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1 **High nitrogen rates do not increase canola yield and may affect soil bacterial functioning**

2 F Becker[1], C MacLaren[1, 2], CJ Brink[3], K Jacobs[3], MR le Roux[1], PA Swanepoel[1*]

3 [1]Department of Agronomy; Stellenbosch University

4 [2]Sustainable Agricultural Sciences, Rothamsted Research

5 [3]Department of Microbiology, Stellenbosch University

6 *Corresponding Author: pieterswanepoel@sun.ac.za; +27218084668

7 **ORCID IDs**

8 CM: 0000-0002-6700-3754; KJ: 0000-0003-3972-5343; CB: 0000-0003-2607-6831; MR: 0000-

9 0002-6473-237X PS: 0000-0002-6481-0673

10

11 **Author contributions:** PS and MR designed the study. FB undertook fieldwork, and FB and CB

12 completed the laboratory tests. CM analysed the data, and FB, CM and PS wrote the article with

13 contributions from CB and KJ. All authors provided critical revisions to the final version.

14

15 **Core ideas**

16 - Over-fertilization of N is common in canola in South Africa

17 - We evaluated N fertilization rates and application timings for canola

18 - Canola growth and yield were generally not affected by N rates or timing

19 - A low N rate favoured soil bacterial communities more likely to mineralize N

20 - A low N rate (60 to 90 kg ha^{-1}) split into two or three applications can sustain yield and

21 have minimal effects on soil bacterial functioning

22

23 **Abbreviations**: ARISA = Automated Ribosomal Intergenic Spacer Analysis; CLPP = Community

24 level physiological profiling: carbon source utilization; DAE = days after emergence; LAI = leaf

25 area index; NMS = non-metric multidimensional scaling; OTU = operational taxonomic unit

26 ***Abstract***

27 Nitrogen fertilization has a fundamental role in agricultural productivity. However, injudicious N
28 applications to crops are common. It is important to ensure the minimum N required for
29 satisfactory crop growth is applied but that excess amounts are avoided due to potential impacts
30 on agroecosystem functioning. Nitrogen at 0, 60, and 150 kg ha^{-1} was applied as limestone
31 ammonium nitrate to plots arranged in a randomized complete block design, on three farms to
32 determine the impact of rate and temporal distribution of fertilizer on canola (*Brassica napus* L.)
33 production in South Africa, and the effect of N fertilizer application on the composition and
34 diversity of soil bacterial communities. The amount and distribution of N had only minor effects
35 on canola growth ($P < 0.05$) and no effects on yield or harvest index. Splitting fertilizer into two
36 or three applications throughout the season resulted in more mineral N available in the soil later
37 in the season. Increasing the N rate from 60 to 150 kg ha^{-1} had a significant impact on bacterial
38 community composition. The lower rate favored bacteria that are more able to break down N-
39 containing carbon sources. No effects of fertilizer amount or distribution were observed on either
40 N fixation potential (number of nifH gene copies) or bacterial community diversity. Overall, a
41 low rate of N fertilizer split into multiple applications is recommended for canola production, as
42 higher rates do not increase yield and may have a detrimental impact on soil carbon and nitrogen
43 cycling.

44 **Introduction**

45 The need for sustainable agricultural production systems is well recognized, with substantial
46 increases in the human population posing huge challenges for future agricultural production within
47 Earth's environmental limits (Tilman et al., 2002; Rockström et al., 2017). Environmental
48 degradation associated with agriculture is exacerbated by external inputs, in particular with
49 injudicious inorganic fertilizer applications. Inorganic N fertilizer application is a primary
50 approach to intensify crop production and ensure food security (Liu et al., 2011), but it also has a
51 large C footprint and causes eutrophication and algal blooms when it leaches from agricultural
52 land to waterways (Seitzinger and Phillips, 2017). In addition, fuel and energy use during the
53 manufacturing and transport processes of fertilizers may also contribute substantially to
54 environmental erosion (Shibata et al., 2017). Although N fertilization has a fundamental role in
55 agricultural productivity, it is important to ensure the minimum N required for satisfactory crop
56 growth is applied.

