# Effects of land-use change and wetting frequency on soil carbon and nitrogen dynamics and the microbial community.

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**Abstract**

Afforestation of agricultural land is proposed as a way to reduce the concentration of atmospheric $CO_2$ and potentially mitigate climate change. Given the future climates in many regions are predicted to be drier, we need to understand how C sequestration is affected by prolonged dry periods. We present results of a study characterising C and N dynamics and the microbial community composition in soil collected from two tree plantings and their adjacent pastures under two different wetting frequency treatments. While the concentration of soil C was similar in pasture and tree planting soils, heterotrophic respiration was significantly lower in soil from pastures than tree plantings. Although there was little difference in the composition of the soil microbial community among any of the soils or treatments, differences in N cycling could indicate a difference in microbial activity, which may explain the differences in heterotrophic respiration between pasture and tree planting. Soils from pastures and tree plantings responded similarly to a reduction in wetting frequency, with a decrease in microbial biomass (measured as total PLFA), and a similar reduction in heterotrophic respiration from the soil. Additional field-based rainfall exclusion experiments are required to determine the overall effect of afforestation on soil microbial community change and C and N cycling under a drying climate.

48 **1. Introduction**

49 Afforestation of agricultural land is an important way of reducing levels of atmospheric $CO_2$
50 to mitigate climate change (IPCC, 2007). Globally forests store large amounts of carbon (C)
51 in their soil, biomass and litter and deadwood (373, 383 and 116 Pg, respectively, Pan *et al.*,
52 2011). Mixed-species tree plantings are increasingly established because they have
53 additional ecological benefits over single-species tree plantations, such as habitat
54 restoration and increasing biodiversity (Brooks and Lake, 2007; Munro *et al.*, 2009). The
55 mechanisms that underpin soil C sequestration after land-use change are largely unknown
56 because many of the drivers and processes that affect sequestration are slow (up to
57 decades or centuries) and/or difficult to study. Soil moisture dynamics are a key factor
58 affecting the sequestration of C in soils (e.g., Austin *et al.*, 2004). Precipitation patterns are
59 predicted to change to prolonged dry periods under future climates (IPCC, 2007), affecting
60 the wetting cycles of soil. In turn, this will alter C and N cycling in the soil (e.g., Borken and
61 Matzner, 2009) and may affect the potential of afforestation to increase C sequestration in
62 soils.

63 Soil C cycling is affected predominantly by moisture (Borken and Matzner, 2009),
64 temperature (e.g. Frey *et al.*, 2008), and the quantity and quality (e.g. C:N ratio, lignin
65 content) of organic matter (e.g. Bending *et al.*, 2002), through their impact on the activity
66 and composition of the soil microbial community. Wetting regimes (frequency and amount
67 of rainfall) strongly affect the activity and mortality of the microbial population (Borken and
68 Matzner, 2009). When the soil dries, microbes produce osmolytes in order to reduce their
69 internal water potential and prevent dehydration (Halverson *et al.*, 2000). Soil microbes that
70 are less adapted to water stress may die over the dry period. When the soil is rewetted, the

71 surviving microbes rapidly excrete the osmolytes, before water enters the cells by osmosis
72 and causes the microbial cells to rupture (Fierer and Schimel, 2003). A pulse in $CO_2$ emission
73 is caused by rapid mineralization of the excreted osmolytes in the soil, as well as
74 mineralization of microbial biomass from microbes that died during the dry period or re-
75 wetting of the soil (Steenwerth *et al.*, 2005; Sponseller, 2007). The magnitude of this $CO_2$
76 pulse is dependent on the specific microbial community (e.g. bacterial vs fungal dominated)
77 and how it responds to wetting and drying cycles (Aanderud and Lennon, 2011). In addition,
78 swelling and shrinking of the soil may breakdown aggregates, which exposes organic
79 material that was previously protected from microbial decomposition or chemical oxidation
80 (van Gestel *et al.*, 1991), which may contribute to the observed pulse of $CO_2$ after re-wetting
81 of soil (Fierer and Schimel, 2003).

82 Afforestation of agricultural land can alter biomass, activity and composition of soil
83 microbes (e.g., Singh *et al.*, 2007; Carson *et al.*, 2010). For example, the soil microbial
84 communities of afforested soils and mature forests typically contain larger fungal biomass
85 compared with pastures. This difference is attributed to fungi decomposing woody material
86 quicker than bacteria (e.g., Fierer *et al.*, 2009; Macdonald *et al.*, 2009) and forest
87 ecosystems generally experience lower levels of soil disturbance, favouring fungi over
88 bacteria (Six *et al.*, 2006). Furthermore, fungi are thought to sequester more C than
89 bacteria, due to the higher C use efficiency of fungi (Bailey *et al.*, 2002b; Jastrow *et al.*,
90 2007) and are thought to be less sensitive to drought stress than bacteria (Schimel *et al.*,
91 2007; Blazewicz *et al.*, 2014). Whether a drier climate leads to less C sequestration after
92 afforestation of pastures has yet to be determined.

93 There is evidence that C sequestration after afforestation is higher in low rainfall
94 areas (Guo and Gifford, 2002; Jackson *et al.*, 2002). A doubling of the length of a dry-period

95 was found to reduce soil respiration flux after re-wetting by approximately 17% (Fay *et al.*,
96 2000). Because of the changes in quantity and quality of organic matter inputs into the soil
97 after afforestation of pastures, and consequently changes in soil microbial communities, soil
98 from pastures and adjacent tree plantings may respond differently to wetting and drying
99 cycles (Gordon *et al.*, 2008; Zhao *et al.*, 2010). Research on soil responses to prolonged dry
100 periods, specifically comparing afforestation, secondary forest or primary forest soils, with
101 adjacent agricultural land are rare (e.g., Fierer and Schimel, 2003).

102 In order to assess the potential of afforestation to sequester C under drying
103 climates, which are predicted for many regions of the world, including southern Australia,
104 we need to understand how this could alter the relative C sequestration potential of pasture
105 and tree plantings in the future. Here we present results of an incubation study that aimed
106 to answer the following questions:

107 1) How does wetting frequency affect soil C and N concentration under a reduced
108 wetting frequency?
109 2) Does afforestation of pastures change the soil microbial community composition,
110 biomass, and activity (respiration)?

111 Afforestation of pasture was expected to increase fungal biomass. In turn, we
112 expected lower heterotrophic respiration in soils from tree plantings than the pastures due
113 to the larger mass of woody inputs and the higher C use efficiency of fungi than bacteria
114 (Bailey *et al.*, 2002b; Jastrow *et al.*, 2007). Finally, the lower sensitivity of fungi to wetting
115 and drying cycles than bacteria (Schimel *et al.*, 2007; Blazewicz *et al.*, 2014) suggested that
116 there would be a smaller reduction in respiration with a reduced wetting frequency from
117 the afforested soil than the pasture soil. These predictions were tested in a 100-day
118 laboratory-based incubation study in which soils collected from tree plantings and their

adjacent pastures at two farms were subjected to a historical wetting frequency and half this frequency to represent a future drier climate.

## 2. Materials and methods

### *2.1 Soil collection*

Soil was collected for the incubation experiment in the austral winter (June 2012), from two mixed-species, restoration tree plantings and their adjacent pastures. All sites were riparian, with the pasture plots immediately downstream of the tree planting (Fig. 1). The sites were located around Benalla (-36.74 °S, 145.99 °E and -36.70 °S, 145.88 °E), Victoria, Australia. Tree plantings were established in 1994 and 1990 (i.e. this study was conducted 18 and 22 years after planting) respectively, and both were dominated by the tree species *Eucalyptus camaldulensis* Dehnh. and *Acacia melanoxylon* R.Br. Soils were sandy loam sodosols with pHs of 5.3 ± 0.09 in the 18-year-old planting and 4.9 ± 0.08 in the adjacent pasture and 4.5 ± 0.1 in the 22-year-old planting and 4.1 ± 0.5 in the adjacent pasture. The climate across the region is temperate with seasonal changes in mean monthly maximum temperature (12.3–29.7 °C) and minimum temperature (4.1–15.3 °C), and an annual precipitation of 650 mm that is winter-dominant, ranging from 30-80 mm $month^{-1}$ (Bureau of Meteorology, 2013).

