0.1; Fig. 5C and F). There were no significant differences in richness estimates (Fig. S7) or

504   Faith's PD (Fig. S8) attributable to tillage treatments at either site.

505   *3.4 Community composition differed slightly between microaggregate fractions*

506   There was a significant effect of soil fraction on bacterial community composition at both sites

507   ($R^2 = 0.03$ and $p < 0.05$ for Arlington and $R^2 = 0.09$ and $p < 0.001$ for Lancaster,

27

508 PERMANOVA; Fig. 4). Pairwise testing demonstrated significant differences between bulk soil

509 and the free microaggregate fraction, and between bulk soil and the occluded microaggregate

510 fraction at Lancaster only ($p < 0.01$), whereas pairwise testing amongst soil fractions was not

511 significant at Arlington. Sample dispersion was homogeneous (i.e., beta diversity was similar)

512 across soil fractions at both treatment and plot scales at both sites (Fig. 5). There was no

513 interaction effect of tillage treatment × soil fraction on community composition at either site.

514 Richness estimates demonstrated a significant effect of soil fraction ($p < 0.05$, Fig. S7) at

515 Lancaster only; the richness estimate for the occluded microaggregate fraction was 8% lower

516 than that of the bulk soil ($p < 0.05$, Tukey's HSD). Faith's PD was also affected by fraction ($p <$

517 0.05, Arlington, and $p < 0.001$, Lancaster; Fig. S8) by which the occluded microaggregate

518 fraction was significantly lower than that of bulk soil at both sites ($p < 0.05$ and $p < 0.001$,

519 respectively, Tukey's HSD), and the free microaggregate fraction was also lower than bulk soil

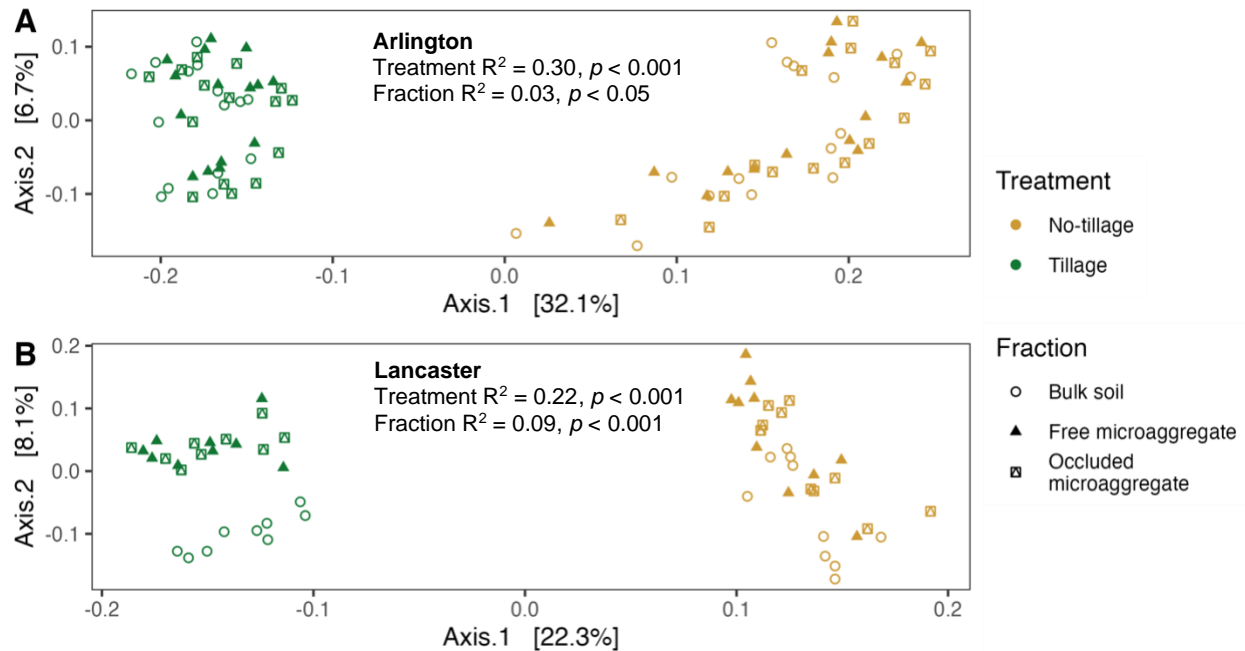520 at Lancaster ($p < 0.01$, Tukey's HSD).

28

521

Figure 4. Principal coordinates analysis of Bray-Curtis dissimilarities of Hellinger-transformed community relative abundances, by tillage treatment for Arlington, WI (**A**), and Lancaster, WI (**B**). Each point represents the community of one sample-fraction. Soil fractions are as follows: Bulk soil = whole soil; Free microaggregate = microaggregate fraction from bulk soil, 53–250 µm; Occluded microaggregate = microaggregate fraction occluded within macroaggregate fraction, 53–250 µm. Displayed statistics are from PERMANOVA.