57 Canola (*Brassica napus*) was introduced into crop rotation systems of South Africa in 1994
58 to increase crop diversity (BFAP 2018). Canola production in South Africa is growing, and
59 according to predictions made by the Bureau for Food and Agricultural Policy (BFAP 2018), will
60 increase to ca. 200,000 tons by 2027. Canola has a higher N demand than most other cash crops
61 (Ma and Herath, 2015), but dependence on inorganic N fertilizers to increase canola production is
62 not sustainable and, therefore, attention should be shifted to retaining and fixing N on-farm through
63 optimising soil biological activity. Moreover, due to the lack of robust guidelines tailored for the
64 South African climate and soil conditions, N fertilization is often applied injudiciously, which may
65 have negative impacts on the finely balanced interactions in the soil environment. There is a need
66 to understand how much inorganic N is necessary to produce satisfactory canola yields in the
67 region, and how different fertilization strategies affect soil biological function.

68 The relationship between soil microbial diversity, ecosystem functioning, associated
69 services, and management practices (e.g. N fertilization) is under increasing scrutiny to elucidate
70 the complexities that underpin the productivity of agroecosystems (Brussaard et al., 2007;
71 Hartmann et al., 2015; Hartman et al., 2018). Increased biodiversity in the microbial community
72 may enhance the functional capacity of the soil ecosystem (Bender et al., 2016). Thus, the use of
73 agricultural management practices that can maintain soil functional diversity is advocated in order
74 to build inherent resilience to environmental shocks. The same motive has driven the
75 implementation of crop rotation systems in the Western Cape (Venter et al., 2017).

76 It is widely acknowledged that N fertilizer may affect soil biodiversity and on-farm
77 ecological functioning, potentially decimating the ecosystem services provided by the soil
78 microbial community (Bisset et al. 2011; Gordon et al., 2016; Hartmann et al., 2015; Jackson et
79 al., 2017). For example, the soil N and C cycles are mediated by soil bacteria, which are involved
80 in the build-up and decomposition of soil organic matter (Jackson et al., 2017), and in the
81 conversion of N between its organic and inorganic forms (Kuypers et al., 2018). It has been shown
82 that microbial communities can be sensitive to fertilizers, particularly at the rates applied in current
83 agroecosystems (Gordon et al., 2016; Hartmann et al., 2015). This suggests that N fertilizer
84 applications may interfere with the capacity of the microbial community (including beneficial
85 bacteria) to cycle N and C in ways that are beneficial to both crop growth and C sequestration.
86 Thus, optimising N fertilizer applications should have benefits for both protecting the off-farm
87 environment and sustaining the capacity of farm soils to produce crops.

88 Our understanding of the effect of N fertilization of canola on soil bacterial communities
89 is currently limited. The overall aim of this study was to evaluate different N fertilization rates and
90 N distribution for canola production in South Africa, but it specifically seeks to determine the
91 effect of fertilizer N application on the composition and diversity of soil bacterial communities.
92 The relationships between different N fertilizer application strategies, the soil bacterial

93 community, and canola growth and yield were assessed. The effects of both the quantity of N
94 fertilizer and whether it was all applied at once or distributed throughout the season (at planting
95 and as a top-dressing) were investigated.
96
97 **Materials and methods**

98 ***Trial location***

99 Trials were conducted in 2016 at three farms in the winter rainfall area within South Africa's
100 Western Cape province: Langgewens Research Farm (33°16'36.6"S, 18°42'11.4"E), Roodebloem
101 Experimental Farm (34°13'29.5"S 19°31'47.3"E) and Altona, a commercial farm (33°42'15.6"S,
102 18°38'12.3"E). Langgewens and Altona are located in the Swartland region, and typically receive
103 440 mm and 690 mm of rain per year with 85-90 % of rainfall falling in the colder winter months
104 (April-September). Roodebloem is located in the southern Cape region and receives 585 mm of
105 rain per year, of which 80 % falls in the winter months. In 2016, annual rainfall and temperature
106 patterns were similar to long-term averages, with the exception that May was unusually dry
107 (records were obtained from weather stations either on or nearby each farm). Soils on Roodebloem
108 Experimental Farm are generally shallow (<400 mm deep), shale-derived soils of a sandy loam
109 texture. The parent material of soils in the Altona and Langgewens districts are mainly derived
110 from greywacke and phyllite with limited pedological development, therefore shallow (<400 mm
111 deep). The soil chemical and physical characteristics of each site is presented in Table 1.
112
113 ***Experimental design***