In the pastures and tree plantings, 10 soil samples of *ca* 500 g were taken randomly, in plots of 20 m x 10 m. The plot in the pasture and adjacent tree planting were approximately 50 - 100 m apart. In the tree planting, soil was sampled at least 1 m away from the stems of trees (Fig. 1). Samples were taken in the top 10 cm of the soil (this was found the most microbial-active zone in these soils, Cavagnaro, unpublished data) using a

142 trowel, after removing any vegetation and litter. The soil was stored immediately at 4 °C in a
143 portable refrigerator for 2 days until further processing in the laboratory.
144
145 *2.2 Incubation pre-treatment*
146 Soil samples were coarsely sieved (5 mm mesh) to remove large pieces of organic matter
147 and stones. Soil moisture was determined from the proportional difference in mass of
148 subsamples of *ca* 10 g of field-moist soil before and after drying at 105 $^{o}$C for 48 h. Each soil
149 sample (10 replicates per land-use at a farm, referred to as a 'soil core' in the statistical
150 model, see below) was then each divided over three 250 $cm^3$ jars, so that each contained ca
151 100 g dry weight equivalent soil (Fig. 1). The soil was compacted in the jar to a bulk density
152 of 1 g $cm^{-3}$ and resulted in a headspace of ca 150 $cm^3$.
153 Soil in each jar was watered to 45% gravimetric moisture content with reverse
154 osmosis water using a spray bottler. This moisture content was chosen to create a 75% field
155 capacity which was found to produce maximum soil respiration in similar soil type and
156 climate (Setia *et al.*, 2011). The jars were acclimatized in a dark incubator at a constant 20 °C
157 for 4 weeks. A lid was placed loosely over the jars in order to minimize evaporation while
158 allowing gas exchange. The jars were weighed, and aerated every other day to check soil
159 moisture and watered back up to 75% field capacity as required. Soil from one of the
160 triplicate jars from a soil core was sampled destructively after the acclimatization period to
161 measure initial total C and total N concentrations, mineral N and soil microbial community
162 composition (see below). The remaining two triplicate jars were subjected to the following
163 wetting frequency treatments (Fig. 1).
164

165 2.*3 Wetting treatments*

166 Using rainfall records from the Australian Data Archive for Meteorology (Bureau of
167 Meteorology, 2013), the average amount of spring rainfall (197 ± 82 mm from September to
168 November) for the Benalla region was calculated over the period 1961-1990. This period is
169 used widely as the reference condition in climate studies of south eastern Australia (Bureau
170 of Meteorology, 2013) and importantly was before the 'Millennial Drought' (1995-2009). To
171 determine the historical rainfall frequency, the years between 1961-1990 that received
172 amounts of rain within the 95% confidence intervals of the mean spring rainfall (91-254
173 mm) were included, giving a total of 18 'average' years. For each average year, daily rainfall
174 was plotted and the number of clustered rainfall events over > 10 mm were counted (i.e.
175 consecutive days of > 10 mm rainfall were considered a single event). The mean frequency
176 of > 10 mm rainfall events in spring was every 19 days, making six wetting events over a
177 100-day period. Six wetting events over 100 days was used as the 'historical' treatment, and
178 a reduction to three wetting events as the 'reduced' treatment, representing a hypothetical
179 future reduction in rainfall events under climate change.
180
181 *2.4 Respiration measurements*
182 After the acclimatization period of 4 weeks, on day 1 of the treatments, soil in the jars was
183 watered up to 45% moisture content if needed, and were left to dry without lids in the
184 incubator. This was 'wetting event 1' and the soils were rewetted either every 19 d
185 (historical) or 48 d (reduced wetting). Soil (heterotrophic) respiration was measured daily or
186 twice daily for the first three days, and less frequently thereafter, for the duration of the
187 experiment (see Fig. 2 for exact timing of sampling events). An initial gas sample was taken

188 from the head space of the jar, with the lid loosely covering the opening, so that air could be
189 drawn into the jar. Before taking the sample, the air in the headspace was mixed by filling
190 and emptying a 5 ml syringe back into the jar. Then, a 2 ml sample was taken and injected
191 into a $CO_2$ gas analyser (LICOR, LI-820, USA). The jar was closed immediately and left for 40
192 min at room temperature (21±1 °C) before taking the next gas sample through a rubber
193 septum in the lid of the jar, again mixing the air before taking the sample. Measured
194 concentrations of $CO_2$ were converted to amount (mg) of $CO_2$ production per hour per kg of
195 dry soil. The masses of the jars were recorded at each $CO_2$ sampling, in order to calculate
196 gravimetric water content of the soil.
197
198 *2.5 Soil analysis*
199 On day 100, 4 days after the final watering event, all jars were destructively sampled.
200 Briefly, 7 g of air-dried soil was extracted with 2M KCL, using a modified method reported in
201 Miranda *et al.* (2001) for $NO_3^-$-N (including $NO_2^-$-N) and Forster (1995) for $NH_4^+$-N. For
202 potential mineralizable nitrogen (PMN) determination, 10 ml deionized water was added to
203 7 g of air-dried soil in a 50 ml centrifuge tube. The head space of the tube was filled with $N_2$
204 to ensure anaerobic conditions (Waring and Bremner, 1964). The soil was incubated for 7
205 days at 37 °C in the dark. Then, 10 ml of 4M KCL was added to each tube and analysed for
206 $NH_4^+$-N, as described above. The rate of PMN of the soil was expressed as the difference
207 between the $NH_4^+$ extracted from air-dried soil, and $NH_4^+$ extracted after the 7-day
208 incubation. Sub-samples of air-dried soil were ground to a fine powder using a mill and
209 analysed for total C and total N using dry combustion in an ANCA GSL 2 elemental analyzer
210 (Sercon Ltd., UK).
211

212

213 *2.6 Microbial community composition*

214 Changes in the soil microbial community composition among wetting frequency treatments
215 were studied by measuring phospholipid fatty acid (PLFA) profiles of the soils. Five of the ten
216 replicates from the initial sampling, five from the final sampling or the historical and five
217 from the final sampling of the reduced wetting treatment, were selected randomly for PLFA
218 analysis using the procedures of Bossio *et al.* (1998) with slight modifications (Ng *et al.*,
219 2014). Briefly, PLFAs were extracted from 4 g freeze-dried, ground soil samples, using a
220 solvent containing citrate buffer (0.15 M, pH 4.0), chloroform and methanol, followed by
221 trans-esterification of the polar lipid fraction containing the phospholipids. Separation of
222 PLFAs was done using gas chromatography (HP-5ms column, Agilent Technologies, USA).
223 Peaks were identified and quantified by comparing with Supelco Bacterial Acid Methyl Ester
224 (BAME) standard mix (product number 47080-U, Supelco, USA). The data were normalized
225 by sample mass, expressed as nmol $g^{-1}$ dry soil and then range standardized scaling values
226 between 0 and 1. Nomenclature of PLFAs followed that of Frostegård and Bååth (1996). The
227 PLFA marker 18:2ω6,9c was used as an indicator of fungal biomass, and the PLFA markers
228 i15:0, a15:0, i16:0, i17:0, 17:0cy, 17:0 and 19:0cy, as indicators of bacterial biomass
229 (Frostegård and Bååth, 1996).

230

231 *2.7 Statistical analysis*

232 All statistical analyses were performed in R, version 2.15.3 (R Core Team, 2013).
233 Heterotrophic respiration was plotted as cumulative $CO_2$ production over time (Fig. 1). The
234 final amount of $CO_2$ that was produced over the 100-day incubation period (i.e. cumulative
235 amount of $CO_2$ calculated from day 0 to day 100) was used in modelling treatments and
236 land-use (see description of mixed model below). The $CO_2$ measurements in the reduced

237 watering treatment, just before the second watering event on day 48 were erroneous due
238 to equipment malfunction. As its absence caused a large overestimation in the cumulative
239 amount of $CO_2$, these measurements were estimated from the mean of the previous four
240 readings. The size of the $CO_2$ peak just after wetting was assessed by calculating $CO_2$
241 respiration rates for the three days after each wetting event. This was done by calculating
242 the linear slope of the curves in Fig. 2 for each site X land-use combination in the three days
243 after a wetting event.

244 Data were analysed as linear mixed effects models using the *lmer()* function in the
245 *lme4* package (Bates *et al.*, 2013). *Site* and *soil core* were included as random factors, and
246 *land-use* and *treatment* were included as fixed factors. Levels of the factor *land-use* were
247 *pasture* and *tree planting*, and levels for the factor *treatment* were *initial*, *historical final* and
248 *reduced final*.

249 Data was checked visually for normality using box plots and Q-Q plots, and
250 transformation of the data was deemed unnecessary. For each measured variable, the
251 model that best described the data was found by systematically excluding a fixed factor
252 from the full model:

253 *Y = site + soil core + land-use + treatment + land-use * treatment*

254 The models were then assessed by comparing the Akaike Information Criterion (AIC,
255 Sakamoto *et al.*, 1986) value for each of the models. The model with the lowest AIC value
256 was considered the best-fit model. Please note that the *lmer*() function calculates model
257 parameter estimates based on maximum likelihood, not observed and expected mean
258 squares and error strata. Therefore, traditional *P*-values are not calculated. Differences
259 among the levels of the factor were evaluated by determining the 95% confidence interval
260 (95% CI) of the estimated difference between the levels. If this interval did not include zero,

differences between the levels of the factor were considered significant. When a significant interaction was found between *land-use* and *treatment*, the model containing *treatment* was run separately for the land-uses *pasture* and *tree planting*.

To test if there was a difference between the historical and the reduced wetting treatment in moisture content to which the soil dried in between wetting events, an adjusted model was used. The moisture content (expressed as % field capacity) of the soil just before each wetting event (i.e. the lowest moisture content that was reached) was the response variable, and the corresponding term *wetting event* was added to the model as a random variable:

*Y = site + soil core + wetting event + land-use * treatment*

To test if the response to a reduction in wetting frequency on cumulative $CO_2$ emission was different between the pasture and tree planting soil, cumulative $CO_2$ emission in the reduced wetting treatment were expressed as a percentage of the cumulate $CO_2$ emission in the historical wetting treatment. This variable was then tested using a simpler model, as soil core and treatment were obsolete here:

*Y = site + land-use*

Relative values were compared because pasture and tree planting soil had different cumulative $CO_2$ emissions in under the historical wetting frequency treatments.