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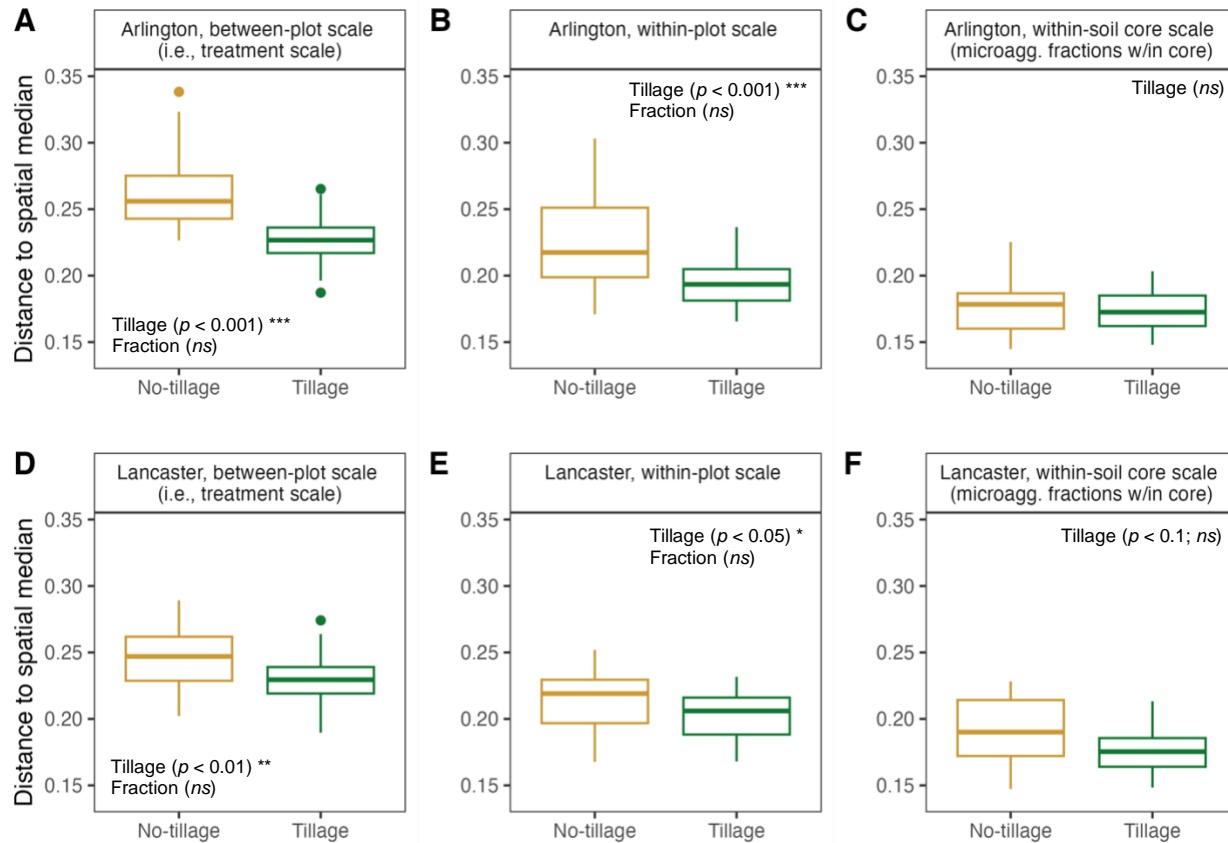
Figure 5. Dispersion of community compositional data as Bray-Curtis dissimilarities, represented as distance to spatial median at the between-plot scale (i.e., treatment scale; **A** and **D**); within-plot scale (**B** and **E**); and soil core scale (free vs. occluded microaggregate fraction samples within a soil core; **C** and **F**) at Arlington, WI (**A**, **B**, and **C**) and Lancaster, WI (**D**, **E**, and **F**). Data presented in A, B, D, and E represent bulk soil and both microaggregate fractions together.

3.5 Weighted mean predicted 16S rRNA gene copy number increased with tillage

At Arlington, there was small but statistically significant 7% increase in the weighted mean predicted 16S rRNA gene copy number with tillage ($p < 0.001$; Fig. 6). Fraction was also significant ($p < 0.001$), and there was a significant interaction effect of tillage and fraction ($p < 0.05$). The weighted mean predicted 16S gene copy number was lower in the occluded

30

541   microaggregate fraction relative to the bulk soil or free microaggregate fraction in the tillage

542   treatment, whereas weighted mean predicted 16S gene copy number was similar across fractions

543   of the no-tillage treatment. At Lancaster, there was a significant 10% increase in the weighted

544   mean predicted 16S gene copy number with tillage ($p < 0.001$), and no significant effect of

545   fraction or interaction effect.