114 The trials followed a crossed full factorial design, with treatments receiving either 60 kg ha^{-1} or
115 150 kg ha^{-1} of N, of which 20 kg ha^{-1} was applied at planting and the remainder distributed in
116 either one, two or three applications later in the season (at 30, 60 and 90 days after emergence;
117 DAE). A null control was also included, which received no N at any point in the season, so the

118 trial included seven treatments in total (Table 2). These were laid out in a randomized complete
119 block design, with four replicates at each of the three farms. Plots were 2.75 x 5 m. Half of each
120 plot was intended for destructive measurements (sampling of plants), while the other half was used
121 for yield determination. Measures of canola plant production and soil bacterial community
122 composition and function were taken in each plot at various time points throughout the season
123 (Table 3). The methods for each of these are detailed in the following sections.
124

125 ***Trial management***

126 Weeds were eradicated prior to planting with paraquat. A fine seedbed was created using a 21 tine
127 vibro flex to a depth of 150 mm, during which the pre-emergence herbicide Trifluralin [2,6-
128 Dinitro-N,N-dipropyl-4-(trifluoromethyl)aniline] was applied so that it could be incorporated into
129 the soil. The insecticide chlorpyrifos (O, O-diethyl O-3, 5, 6-trichloropyridin-2-yl
130 phosphorothioate) was applied just before planting. Phosphorous, in the form of double
131 superphosphate, was applied at a rate of 20 kg ha^{-1} on the day of planting, according to
132 recommendations from soil tests done prior to establishment of the trial. No potassium was
133 required according to soil test results (Table 1). Nitrogen was applied in the form of limestone
134 ammonium nitrate (LAN) as specified in Table 3.

135 Canola was sown using a Wintersteiger disc plot planter with 170 mm row spacing. The
136 triazine tolerant canola cultivar, Hyola 555 TT was planted at 4 kg ha^{-1} on 9 May 2016 at
137 Langgewens, 5 May 2016 at Altona, and 4 May 2016 at Roodebloem. The preceding crops on
138 Langgewens, Altona and Roodebloem were wheat (*Triticum aestivum*), annual medics (*Medicago*
139 spp.), and oats (*Avena sativa*), respectively. Methiocarb [(3,5-dimethyl-4-methylsulfanylphenyl)
140 N-methylcarbamate] was applied just after planting to control snails, slugs and millipedes. At 30
141 DAE, atrazine (6-chloro-4-N-ethyl-2-N-propan-2-yl-1,3,5-triazine-2,4-diamine) and chlorpyrifos

142 were applied to control grass weeds and insects, respectively. Methiocarb was also applied at 30
143 DAE.
144
145 ***Soil nitrogen content analysis***
146 Soil cores (ø 45 mm) were taken to a depth of 150 mm. Three sub-samples were taken from each
147 plot and combined to form a single composite sample per plot, then air-dried at room temperature,
148 and sieved with a 1 mm sieve. Samples were taken 30, 60, 90 DAE and at when the canola reached
149 physiological maturity (approx. 150 DAE). Soil samples were analysed for ammonium and nitrate
150 content using the indophenol-blue (Keeney et al., 1982) and salicylic acid methods (Cataldo et al.,
151 1975) respectively. Total soil mineral N (kg ha^{-1}) was calculated as ammonium plus nitrate, which
152 is the N readily available to plants. To convert the total mineral N concentration (mg kg^{-1}) to stock
153 (kg ha^{-1}) a bulk density of 1400 kg m^{-3} was used, which is the average bulk density for the region
154 reported by (de Clercq et al., 2013).
155
156 ***Soil bacterial community analysis***
157 Soil cores (ø 45 mm) were taken to a depth of 150 mm using a stainless steel pipe and a hammer.
158 The pipe was washed and sterlized with 70 % ethanol between sampling of different plots. Samples
159 were collected at canola physiological maturity only.
160
161 *Automated Ribosomal Intergenic Spacer Analysis (ARISA)*
162 The bacterial community composition within each sample was determined with Automated
163 Ribosomal Intergenic Spacer Analysis (ARISA) (Ranjard et al., 2001). DNA was extracted from
164 0.25 g of soil using the Zymo research soil microbe DNA MicroPrep™ kit (Zymo research USA).
165 Extracted and purified DNA was separated on a 1 % agarose gel stained with ethidium bromide to
166 visualize under ultraviolet light.