To explore dissimilarities in PLFA communities among sites, land-uses and under the historical or reduced wetting treatment, non-metric multidimensional scaling (NMDS) was performed using the metaMDS function within the vegan package (Oksanen *et al.*, 2013). The dissimilarity in PLFA communities among the samples was estimated using the Bray–Curtis metric (Bray and Curtis, 1957). Differences in PLFA communities between sites, land-uses and treatments, were tested using the *adonis* function within the *vegan* package.

285 Differences between land-uses were also tested separately within each site, and differences
286 between treatments were tested separately for each site and land-use combination (e.g.
287 site 1 x pasture).
288
289
290 **3. Results**

291 *3.1 Soil moisture*

292 Soil moisture in the historical wetting frequency treatment (expressed as % field capacity)
293 reached a minimum of 29 ± 0.7% field capacity after each of the first four wetting events,
294 and a minimum of 18 ± 0.7% field capacity after the fifth wetting event (Fig. 2a). In the
295 reduced wetting frequency treatment, soil moisture content reached a minimum of 18 ±
296 0.8% field capacity (Fig. 2b), which was significantly lower than in the historical treatment.
297

298 *3.2 Heterotrophic respiration*

299 The cumulative amount of $CO_2$ respired over the 100-day incubation period (Table 1, Fig. 3),
300 was significantly lower in the pasture soil compared with the tree planting soil. Irrespective
301 of land-use, there was significantly less cumulative $CO_2$ emission in the reduced wetting
302 treatment compared with the historical wetting treatment. There was no significant
303 difference between land-uses, when comparing the difference in cumulative $CO_2$ emission
304 between the historical and reduced wetting treatment ('relative $CO_2$', Table 1). The pasture
305 and tree planting soils had similar reductions in respiration under the reduced wetting
306 treatment (63.9 ± 14.1 and 59.5 ± 14.1 mg $CO_2$ $kg^{-1}$ dry soil, respectively).

In the reduced wetting treatment, a levelling of the slope can be observed between the second and third wetting event (see Fig. 3b, around day 70). This indicates a reduction in $CO_2$ emission over time, to approximately zero. It should be noted that the measuring point on day 48, just before the second wetting event, was the estimated data point, based on the average of the previous measuring points (see Materials and methods). This point is likely an overestimate and soil $CO_2$ emissions might have also dropped to approximately zero between the first and second wetting event. In the historical wetting treatment (Fig. 3a), the slope did not level-off between wetting events and respiration continued throughout the period between all wetting events.

*3.3 Total carbon and nitrogen*

Concentration of total N was marginally higher (albeit not significantly, as the 95% confidence interval included 0) in the pasture soil compared with the tree planting soil. There was no significant difference in concentration of total C among any of the treatments (Tables 1 and 2). The C:N ratio of the pasture soil was significantly lower compared with the tree planting soil (Tables 1 and 2). Over the 100-day incubation period, the total concentration of N increased marginally but did not differ between the treatments (Tables 1 and 2). Consequently, the C:N ratio of soil was lower in both treatments at the end of the incubation but did not differ significantly between the historical and the reduced wetting treatment (Tables 1 and 2).

*3.4 Mineral nitrogen*

The concentrations of both $NO_3^-$ and $NH_4^+$ did not differ between tree planting and pasture soils. The concentration of $NO_3^-$ was significantly higher in the historical treatment

331 compared with the reduced wetting treatment whereas the concentration of $NH_4^+$ did not
332 differ significantly between treatments (Tables 1 and 2). Both $NO_3^-$ and $NH_4^+$ increased
333 during the 100-day incubation. In contrast, PMN was significantly higher in the tree planting
334 soil compared with the pasture soil (Tables 1 and 2). In the tree planting soil, PMN
335 decreased significantly during the 100-day incubation, from 61.2 ± 6.49 μg $g^{-1}$ dry soil in the
336 initial sample, to 49.0 ± 3.79 and 47.5 ± 4.57 μg $g^{-1}$ dry soil in the historical and reduced
337 wetting treatment, respectively. In the pasture soil, there was no change in PMN during the
338 100-day incubation (Fig. 4).

339

340 *3.5 Soil microbial community*

341 The total amount of PLFA decreased significantly during the 100-day incubation period in
342 both treatments (Table 1). At the end of the incubation, the total amount of PLFA was
343 significantly lower in the historical wetting treatment compared with the reduced wetting
344 treatment (Tables 1 and 2). The amount of fungal PLFA did not differ significantly between
345 the treatments, but the amount of bacterial PLFA was significantly lower in the historical
346 wetting treatment compared with the start of the incubation. In addition, the F:B ratio was
347 significantly higher in the tree planting soil compared with the pasture soils, driven by a
348 significantly higher amount of fungal PLFA in the tree planting soil, rather than a decrease in
349 total bacterial PLFA (Tables 1 and 2).

350 The overall soil microbial community composition was significantly different
351 between the two farms (Fig. 5, $R^2 = 0.31$, $P < 0.01$) whereas land-use explained little
352 variation in composition ($R^2 = 0.05$, $P < 0.01$). There was no difference in microbial
353 community composition between the historical and reduced wetting treatment, nor when

354 compared with the start of the incubation ($R^2$ = 0.04, $P$ = 0.08). No significant differences
355 were found when comparing land-use per farm, and treatment per farm and land-use.
356
357 **4. Discussion**

358 Two decades after afforestation, there were substantial differences in soil under the tree
359 plantings and their adjacent pastures. The soils differed in C:N ratio, PMN and heterotrophic
360 respiration (Tables 1 and 2, Fig. 3). Despite these differences, the soils responded in a similar
361 way to a reduction in the frequency of wetting and drying cycles. Soil under pastures and
362 tree plantings respired significantly less $CO_2$ in the reduced wetting treatment than the
363 historical wetting treatment, and the magnitude of the reduction in heterotrophic
364 respiration was similar in both land-uses (Tables 1 and 2).

365

366 *4.1 Pasture versus tree planting soils*

367 We hypothesised that heterotrophic respiration from tree planting soil would be less than
368 from the pasture. This is because we expected the woody organic matter inputs in the soil of
369 the tree planting to decompose slower than those in the pastures (Aerts and Chapin, 2000),
370 and because of a shift to a larger fungal biomass in the tree planting soil with higher C use
371 efficiency (Bailey *et al.*, 2002b; Jastrow *et al.*, 2007). However, the pasture soils emitted
372 significantly less cumulative $CO_2$ (over the 100-day incubation) than the tree planting soil
373 (Fig. 3, Table 1). An incubation study comparing pastures and 25-year-old *Pinus radiata*
374 plantings, did find higher $CO_2$ emission from the pasture soils, which were associated with a
375 larger microbial biomass and larger labile organic C pool under pasture (Saggar *et al.*, 2001).

376 A different incubation found higher $CO_2$ respiration in soil from under a *Quercus agrifolia*
377 (oak) forest compared with pasture soil (Fierer and Schimel, 2002). However, in that study
378 the forest soil contained a significantly higher amount of C which would explain the higher
379 $CO_2$ emission in the forest soil.
380 In our study, the total C concentration was the same in soil from both land-uses
381 (Tables 1 and 2). The C:N ratio was significantly higher in the tree planting soil (12.5 vs 14.6,
382 Table 2) due to a small, non-significant decrease in N content, suggesting the presence of
383 more difficult to decompose organic matter (Enriquez *et al.*, 1993). Therefore, it was
384 unlikely that there was a larger labile organic matter pool in the tree planting soil to explain
385 the higher $CO_2$ emissions (Birch, 1958; Inglima *et al.*, 2009). Differences in soil organic
386 matter quality between the pasture and tree planting site can affect the soils water
387 retention capacity (e.g., Rawls *et al.*, 2003) and consequently microbial activity and
388 respiration. However, the pasture and adjacent tree planting soils dried at similar rates (Fig.
389 2) and so the difference in heterotrophic respiration was not explained by different
390 moisture levels of the soil.
391 The F:B ratio was significantly higher in the tree planting, due to a significant
392 increase in fungal biomass in the tree planting compared with the pasture soil (Tables 1 and
393 2). This was consistent with our hypothesis and other studies (e.g., Fierer *et al.*, 2009;
394 Macdonald *et al.*, 2009). However, the fungal biomass was still only a small fraction of the
395 total microbial mass (3.8 %). Their relative biomass was likely too small to significantly
396 reduce heterotrophic respiration. Considering the microbial community as a whole (e.g. all
397 PLFA markers, not only the strict fungal and bacterial markers), there was negligible
398 difference in microbial communities of the pasture and tree planting soils (Fig. 5). This was
399 in contrast to another study in the same region that found significant differences in

microbial community composition between pasture, tree plantings of 10, 20 and 30 years old and remnant forests (Fitzpatrick, 2012). Microbial activity, rather than community composition may explain the higher heterotrophic respiration in the tree planting soil compared with the pasture soil. There was a significantly higher amount of PMN in the tree planting soil (Table 1), which could indicate that the N mineralizing microbial community was more active (and thus respired more) in the tree planting than in the pasture soil (Fig. 6).