546

547   **Figure 6**. Weighted mean predicted 16S rRNA gene copy number. These data represent taxa for
548   which a gene copy number was available in the rrnDB (Stoddard et al., 2015). Bulk soil = whole
549   soil; Free micro. = microaggregate fraction from bulk soil, 53–250 µm; Occluded micro. =
550   microaggregate fraction occluded within macroaggregate fraction, 53–250 µm. Boxplots with the
551   same letter (within site) are not statistically different.

31

552   *3.6 Influence of homogenizing dispersal increases with tillage*

553   Using the full community comparison approach, community assembly was dominated by

554   homogeneous selection, with > 95% of pairwise comparisons across each set of samples

555   (representing site, tillage treatment, and fraction combinations), when null models were

556   comprised of site-wide data. This outcome is consistent with the intensity and uniformity of

557   management in monocrop systems (Figuerola et al., 2015).

558   We next performed the community-wide approach separately for each set of samples (site ×

559   treatment × fraction combination) in order to further scrutinize community assembly processes.

560   This approach, critically, employed narrower null models that were more specific to the pairwise

561   comparisons. The results from that analysis demonstrate evidence for homogenizing dispersal

562   amongst other processes, while still emphasizing the importance of homogeneous selection in

563   these systems (see Fig. S9, for full results using treatment-specific null models).

564   Going forward, and in the Discussion section, we will focus on the results from the OTU

565   binning-based approach (Ning et al., 2020), which indicated that homogeneous selection had a

566   ~14% relative influence across treatments and fractions, for both within-plot and between-plot

567   comparisons at Arlington (Fig. 7A and B), with the between-plot comparisons demonstrating a

568   significant decrease in homogeneous selection under tillage relative to no-tillage ($p < 0.05$). The

569   influence of homogenizing dispersal significantly increased with tillage ($p < 0.001$), from 25% to

570   46% in bulk soil for within-plot comparison; and from 12% to 28% for between-plot

32

571 comparisons. There was also a large proportion of undominated comparisons— 30–60% at the

572 within-plot scale and 50–70% at the between-plot scale.

573 When using the OTU binning-based approach at Lancaster (Fig. 7C and D), the within-plot

574 comparisons demonstrated similar trends as those at Arlington regarding influences of

575 homogeneous selection (~15%), homogenizing dispersal (~40%), and undominated (~45%).

576 Unlike Arlington, there was no significant effect of tillage on the influence of homogenizing

577 dispersal at the within-plot scale, whereas homogenizing selection significantly increased from

578 10% to 15% of relative abundance in the bulk soil ($p < 0.05$). In the between-plot comparisons,

579 the influence of homogenizing dispersal significantly increased with tillage in the bulk soil and

580 occluded microaggregate fraction ($p < 0.001$), whereas homogeneous selection experienced a

581 small decrease with tillage in the free microaggregate fraction, and a small increase with tillage

582 in the occluded microaggregate fraction ($p < 0.05$, and $p < 0.01$, respectively). For the between-