167 The polymerase chain reaction (PCR) reactions were performed on the purified DNA using
168 ITSReub (5'-GTCGTAACAAGGTAGCCGTA-3') and FAM (carboxy-fluorescein) labelled
169 ITSF (5'-GCCAAGGCATCCACC-3') primer set for the 16S rRNA intergenic spacer region to
170 determine bacterial diversity using ARISA (Cardinale et al. 2004, Slabbert et al., 2010b). PCR
171 reactions were done using a 2720 Thermal Cycler (Applied Biosystems, USA). The reaction
172 mixture contained 0.5 µl purified genomic DNA, 500 nM of each primer, 4.1 µl PCR grade water
173 (nuclease free) and 5 µl KapaTaq readymix (Kapa Biosystems, South Africa) for a total volume
174 of 10 µl. The PCR consisted of an initial denaturing step of 5 minutes at 94°C, followed by 40
175 cycles at 94°C for 45 s, 56°C for 50 s and 72°C for 70 s. The reaction was completed with a final
176 extension at 72°C for 7 minutes and then cooled and held at 4°C. All the samples were done in
177 triplicate and pooled to compensate for PCR bias.
178 The PCR products of the pooled samples were run on an ABI 3010xl Genetic Analyser to
179 obtain an electropherogram of different fragment lengths and fluorescent intensities. Bacterial
180 ARISA samples were run along the LIZ 1200 size standard which is designed for sizing DNA
181 fragments in the 100 – 1200 base pair (bp) range. Fluorescence intensities were converted to
182 electropherograms using the Genemapper 5 software. The peaks on the electropherogram represent
183 different fragments of different sizes, termed operational taxonomic units (OTUs), and the heights
184 of the peaks indicate relative abundance of the fragments. The lengths were calculated by plotting
185 a best fit curve using the size standard and extrapolating the fragment size from the sample. Only
186 fragment sizes between 100 and 1000 base pairs and peak heights above 150 fluorescent units were
187 used for analysis as OTU's. A bin size of 3 bp was used to minimize inaccuracies of the ARISA
188 profile (Brown et al., 2005; Slabbert et al., 2010b).
189
190 *Community level physiological profiling (CLPP): carbon source utilization*

191 The CLPP was done by determining the carbon source utilization of the soil bacterial community.
192 Soil samples were diluted in distilled nuclease free water and inoculated, in triplicate, into Biolog
193 EcoPlates^{TM} (Biolog Inc., USA). The plates contain 31 different C sources in different wells and
194 a control well containing no C source. Plates were incubated at 28°C. Utilization of the C sources
195 by microbial populations reduce the tetrazolium dye inside the plate wells that cause a colour
196 change. This colour change was measured twice daily over a period of 5 to 10 days with a
197 spectrophotometer at 590 nm to determine the average well colour development (AWCD).
198
199 *Nitrogen fixation capacity: number of nifH gene copies*
200 The *nifH* PCR product from a *Burkholderia* strain was used for preparation of the standards as
201 well as a positive control. The PCR product was purified using GeneJET PCR Purification Kit
202 (Thermo Scientific). The PCR product size of 380 bp was verified by electrophoresis on a 1 %
203 agarose gel. The purified PCR products were quantified using a µLITE (Biodrop, Cambridge, UK)
204 and the *nifH* gene copy number was determined using the fragment length, molecular weight and
205 Avogadro's number. The known concentration of the PCR product was used to prepare a standard
206 curve, in triplicate, to measure *nifH* gene copy numbers. The *nifH* gene copy numbers of the soil
207 samples were quantified by using quantitative PCR (qPCR) using the nifH F1 and nifH 438r
208 primers (Boulygina et al., 2002; De Meyer et al., 2011). The qPCR assays were performed using
209 the LightCycler 96 (Roche) with a SYBR Green 1 fluoroprobe as the protocol suggested (Brink et
210 al., 2019).
211 A standard curve was generated for every qPCR run ranging from 1x10^10 to 1x10^0 gene
212 copies µL^{-1}. Each run also included a positive control as well as a negative control. The same soil
213 DNA samples used for ARISA was used for qPCR and was run in duplicate. The reaction volume
214 contained 3 µL nuclease free water, 2 µL nifH F1 (100 nM) and nifH 438r (100 nM) primers, 10
215 µL SYBR Green I Master Mix (2x) and 5 µL sample DNA as described by manufacturers