PMN was previously found higher in tree plantings sites in the same area (Smith *et al.*, 2012), but the opposite was found in pastures and afforested pastures in south-western Australia and New Zealand (Saggar *et al.*, 2001; O'Connell *et al.*, 2003). We also found lower $NO_3^-$ in the tree planting soil compared with the pasture soil (Tables 1 and 2). Because $NH_4^+$ remained at similar levels, a reduction of nitrification is less likely and an increase in $NH_4^+$ is expected, based on the higher rate of PMN. Therefore, we expect that it indicates higher denitrification (i.e. $N_2O$ emission) in tree planting soil (Fig. 6). *In situ* measurements often find higher $N_2O$ emission in pasture than forest (Barton et al 1999). An *in situ* study over a range of climates in Australia found lower $N_2O$ emissions in tree plantings with full canopy cover (5 – 23 years-old), but a slightly higher $N_2O$ emission in young developing tree plantings (i.e. before canopy closure) compared to adjacent pastures (Allen *et al.*, 2009). Nitrogen cycling is highly dependent on soil moisture and temperature (Allen *et al.*, 2009), so microclimate differences between pastures and tree plantings (see section *4.4 Microclimate*) are likely to play an important role in $N_2O$ emission rates. Tree density and associated canopy cover could be an important determinant of $N_2O$ emission after afforestation if tree densities are low and their microclimate resembles the pasture microclimate. Therefore, based on our findings, $N_2O$ emissions in the tree planting could

potentially exceed $N_2O$ emissions in the pasture. However, this hypothesis remains to be tested.

*4.2 Wetting frequency*

Cumulative $CO_2$ emission over the 100-day incubation period was significantly lower in the reduced wetting treatment compared with the historical wetting treatment for both pasture and tree planting soil (Fig. 3). There was a non-significant trend towards a larger reduction in the pasture soil than the tree planting soil with a 29 ± 4.4% and a 19 ± 3.6% reduction of cumulative $CO_2$ in the reduced wetting frequency treatment, relative to the historical wetting frequency treatment, in pasture and tree planting soil respectively. A reduction in cumulative $CO_2$ emissions in the reduced wetting frequency treatment is consistent with soil microbial activity and respiration being positively influenced by soil moisture (Harper *et al.*, 2005; Borken and Matzner, 2009). Total microbial biomass (defined as total PLFA, e.g., Bailey *et al.*, 2002a) decreased presumably due to water stress, without sufficient time to recover between the wetting events, resulting in decrease respiration over time (Butterly *et al.*, 2009). Total PLFA mass was lower in the historical treatment compared with the reduced wetting treatment (Table 1, 2), which was likely a result of the higher wetting stress in this treatment.

While bacterial biomass decreased during both wetting treatments, fungal biomass did not change during the 100-day incubation, in either wetting frequency treatment (Tables 1 and 2). This supports other studies showing that fungi are less sensitive to drought stress

than bacteria (Schimel *et al.*, 2007; Blazewicz *et al.*, 2014). In contrast, microbial biomass increased during multiple wetting and drying events in a two-month-incubation experiment with grassland soil in California (Xiang *et al.*, 2008). Overall, there was no substantial change in microbial community composition between the wetting and drying treatments or compared with the microbial community composition in the initial soil samples before the incubation (Fig. 5). As the microbial community sampled here was already subjected and likely adjusted to wetting and drying cycles in the field, a prolonged dry period may not affect the microbial community much and a 100-day incubation period may not be long enough to find measurable changes. Few studies have investigated microbial community compositions under differing wetting and drying frequencies (Fierer *et al.*, 2003; Zeglin *et al.*, 2013). For example, a shift in microbial community composition was observed between a 'historical' and 'reduced' wetting regime in a long-term (14-yr) rainfall exclusion experiment (Zeglin *et al.*, 2013). Slight changes in microbial communities were found after 2 months in incubated oak soils that were exposed to higher wetting frequencies but not in grassland soil (Fierer *et al.*, 2003).

*4.3 Total C, and total and mineral N*

Regardless of land-use, the C:N ratio of the soil decreased over time in both wetting frequency treatments. This was largely due to a marginal increase in total N concentration, rather than a decrease in soil C (Tables 1 and 2). As only demineralized water was added to the soil, the increase in soil N concentration may have been caused by free living N-fixing bacteria (Son, 2001).

468 During the 100 day incubation period, PMN in the tree planting reduced significantly
469 under both treatments (Tables 1 and 2). In the pasture soil, PMN did not change during the
470 incubation (Fig. 4). As discussed earlier, higher PMN in the tree planting indicates faster N
471 mineralization and thus higher microbial activity. Because of their higher activity in the tree
472 planting, the wetting and drying treatments may have affected them more than in the
473 pasture soil and this could explain the reduction in PMN in the tree planting but not pasture.
474 The significant increase in $NO_3^-$ and $NH_4^+$ in both wetting treatments compared with the
475 initial sample (Tables 1 and 2) may be explained by a reduction in nitrification and
476 denitrification in the drying soil during the incubation (Jackson *et al.*, 2008). When
477 comparing the wetting treatments, we found lower levels of $NO_3^-$ in the reduced wetting
478 treatment compared with the historical wetting treatment (Tables 1 and 2). As
479 denitrification occurs under wet, anaerobic conditions (Jackson *et al.*, 2008), we would
480 expect to see an increase in $NO_3^-$ in the reduced wetting treatment, as denitrification has
481 less chance to occur in this treatment. Instead, we may explain the lower levels of $NO_3^-$ in
482 the reduced wetting treatment by the significantly larger microbial biomass (i.e. total
483 amount of PLFA) in this treatment (Tables 1 and 2). The larger microbial biomass would have
484 a higher demand for N, suggesting more $NO_3^-$ was assimilated by the microbial community
485 in the reduced wetting treatment, reducing its content in the soil (Mikha *et al.*, 2005; Zeglin
486 *et al.*, 2013). In contrast, a decrease in $NO_3^-$ was found when soil from pasture and *Quercus*
487 *agrifolia* (oak) forest was subjected to higher frequencies of wetting and drying cycles
488 (Fierer and Schimel, 2002). However, a larger microbial biomass was found under the higher
489 frequency wetting treatment in these soils, supporting the same hypothesis of reduced $NO_3^-$
490 because of increased microbial assimilation. No difference in $NO_3^-$ was found among

491 different wetting frequencies in a soil cropped with wheat and maize rotation, while a larger
492 microbial biomass was found under higher wetting frequencies (Zhao *et al.*, 2010).
493
494 *4.4 Microclimate*
495 Differences between pasture and tree planting soil found in laboratory/incubation
496 experiments must be extrapolated to field scenarios with caution. The difference in
497 vegetation structure between pastures and tree plantings produce different microclimates
498 (Chen *et al.*, 2003; Kellman *et al.*, 2007). For example, trees increase shading relative to
499 pasture (Vetaas, 1992), which reduces soil temperature, while the higher evapotranspiration
500 of trees may reduce soil moisture compared with the pasture soil (Ellison *et al.*, 2012).
501 Differences in microclimate may explain why soil respiration is generally higher under
502 pasture than (adjacent) forest or woodland soils, when measured in the field (e.g. Raich and
503 Tufekciogul, 2000; Garcia-Montiel *et al.*, 2003; Salimon *et al.*, 2004). However, deep roots of
504 trees may uptake water in the dry seasons, enabling year-round autotrophic respiration and
505 causing higher annual $CO_2$ emission in evergreen forest compared with pastures (Davidson
506 *et al.*, 2000). We do not know if the pasture soils that were sampled in this study would emit
507 more $CO_2$ than the tree planting soils, when measuring $CO_2$ emission in the field after a
508 natural rainfall event. The contradiction between our results and many field measurements
509 could suggest that field respiration rates are determined predominantly by differences
510 microclimate and autotrophic respiration between the vegetation of different land-uses,
511 and less by soil microbial composition. However, this remains to be investigated.
512
513 **5. Conclusions**

514 While the concentration of soil C did not differ between the pasture and tree planting soil,
515 heterotrophic respiration was higher in soils from tree plantings than from the adjacent
516 pastures. This was inconsistent with the predicted decrease in heterotrophic respiration due
517 to more woody inputs and fungal biomass in soils following afforestation. There were only
518 small increases in the mass of fungi relative to bacteria, and negligible changes to soil
519 microbial composition following afforestation of pastures. Differences in the activity of
520 certain groups of microbes, rather than different microbial community composition may
521 explain the higher respiration after afforestation, as well as differences found in N-cycling.
522 Soils from the pastures and tree plantings showed a similar decrease in microbial biomass,
523 and related reductions in soil respiration under a reduced wetting frequency. Under field
524 conditions, afforestation, relative to pastures, would increase shading, which reduces soil
525 temperature, and reduce soil moisture by increased evapotranspiration. Field-based rainfall
526 exclusion experiments are required to determine the overall effect of afforestation on soil
527 microbial community change and C and N cycling under a drying climate.
528

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725 **Figures**

726

727 Figure 1. Schematic overview of soil sampling and treatment design. At a farm, ten soil
728 samples (x) were taken in both the pasture and adjacent tree planting. Each soil sample was
729 divided over three jars: one jar was sampled after an acclimatization period, and is referred
730 to as 'initial' sample, one jar was sampled after 100-days incubation under a historical
731 wetting treatment ('historical' sample), and one jar was sampled after 100-days incubation
732 under reduced wetting treatment ('reduced' sample). Water drops represent the number of
733 wetting events over the incubation period.