583 plot comparisons, most were undominated— 60–80%.
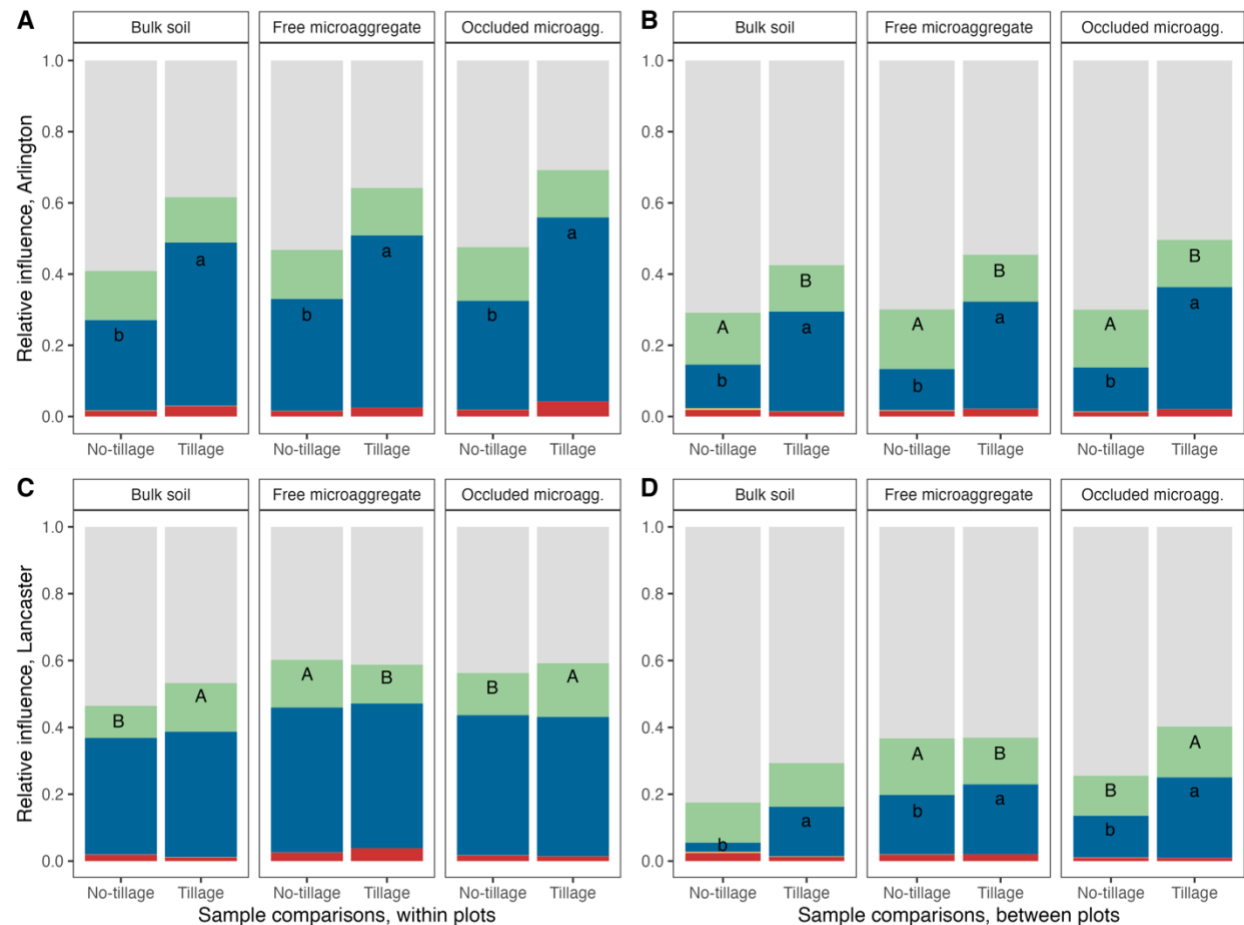
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Figure 7. The dominant community assembly processes, by tillage treatment, within bulk soil, free microaggregate, and occluded microaggregate fractions at Arlington, WI (**A** and **B**); and Lancaster, WI (**C** and **D**). Sample comparisons were made within-plot (**A** and **C**) or between-plot (**B** and **D**). Community assembly processes were assigned within phylogenetically related bins of OTUs for pairwise comparisons of samples using a null modeling approach, and weighted by the relative abundance of OTUs in that bin to comprise the full community (Ning et al., 2020). As detailed in the text, first the influence of selection was determined using the β-mean nearest taxon distance, and then the influence of dispersal was determined using the modified Raup-Crick metric based on Bray-Curtis dissimilarity. For homogeneous selection and homogenizing dispersal (the processes with > 5% influence), different letters signify a statistically significant difference in the influence of that process due to tillage (within site and fraction).

34

597 3.7 Taxonomic differences attributable to tillage

598 The most common phyla across the datasets were *Actinobacteria*, *Acidobacteria*, and

599 *Proteobacteria*, which together comprised about 60% of all reads at each site in both tillage

600 treatments. At Arlington, there was generally a consistent phylum-level response across the bulk

601 soil, free microaggregate, and occluded microaggregate fraction by which tillage resulted in

602 significant increases in relative abundances of *Actinobacteria*, *Armatimonadota*, *Chloroflexi*,

603 *Cyanobacteria*, *Firmicutes*, *Gemmatimonadetes*, and *Methylomirabilota*, and significant

604 decreased relative abundances of *Acidobacteria*, *Myxococcota*, *Proteobacteria*, and

605 *Verrucomicrobia* (see Fig. S10 for relative abundances and *p* values). At Lancaster, tillage

606 resulted in significant increases in relative abundances of *Chloroflexi*, *Cyanobacteria*, and

607 *Gemmatimonadetes*, and significant decreases in relative abundances of *Crenarchaeota* and

608 *Verrucomicrobia* (see Fig. S11 for relative abundances and *p* values).

609 We also identified key taxa associated with no-tillage and tillage treatments, based on differential

610 abundance. Across both sites, we identified a total of 1658 taxa that were enriched with tillage

611 (relative to no-tillage), and 1602 taxa that were enriched in no-tillage (relative to the tillage

612 treatment). See Supplementary Table S2 for a complete list of enriched taxa, with coefficients of

613 differential abundance (μ) and sequences. We focused on the taxa with the biggest responses (μ

614 > 1.0), and only considered enriched taxa with mean relative abundances greater than 0.001

615 (0.1%), which resulted in 15 and 9 focal taxa enriched with tillage and no-tillage, respectively, at

616 Arlington, and 9 and 4 focal taxa enriched with tillage and no-tillage, respectively, at Lancaster

617 (Figs. S12 and S13). Though some taxa were unique responders within a soil fraction, there were

35

618   numerous taxa that were enriched across bulk soil, free microaggregate, and occluded

619   microaggregate fractions.

620   *3.8 Taxonomic differences between microaggregate fractions*

621   We also identified taxa that were enriched in the microaggregate fractions using differential

622   abundance. We identified a total of 382 taxa across both sites that were enriched in the free or

623   occluded microaggregate fractions relative to the bulk soil. See Supplementary Table S3 for a

624   complete list of enriched taxa, with coefficients of differential abundance (μ) and sequences.

625   Narrowing our focus on taxa with the biggest responses ($\mu > 1.0$), and not overly rare (mean

626   relative abundances greater than 0.001 [0.1%]), there were 8 and 10 taxa enriched in the free

627   microaggregate and occluded microaggregate fractions at Lancaster, respectively, most of which

628   were in the tillage treatment (Fig. S14), and no taxa that fit those parameters at Arlington. There

629   were several *Chloroflexi* OTUs representing the class *Anaerolineae*, and several *Cyanobacteria*

630   OTUs that were relatively enriched in the occluded microaggregate fraction. We did not assess

631   taxa that were depleted in microaggregate communities relative to the bulk soil because the

632   former is inherently a subset of the latter.

633   **4. Discussion**

634   We examined the effects of tillage on soil bacterial community composition and assembly,

635   specifically in the free and occluded microaggregate fractions, and will discuss these findings

636   with respect to soil carbon protection as modulated through changes to soil aggregation. Findings

36

637   generally supported our hypotheses that tillage would homogenize bacterial communities, with

638   community assembly driven by homogenizing dispersal, but only weakly supported the

639   hypothesized distinctions between the free and occluded microaggregate communities. Overall,

640   we found decreased aggregation, soil C, and soil N with tillage (Figs. 2 and 3, Table 1), which

641   agrees with previous work (Frey et al., 1999; Six et al., 1999; Al-Kaisi et al., 2014; Zheng et al.,

642   2018). This supports the paradigm that tillage increases macroaggregate turnover, thus derailing

643   occluded microaggregate formation, and decreasing soil C content through enhanced

644   decomposition and weakened long-term protection (Six et al., 1999; King et al., 2019).

645   *4.1 Tillage decreased soil aggregation and soil carbon*

646   Our work provides further evidence supporting the relationship between soil aggregation and

647   SOC content, while reiterating that tillage reduces aggregation and SOC in surface soil. We

648   found that 90% of the increase in SOC under no-tillage relative to tillage was in aggregate

649   fractions, with the majority ($>75\%$) of this increase specifically in the microaggregate fractions

650   (Figs. 3 and S2). At Arlington, most of the increase in C under no-tillage was attributed to the

651   occluded microaggregate fraction, which reflects previous work (Denef et al., 2004; Six and

652   Paustian, 2014). However, at Lancaster, most of the increase in C was in the free microaggregate

653   fraction, which could reflect post-season sample timing with respect to macroaggregate seasonal

654   dynamics. As roots and hyphae die following crop plant senescence, macroaggregates rapidly

655   destabilize, liberating previously occluded microaggregates into the free microaggregate pool

656   (Perfect et al., 1990; Oades and Waters, 1991), as detailed below in Section 4.6.

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657   As with the difference in SOC accumulation between the microaggregate fractions, the two sites

658   continue to tell somewhat different stories of aggregation and SOC distribution. Arlington

659   exemplifies the "cultivation loop" (sensu Six et al., 1999), by which tillage stimulates

660   decomposition and macroaggregate turnover, thus precluding SOM enrichment and resulting in

661   older, C-depleted microaggregate fractions (Table 1). Simultaneously, under no-tillage,

662   undisturbed macroaggregates foster development of new occluded microaggregates, as indicated

663   by higher C concentrations and wider C:N ratios in the microaggregate fractions (Table 1).

664   On the contrary, at Lancaster, the macroaggregate fraction *under tillage* had a high C

665   concentration, wide C:N ratio, and increased proportion of macroaggregate-occluded POM

666   relative to no-tillage (Table 1, Figs. 2 and S4), indicative of largely undecomposed residue.

667   Substantial residue at Lancaster is a testament to the continuous corn rotation; the residue from

668   the previous crop (corn) was potentially double that of Arlington (where the previous crop was

669   soybean), and of a higher C:N ratio (Ordóñez et al., 2020). Tillage breaks up crop residue,

670   bringing it into direct contact with mineral particles and soil microbiota to nucleate new

671   macroaggregates, which could have enhanced C and POM concentration in the macroaggregate

672   fraction, despite the overall tillage-driven decrease in proportion of macroaggregates. Though

673   counter to how we typically characterize macroaggregates under tillage (e.g., low soil C and

674   POM concentrations), this evidence for less processed SOM in the macroaggregate fraction

675   supports the overall narrative of a shorter mean macroaggregate lifespan under higher turnover

676   with tillage (Elliott, 1986). In contrast, the corn-soy rotation at Arlington resulted in more

677   straightforward soil C trends (e.g., C concentration in no-tillage > tillage; C concentration in

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678 microaggregates > macroaggregates and bulk soil; Table 1 and Fig S3). It would be relevant to

679 repeat these measurements shortly after a fall tillage event to assess if tillage accelerates the

680 decomposition of occluded POM and decreases SOC in the macroaggregate fraction, particularly

681 in a system such as Lancaster where these metrics were high just prior to a fall tillage event.