734

735 Figure 2. Soil moisture, expressed as % field capacity, in a) the historical and b) the reduced
736 wetting frequency treatment. Values are means ± standard error. Symbols differentiate
737 among the 18-year-old planting site (circles), 22-year-old planting site (triangles), pasture
738 plots (white) and tree planting plots (black, *N* = 10).

739

740 Figure 3. Cumulative $CO_2$ (mg $g^{-1}$ dry soil) in a) the historical and b) reduced wetting
741 frequency treatment. Arrows indicate the timing of wetting events. Values are means ±
742 standard error. Symbols differentiate among the 18 year old planting site (circles), 22 year
743 old planting site (triangles), pasture plots (white) and tree planting plots (black, *N* = 10).

744

745 Figure 4. Potential mineralizable N (PMN, means ± standard error) in pasture and tree
746 planting soil, in the initial sample (white bar), historical wetting treatment (grey bar) and
747 reduced wetting treatment (black bar, *N* = 10).

748

749 Figure 5. Non-metric multidimensional scaling (NMDS) ordination of range standardized
750 PLFA masses in soil samples. Symbols differentiate among the 18-year-old planting site
751 (circles), 22-year-old planting site (triangles), pasture plots (white) and tree planting plots
752 (black). Stress value was 0.076.

753 Figure 6. Proposed N cycling in incubated pasture and tree planting soil. The size of the
754 arrows and boxes indicate the relative rate of the N cycling process (arrows) or size of the N
755 pool (boxes).

756

757

**Tables**

Table 1. Best fit models for soil and microbial variables. Inclusion of a factor in the best fit model indicates a significant effect of that factor on the variable. Significant differences between the levels of the factor were assumed when the 95% confidence interval (CI) of the difference between the estimated means did not include zero. Significant differences are indicated in bold. I: Initial sample at start of incubation, H: historical wetting treatment sample at end of incubation, R: reduced wetting treatment sample at end of incubation.

Table 2. Characteristics of soils (mean ± standard error) from different and uses and watering treatments. Different letters indicate significant differences among land-use and/or treatments.

| Variable | Best fit model | 95% CI of the difference between factors: | |
|---|---|---|---|
| | | Land-use | Treatment |
| Minimum soil moisture (% field capacity) | Y = site + soil core + wetting event + treatment | - | R – H: **-8.87, -22.54** |
| Cumulative $CO_2$ emission [a] (mg $kg^{-1}$ dry soil) | Y = site + soil core + treatment + land-use | pasture – planting: **-85.45, -28.25** | R – H: **-90.28, -33.08** |
| Relative $CO_2$ [b] | Y = site | - | - |
| Total N (%) | Y = site + soil core + treatment + land-use | pasture – planting: **0.00, 0.06** | H – I: **0.00, 0.04**<br>R – I: **0.00, 0.04**<br>R – H: -0.02, 0.02 |
| Total C (%) | Y = site + soil core | - | - |
| C:N ratio | Y = site + soil core + treatment + land-use | pasture – planting: **-2.71, -1.49** | H – I: **-1.13, - 0.26**<br>R – I: **-1.22, - 0.34**<br>RF – HF: -0.52, 0.35 |
| $NO_3^-$-N (µg $g^{-1}$ dry soil) | Y = location + replicate +treatment | - | H – I: **294.20, 395.54**<br>R – I: **192.91, 294.25**<br>R – H: **-151.96, -50.62** |
| $NH_4^+$-N (µg $g^{-1}$ dry soil) | Y = location + replicate +treatment | - | H – I: **1.20, 5.14**<br>R – I: **2.75, 6.69**<br>R – H: -0.42, 3.53 |
| PMN (mg $g^{-1}$ dry soil) | Y = location + replicate + treatment + land-use + treatment * land-use | pasture – planting: **-26.78, -10.52** | *planting:*<br>H – I: **4.13, 20.27**<br>R – I: **5.60, 21.73**<br>R – H: -9.53, 6.61<br>*pasture:*<br>H – I: -8.79, 4.53 |

| | | | |
|---|---|---|---|
| | | | R – I: -5.23, 8.10<br>R – H: -3.15, 10.17 |
| Total PLFA (nmol $g^{-1}$ dry soil) | Y = site + soil core + treatment | - | H – I: **-15.56, - 37.58**<br>R – I: **- 4.01, - 26.02**<br>R – H: **0.54, 22.56** |
| F:B ratio | Y = site + soil core + treatment + land-use | pasture – planting: **-0.08, -0.04** | H – I: **0.002, 0.02**<br>R – I: **0.03, 0.005**<br>R – H: -0.01, 0.01 |
| Total fungal biomass (nmol $g^{-1}$ dry soil) | Y = site + soil core + land-use | pasture – planting: **-1.51, - 3.32** | - |
| Total bacterial biomass (nmol $g^{-1}$ dry soil) | Y = site + soil core + treatment | | H – I: **-6.28, - 15.69**<br>R – I: -11.52, - 2.11<br>R – H: -0.53, 8.88 |

[a]: cumulative $CO_2$ at the end of the 100-day incubation.

[b]: $CO_2$ emission in reduced wetting treatment expressed as percentage of historical wetting treatment. This model was only tested with the factors *location* and *land-use*. The factors *soil core* and *treatment* have become obsolete in this analysis.

Table 2.

| | Land-use | | Treatment | |
|---|---|---|---|---|
| | Pasture | Tree planting | Initial | Historical final |
| Total C (%) | 2.94 ± 0.16 | 3.07 ± 0.18 | 2.97 ± 0.20 | 3.05 ± 0.21 |
| Total N (%) | 0.27 ± 0.01 | 0.24 ± 0.01 | 0.24 ± 0.01 | 0.26 ± 0.02 |
| C:N ratio | 12.5 ± 0.22[a] | 14.6 ± 0.19[b] | 14.0 ± 0.33 [a] | 13.3 ± 0.27[b] |
| $NO_3^-$ (μg $g^{-1}$ dry soil) | 513 ± 33.9[a] | 488 ± 39.4[b] | 206 ± 17.8[a] | 550 ± 39.2[b] |
| $NH_4^+$ (μg $g^{-1}$ dry soil) | 10.29 ± 0.70 | 9.70 ± 1.36 | 6.05 ± 0.24[a] | 9.22 ± 1.32[b] |
| PMN (μg $g^{-1}$ dry soil) | 29.58 ± 3.50[a] | 48.23 ± 2.91[b] | 45.6 ± 5.16 | 38.4 ± 3.45 |
| Total PLFA (nmol $g^{-1}$ dry soil) | 93.8 ± 6.89 | 104 ± 8.63 | 113 ± 11.41[a] | 86.2 ± 7.87[b] |
| F:B ratio | 0.04 ± 0.002[a] | 0.10 ± 0.006[b] | 0.06 ± 0.005[a] | 0.07 ± 0.007[b] |
| Total fungal biomass (nmol $g^{-1}$ dry soil) | 1.54 ± 0.17[a] | 3.95 ± 0.39[b] | 2.88 ± 0.55 | 2.47 ± 0.35 |
| Total bacterial biomass (nmol $g^{-1}$ dry soil) | 42.2 ± 3.54 | 41.9 ± 3.93 | 48.0 ± 5.37[a] | 37.0 ± 3.91[b] |

Figures

Figure 1.

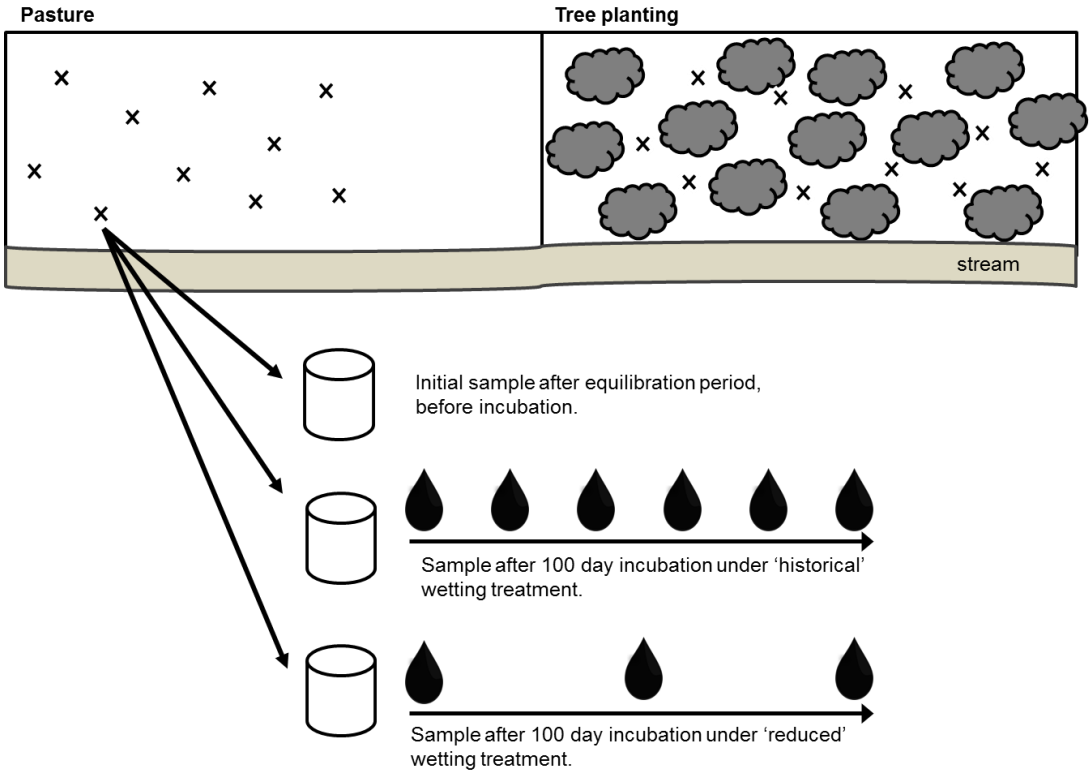


Figure 2.