682 The overall weak aggregation at Lancaster (Fig. 2), with less than 15% of soil in aggregates,

683 lends support to a recently proposed paradigm shift that suggests soils under tillage may not be a

684 relevant application of the physicogenic aggregate, but instead represent engineered, loosely

685 arranged soil fragments that largely lack natural biopore networks (Or et al., 2021).

686 *4.2 Tillage homogenized bacterial communities via dispersal*

687 Tillage had a significant effect on bacterial community composition at both sites (Fig. 4), as

688 observed by others (Srour et al., 2020; Bhattacharyya et al., 2021), which resulted in more

689 homogeneous communities at both within-plot and between-plot scales, confirming hypothesis

690 H3 (Figs. 4 and 5). At the within-plot scale, decreased compositional differences with tillage

691 (Fig. 5) may be driven by homogenizing dispersal at Arlington (Fig. 7A), partially confirming

692 hypothesis H1. At Lancaster, the relatively smaller effect of tillage on community composition

693 (Fig. 4) and community dispersion (Fig. 5) may be attributable to the lack of increased influence

694 of homogenizing dispersal, and only small increases in homogeneous selection (Fig. 7).

695 At the between-plot scale, we might have expected to see increased influences of homogeneous

696 selection and perhaps dispersal limitation with tillage, because management of these plots is

697 similar, yet they are spatially separated. However, like findings at the within-plot scale, tillage

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698   also increased the influence of homogenizing dispersal at the between-plot scale at both sites.

699   Therefore, another tillage-driven mechanism increased the compositional similarity amongst

700   these spatially distinct plots, barring direct organismal dispersal, without increasing phylogenetic

701   similarity (which would have increased homogeneous selection). Because tillage systematically

702   preserves more stable, potentially older microaggregates, we may be observing founder effects

703   that manifest as taxonomic similarity between plots in a field (Rillig et al., 2017). Despite some

704   significant shifts in selection and dispersal, community assembly is largely undominated at the

705   between-plot scale, demonstrating a high level of stochasticity and potential for ecological drift.

706   Despite homogenizing community composition, tillage did not have a significant effect on

707   bacterial richness (Fig. S7). Previous work has found tillage to have contrasting effects on

708   richness with both neutral and negative findings (Constancias et al., 2013; Smith et al., 2016).

709   The tillage practices used at these sites (fall chisel plow plus spring cultivation) are perhaps too

710   infrequent or mild to affect richness estimates, as previous work has found that richness

711   significantly decreased only in soil disturbed at least biweekly (West and Whitman, 2022). It is

712   also possible that sequencing efforts poorly represented the relative richness of these systems and

713   soil fractions (Bach et al., 2018), though the *betta* model that we used for richness estimation is

714   specifically designed to account for unobserved taxa (Willis et al., 2017).

715   We did not observe strong influences of dispersal limitation or variable selection under no-

716   tillage, as was hypothesized (H1). This may be attributed to the generally homogeneous soil

717   environment that is characteristic of intensively managed monocrop systems, regardless of tillage

718   practices.

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719 *4.3 Tillage favors potential for fast growth*

720 Increased weighted mean predicted 16S gene copy number under tillage (Fig. 6) was also noted

721 in a recent global metanalysis (Wilhelm et al., 2023), and is consistent with the idea that pulses

722 of resources (e.g., C liberation or litter incorporation via tillage) select for competitors with fast

723 growth potential in soil bacteria (Schmidt et al., 2018). These studies also found that larger mean

724 estimated genome size correlated with lower soil health ratings and tillage, indicating a need for

725 higher metabolic and regulatory capabilities under environmental instability (Schmidt et al.,

726 2018; Wilhelm et al., 2023). However, the fairly uniform effect on weighted mean predicted 16S

727 gene copy number across soil fractions (Fig. 6), which do differ in chemical composition (Table

728 1), indicate that physical disturbance may also influence fitness as it relates to other aspects of

729 life history strategy, such as chemical signaling, community goods, or secondary metabolites.

730 For example, this could point to a scenario by which oligotrophic organisms, which invest

731 heavily in extracellular enzymes, are at a disadvantage when proximity to these metabolites is

732 disrupted under physical disturbance (Junkins et al., 2022), whereas copiotrophic generalists,

733 which are less reliant on these proximity-based life strategies, might be less hindered by physical

734 disturbances.

735 We might have expected tillage-driven increases in weighted mean predicted 16S gene copy

736 number (Fig. 6) to be accompanied by increases in soil respiration (on a per gram C basis), due

737 to lower carbon use efficiency associated with increased 16S gene copy number (Roller et al.,

738 2016). However, the similar rate of C respiration across tillage treatments at both sites (Fig. S5B)

739 implies that the no-tillage and tillage communities processed C similarly, and/or the small

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740   increase in weighted mean predicted 16S gene copy number, although statistically significant,

741   was not biologically relevant for C mineralization. Soil respiration—on a per gram soil basis—

742   did decrease under tillage at Arlington (Fig. S5A), as a function of decreased bulk soil C

743   concentration (Table 1). The no-tillage samples averaged slightly higher soil moisture content,

744   which also may help explain the slight increases in respiration. Though our measurements of

745   CO_2 evolution from sieved soil do not accurately represent respiration of an intact soil and pore

746   network (Vogel et al., 2022), this analysis still indicates that the C mineralization potential of

747   these soil communities may not be limited by tillage-driven compositional changes.

748   *4.4 Taxonomic differences due to tillage*

749   Some broad, phylum-level compositional differences follow archetypical expectations under

750   tillage: *Firmicutes*, generally thought to include many fast-growing copiotrophs, increased in

751   relative abundance with tillage, as was previously observed (Schmidt et al., 2018), whereas

752   *Verrucomicrobia* include numerous oligotrophic taxa (Bergmann et al., 2011), and decreased

753   under tillage (Figs. S10 and S11). *Firmicutes* had higher mean relative abundances in the bulk

754   soil as compared to microaggregate fractions. There were several taxa that responded to tillage

755   representing the genus *Nocardioides* (*Actinobacteria*). A responder to frequent soil disturbance

756   (West and Whitman, 2022), *Nocardioides* has been negatively correlated with soil health in a

757   global metanalysis (Wilhelm et al., 2023). We also found relative enrichment of *Sphingomonas*

758   and *Geodermatophilus* under tillage, both of which have been identified as key tillage responders

759   (Wilhelm et al., 2023). Under no-tillage, we also found enrichment of the genus *Gaiella*,

760   (*Actinobacteria),* which was one of several identified bioindicators of high biological soil health

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761   ratings (Wilhelm et al., 2023). We also found enrichment of anaerobic taxa (e.g., *Anaerolineae*)

762   (Yamada and Sekiguchi, 2020) in microaggregates (Fig. S14), which have anoxic microsites

763   (Sexstone et al., 1985).

764   The enrichment of *Cyanobacteria* with tillage (Fig. S12) reflects how repeated disturbance to the

765   soil surface suspends biocrust communities in an early successional stage (Belnap and Eldridge,

766   2001). Because *Cyanobacteria* are particularly enriched in microaggregate fractions, where we

767   would not expect photosynthetic organisms to survive or thrive, this enrichment may reflect the

768   presence of relic DNA (Carini et al., 2016) or dormant organisms (Lennon and Jones, 2011),

769   integrated from biocrusts into the soil matrix via tillage, and subsequently protected within

770   microaggregates. The specific *Cyanobacteria* taxa enriched in our study (*Microcoleus* PCC-7113

771   and *Tychonema* CCAP 1459-11BA) were both previously found in soil under organic

772   management, perhaps also due to frequent disturbance (Santoni et al., 2022).

773   *4.5 Evidence for fluidity between the free and occluded microaggregate fractions*

774   Within tillage and no-tillage treatments, the bacterial compositions and community assembly

775   patterns of the free and occluded microaggregate fractions are fairy indistinct from each other (*p*

776   > 0.05, PERMANOVA; Figs. 4 and 7), contrary to hypothesis H2. This indicates that these

777   operationally defined fractions likely have substantial overlap, potentially attributable to

778   wholesale shifts of occluded microaggregates to the free fraction at the end of the temperate

779   annual cropping season, when macroaggregates rapidly degrade with root senescence (Oades and

780   Waters, 1991), as detailed in Section 4.6. Further, the community compositional dispersion of

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781   free and occluded microaggregate fractions within each soil core was unaffected by tillage

782   treatment (Fig. 5C and F), which indicates that the fluidity between the free and occluded

783   microaggregate fractions may not be particularly responsive to disturbance. Since we did not

784   identify bacterial drivers that explain enhanced SOC persistence in the occluded microaggregate

785   fraction, future work could instead focus on the physical and chemical drivers of C storage and

786   persistence in microaggregate fractions (Bailey et al., 2019; Kravchenko et al., 2019), or fungal

787   community drivers (Lehmann and Rillig, 2015).

788   Overall, microaggregate communities were less phylogenetically diverse than bulk soil at both

789   sites (Fig. S8), implying that the microaggregate microenvironment selects for a community

790   adapted to that environment. This is consistent with the PERMANOVA results that demonstrated

791   significant compositional differences between the bulk and microaggregate fractions, though

792   these differences were very small ($R^2 = 0.09$ at Lancaster and $R^2 = 0.03$ at Arlington).