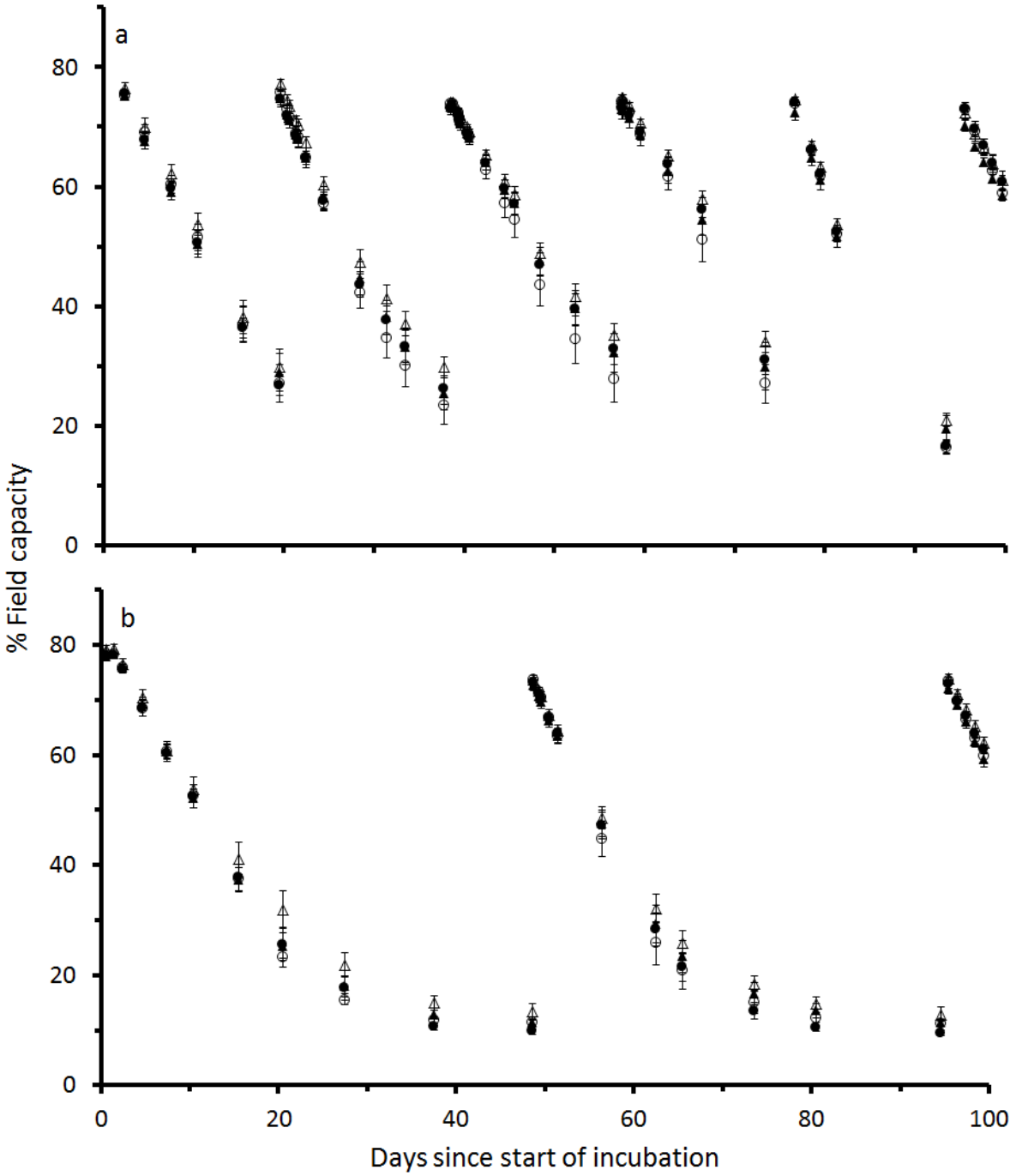

a
b
% Field capacity
0
20
40
60
80
100
Days since start of incubation

Figure 3.

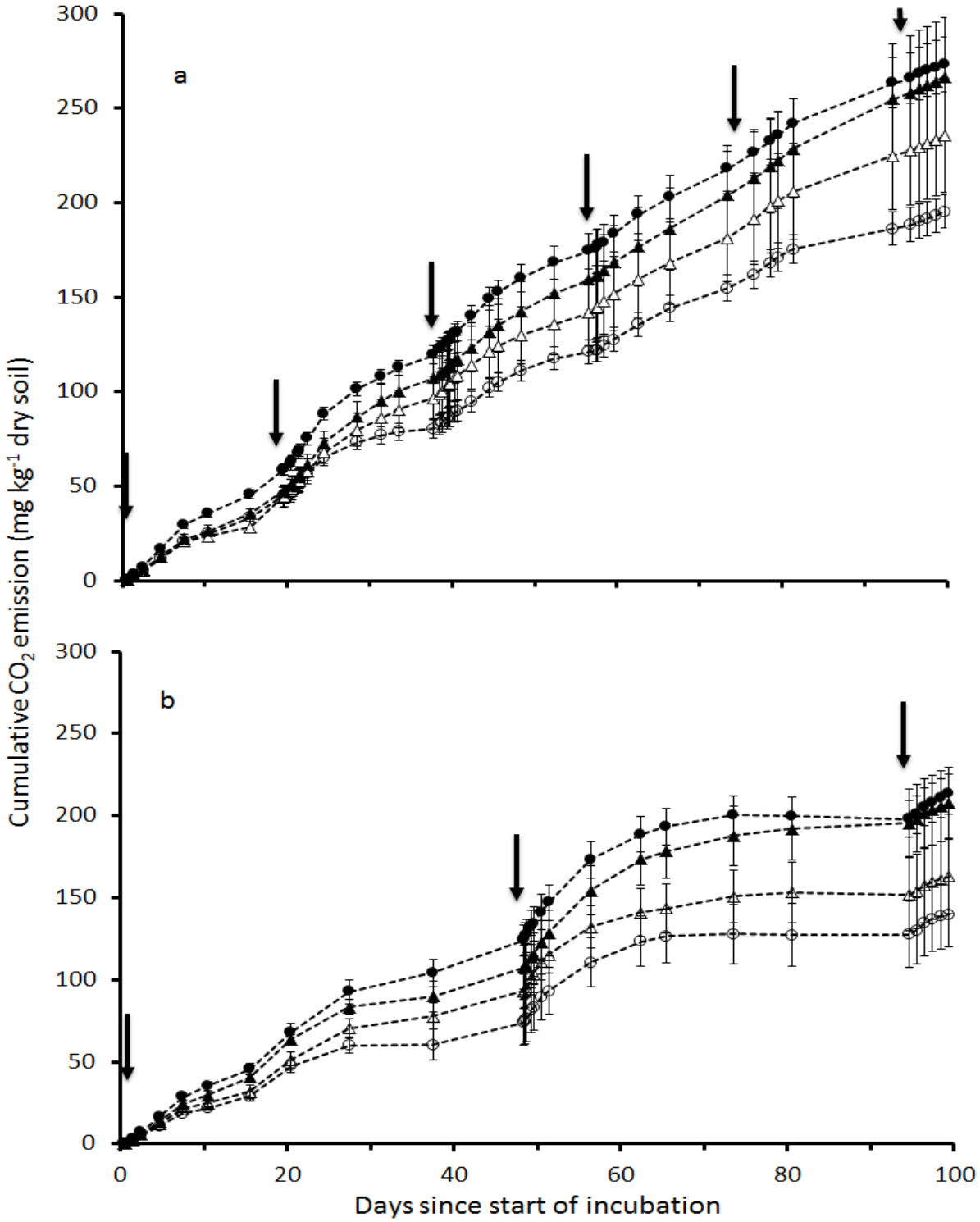

a
b
Cumulative $CO_2$ emission (mg $kg^{-1}$ dry soil)
Days since start of incubation
0
50
100
150
200
250
300
0
20
40
60
80
100

Figure 4.