793   *4.6 Factors that may have moderated the measured impact of tillage*

794   We will briefly consider several nuanced factors in this study. The tillage treatment at both sites

795   included a fall chisel plowing, which is sometimes considered a reduced or even conservation

796   tillage approach because it is shallower and more moderate compared to moldboard or disk

797   plowing, and does not invert the soil (e.g., Zuber and Villamil, 2016). Previous work has found

798   chisel plow tillage to have the same effect as no-tillage on aggregate stability and microbial

799   biomass (Al-Kaisi et al., 2014; Zuber and Villamil, 2016). Several other factors may obscure or

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800   diminish the relative impacts of tillage in this study, including crop-related seasonal

801   macroaggregate dynamics, wet-dry or freeze-thaw cycles, and clay mineralogy.

802   As noted above, macroaggregates rapidly destabilize following crop senescence—which begins

803   four to eight weeks prior to grain harvest—thus potentially diminishing tillage-driven differences

804   in soil aggregation measured post-harvest (Fig. 2) and liberating occluded microaggregates into

805   the free microaggregate pool (Perfect et al., 1990; Oades and Waters, 1991). Similar aggregation

806   patterns across tillage treatments were previously observed by Huang et al. (2010), in which

807   sampling occurred months after corn harvest. Tillage differences may be further diminished by

808   the physically disruptive effects of freeze-thaw and wet-dry cycles at the soil surface, which

809   would impact aggregate stability of otherwise undisturbed soil under no-tillage (LeGuillou et al.,

810   2012; Bailey et al., 2019). These effects are likely variable in tillage vs. no-tillage treatments,

811   given differences in protective surface residues (Cruse et al., 2001).

812   Another contributing factor to differences in aggregation and C concentration between sites may

813   be driven by different mineralogy (Denef et al., 2004). Mollisols, such as the soil at Arlington,

814   are generally recognized to promote organo-mineral complexes. The clay mineralogy of the

815   Plano silt loam at Arlington is interstratified smectite-illite (Liu et al., 1997); the high specific

816   surface area of illite may promote SOC retention, and the expansible nature of smectite may

817   physically protect organic matter (Sarkar et al., 2018). The Fayette silt loam at Lancaster

818   (Alfisol) has been mineralogically characterized as predominantly montmorillonite clay

819   minerals, which is also an expansible layer phyllosilicate (Caldwell et al., 1955), though the low

820   activity (1:1) clay may be influential, as evidenced by the very low level of aggregation even

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821   under no-tillage (Fig. 2), as was noted for a mixed-mineralogy clay in Six et al. (2000). We find

822   support for these literature-based suppositions in the lower concentrations of base cations and

823   lower overall cation exchange capacity measured at Lancaster (Table S1). These mineralogical

824   differences could explain why Arlington has higher proportions of aggregated soil and higher

825   SOC and SOM concentrations than Lancaster, despite similar texture (silt loam) and relatively

826   similar corn-based cropping systems. Yet another consideration might be the lower sand content

827   across the tillage treatment at Lancaster, with 8% sand in contrast to 18% in the no-tillage

828   treatment, potentially contributing to the relatively high C concentrations in the macroaggregate

829   and occluded microaggregate fractions under tillage, though these sand measurements are

830   somewhat anecdotal because they represent composite samples (n = 1 per treatment). Sand

831   content correction in aggregate fractions could have altered our fraction proportions and soil C

832   concentrations, but this adjustment was not performed because our primary objective for

833   aggregate fractionation was to isolate the microaggregate fractions for bacterial community

834   assessment.

835   **5. Conclusions**

836   For the first time, this study demonstrates that tillage homogenizes the soil microbial community

837   through homogenizing dispersal, while supporting previous conclusions that tillage disrupts

838   aggregation and decreases carbon at the soil surface. Counter to our hypothesis, the bacterial

839   communities of the free and occluded microaggregate fractions are highly similar, indicating that

840   microaggregates may readily shift between these operationally defined soil fractions, rather than

841   adhering to purported categories. Tillage may accentuate seasonal changes that characterize

842   temperate annual cropping systems (e.g., crop senescence, freeze-thaw, and wet-dry cycles),

843   which together challenge the strength and longevity of macroaggregates in which occluded

844   microaggregates form and soil carbon is protected. Thus, while our findings reiterate the

845   importance of the occluded microaggregate fraction for soil C persistence, we also suggest that

846   this occluded microaggregate C is subject to an increased rate of turnover upon convergence with

847   the free microaggregate fraction when macroaggregate stability degrades. Conceptually, this

848   underscores how aggregate microhabitats develop and devolve throughout the soil matrix, in

849   concert with microbial activity, forming isolated hotspots driven by resource availability in the

850   patchy soil environment.

851   **Supplementary Information**

852   Supplementary Information can be found online.

853   **Acknowledgements**

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873   *Conflict of Interest*. None declared.

874   *Author contributions*. JW and TW conceived of the project. JL has maintained the tillage

875   experiment in Arlington, WI since 1994. JW collected soil samples, conducted lab work,

876   analyzed the data, and drafted the manuscript. All authors reviewed and edited the manuscript.

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