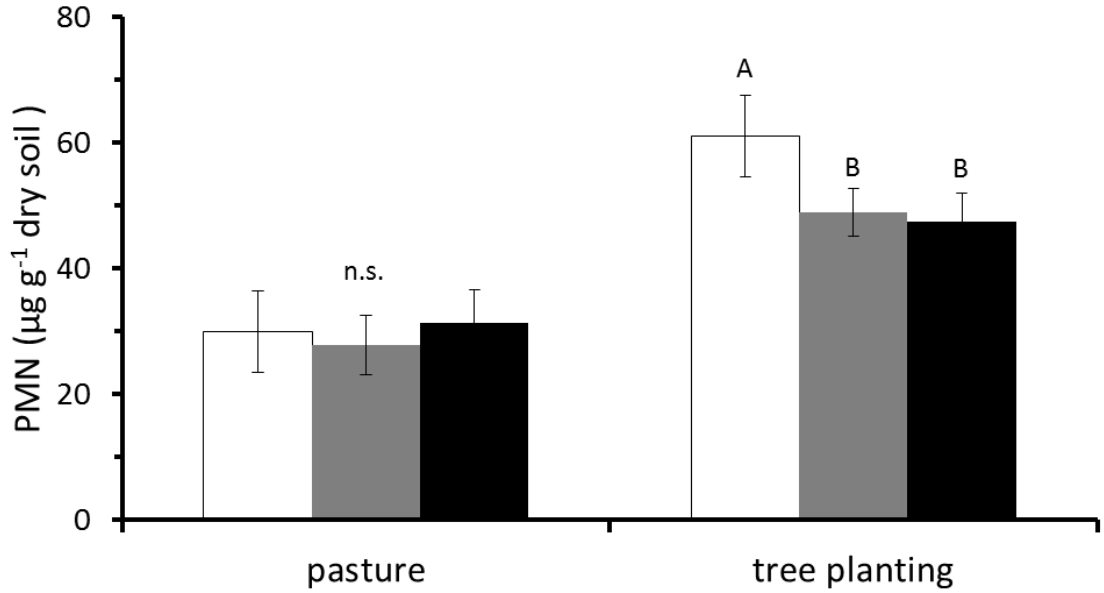



Figure 5.

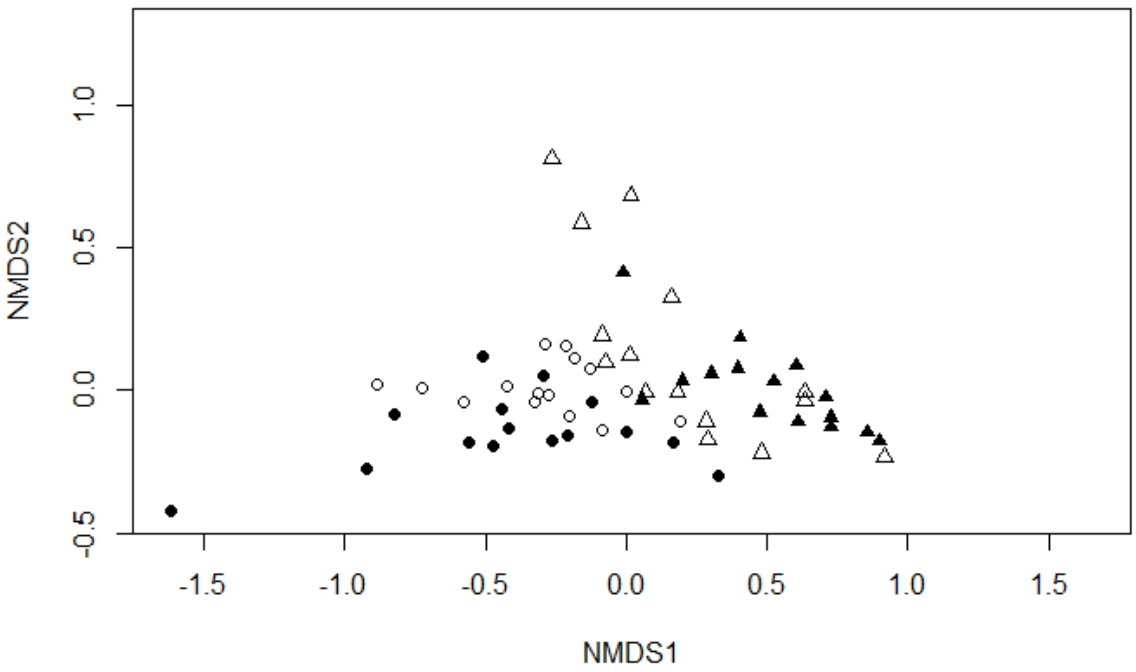
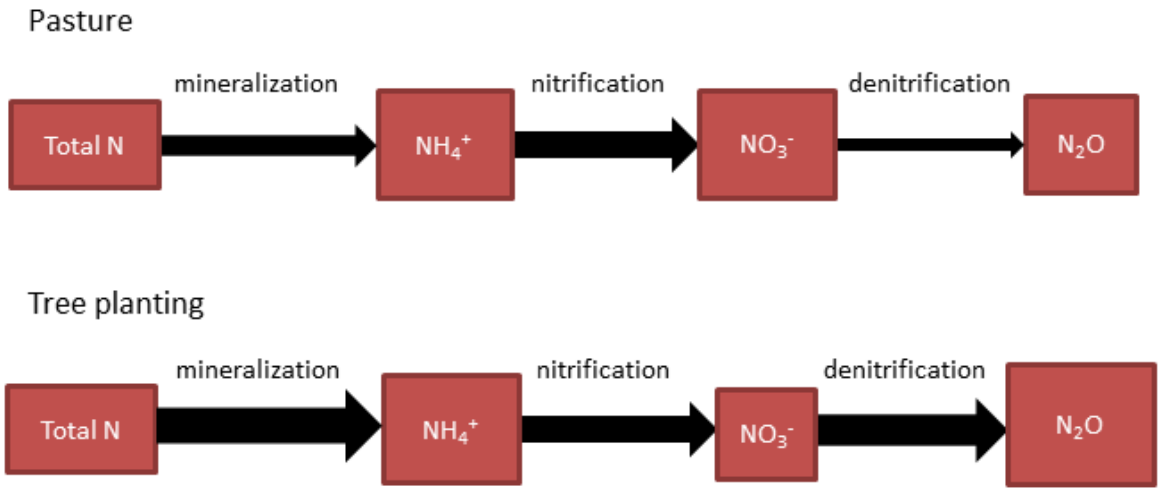


Figure 6.


Pasture
mineralization
nitrification
denitrification
Total N
$NH_4^+$
$NO_3^-$
$N_2O$
Tree planting
mineralization
nitrification
denitrification
Total N
$NH_4^+$
$NO_3^-$
$N_2O$

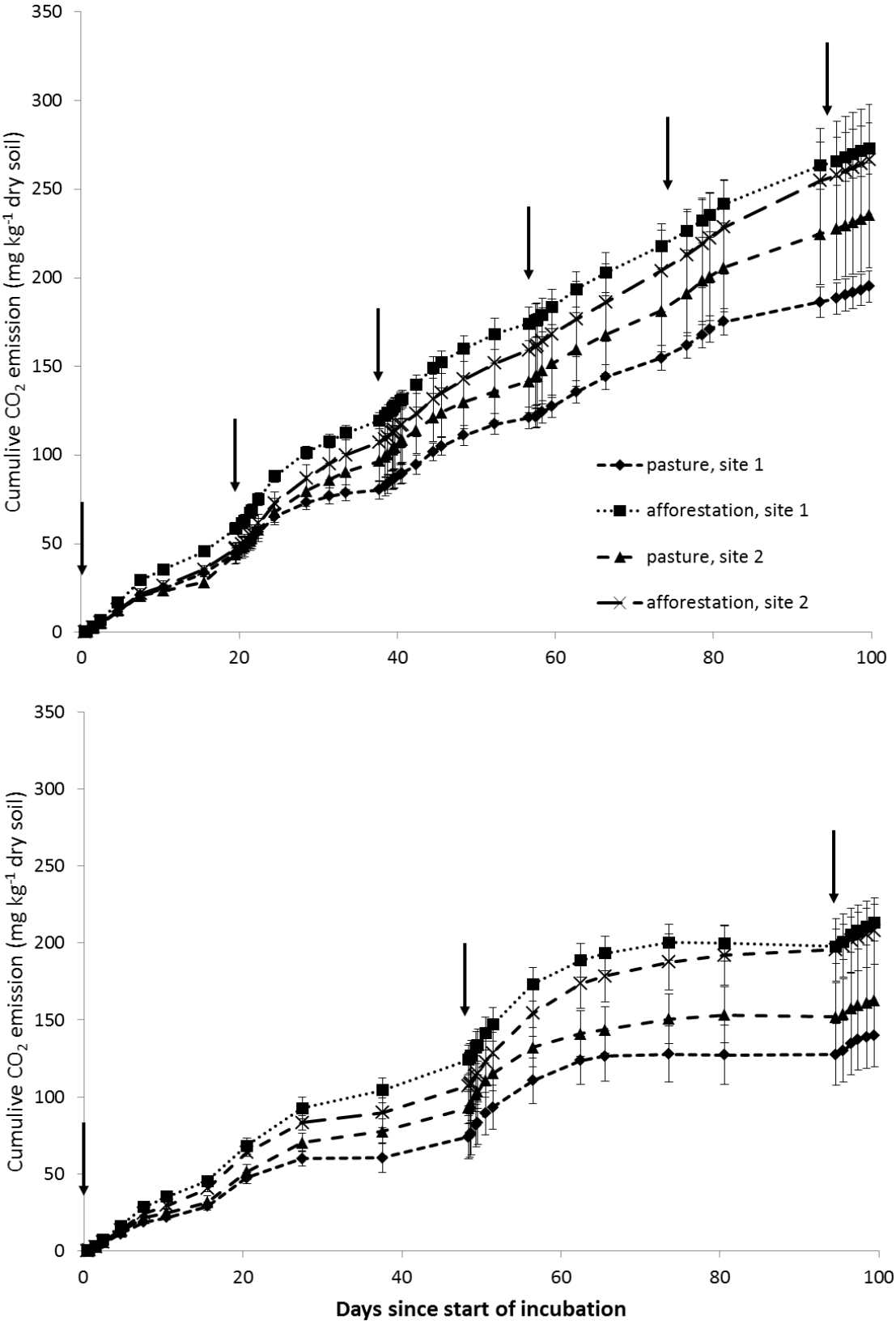
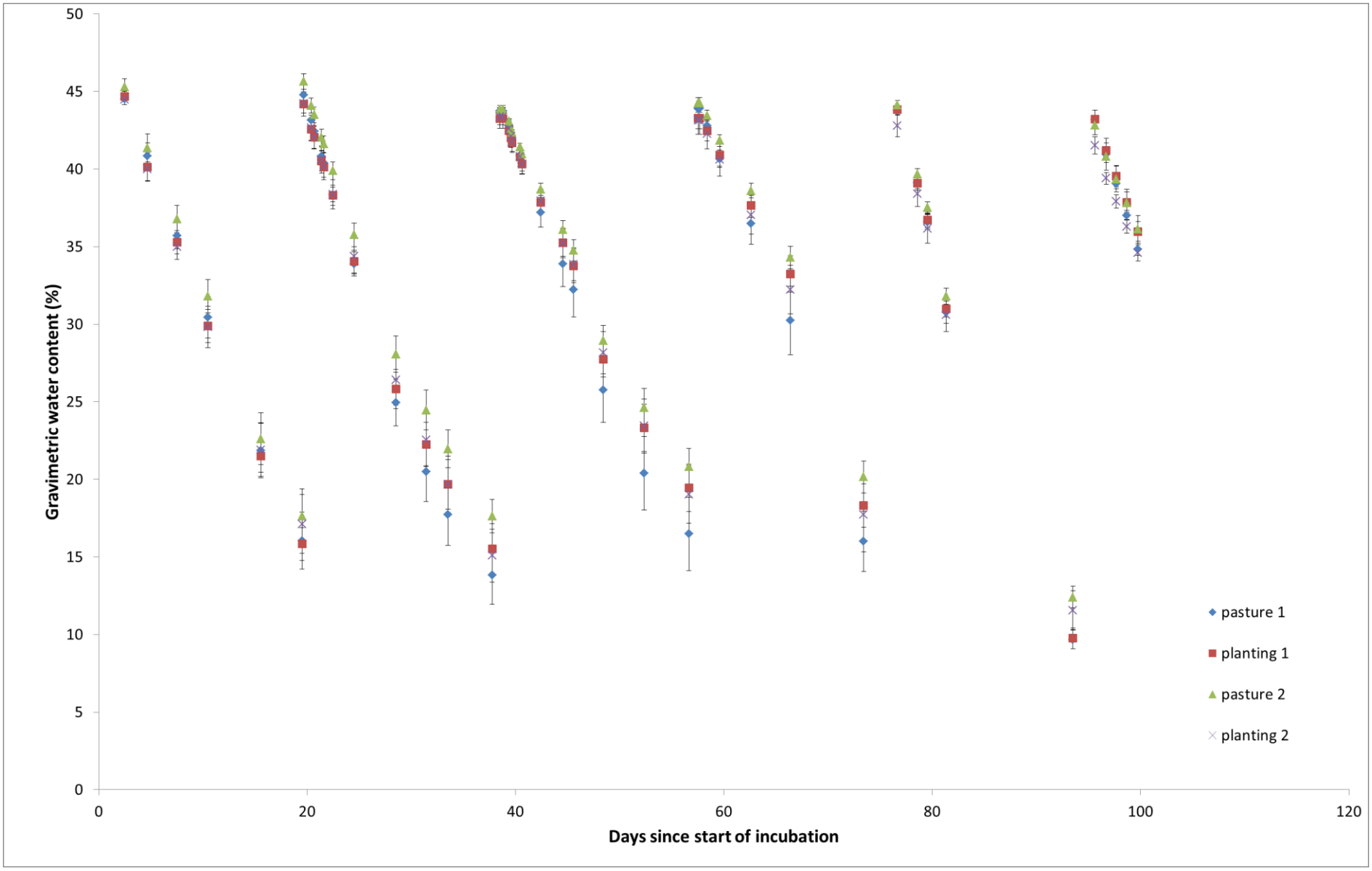



Figure 1. Cumulative $CO_2$ (mg $g^{-1}$ dry soil) over the 100 day incubation period for the normal (a) and reduced (b) wetting treatment. Arrows indicate wetting event. Error bars represent standard errors, N = 10.


Gravimetric water content (%)
50
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
Days since start of incubation
pasture 1
planting 1
pasture 2
planting 2

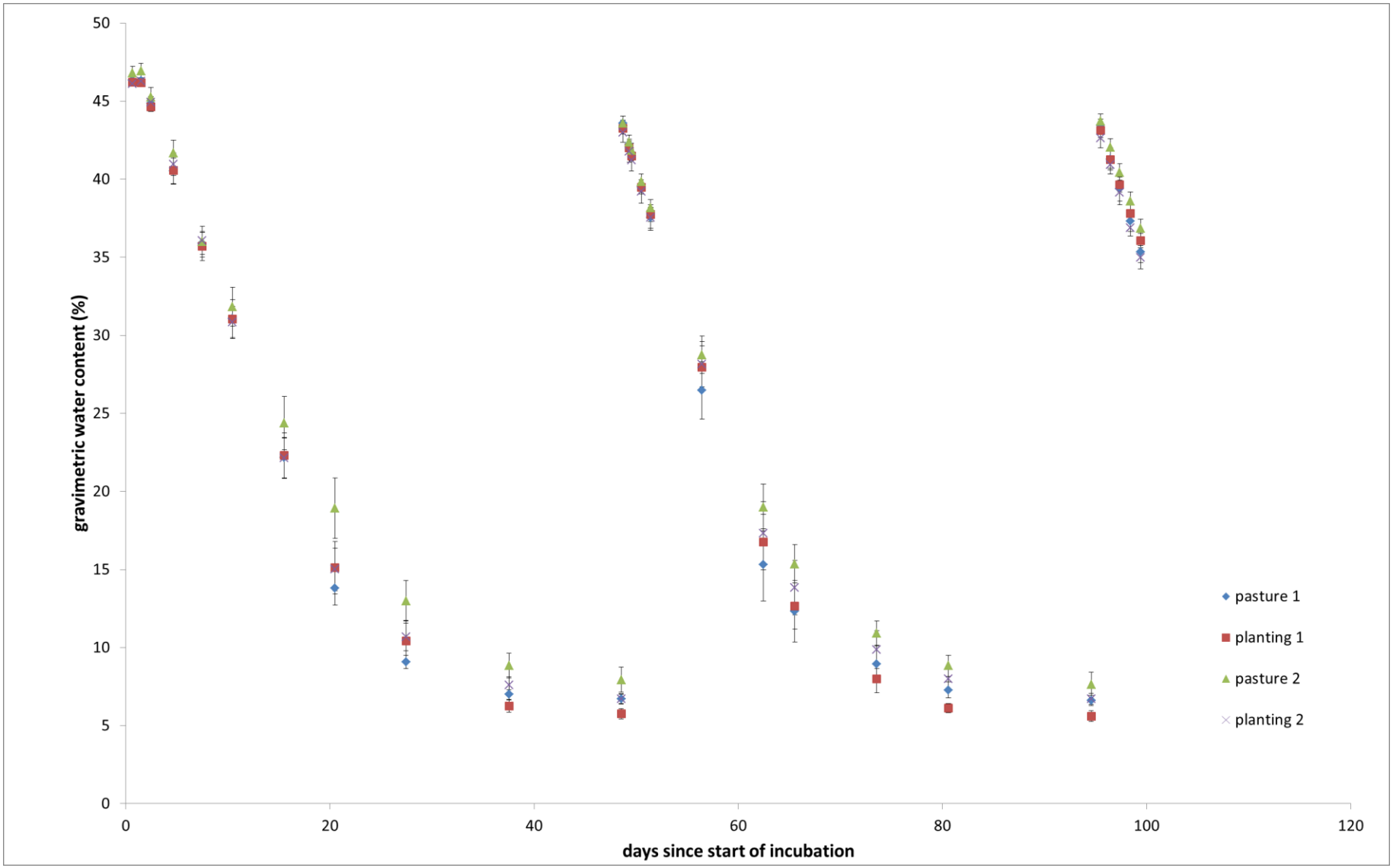

gravimetric water content (%)
50
45
40
35
30
25
20
15
10
5
0
0
20
40
60
80
100
120
days since start of incubation
pasture 1
planting 1
pasture 2
planting 2

Figure 2. Gravimetric moisture content (%) over time in the a) normal and b) reduced wetting frequency treatment. Error bars indicate standard errors, N=10.