216 specifications. The thermal cycle used for qPCR consisted of 95°C for 5 minutes followed by 40
217 cycles of 94°C for 60 s, 60°C for 60 s and 72°C for 30 s. A melt curve analysis was done after the
218 40 cycles to verify specificity of amplicons. This analysis identified the number of nifH gene
219 copies per 5 µL of DNA, which was converted to the copy number per gram soil based on 100 µL
220 DNA per 0.25 g soil.

221

222 ***Canola growth and yield determination***

223 Plant population was determined by counting seedlings within the border of a half square meter
224 quadrat at 30 DAE and converted to plants m^{-2}. Biomass was determined by cutting 10 plants per
225 plot at ground level at 30, 60, 90 DAE and at physiological maturity. The plants were dried in an
226 oven at 70°C for 48 hours and weighed. Biomass per plant was converted to biomass m^{-2} by using
227 the particular plot's plant population. An additional ten plants per plot were sampled to determine
228 leaf area index (LAI). Leaf area index was measured at 60 and 90 DAE using a LI-COR 3100 leaf
229 area meter. The LAI describes the potential surface area of leaves available for capturing light and
230 thus photosynthetic capacity, and so a higher LAI gives a plant the capacity for higher biomass
231 accumulation and yield potential (Viña et al., 2011).

232 Canola seed was harvested at physiological maturity on 7 November 2016 at Langgewens,
233 9 November 2016 at Altona and 4 November 2016 at Roodebloem with a Hege plot harvester. The
234 harvested seed were cleaned by using sieves and weighed to determine the yield per plot (ton ha^{-}
235 ^{1}). The harvest index, or proportion of aboveground biomass, was also calculated:

236

237
$$Harvest\ index\ (\%) \frac{Dry\ mass\ of\ harvest\ component}{Total\ biomass\ at\ harvest}\ x\ 100$$

238

239 Ten plants per plot were dried, ground and passed through a 1 mm sieve, then analysed for % N
240 content using the Kjeldahl method (AOAC, 2000).

241

242 ***Data analysis***

243 Prior to analyses, OTU heights were normalized to the lowest height total before analysis, by
244 dividing each value within the sample by the total height representing relative abundance of the
245 DNA fragments, and multiplying each value with the lowest height total (so that the number of
246 OTUs is underestimated rather than overestimated) (Slabbert et al 2010b) . The OTU Shannon
247 diversity was calculated according to the following formula:

248 $$-\sum_{i} p_i ln\, p_i$$

249

250 where *p* is the proportion of biomass in species *i,* and *ln* is the natural logarithm. The Shannon
251 index is an acceptable diversity measure for OTU data (Hill et al 2003).

252 Linear mixed regression models were used to assess differences in soil mineral N, canola
253 biomass, leaf area index, yield, harvest index, bacterial community diversity, and bacterial N
254 fixation capacity. The amount and distribution of N and their interaction were set as fixed effects,
255 while replicate nested in farm was set as a random effect. Farm was included as a random effect
256 to explore whether there was any independent effect of the N fertilizer treatments when site was
257 accounted for. Site itself was not a factor of interest, as sites were selected for logistical reasons
258 and not due to any particular characteristics nor prior knowledge of typical canola yields or
259 microbial communities. The negative control was included by structuring the model to test for the
260 difference between the control and all treated plots, and to test for differences between treatments
261 nested within all treated plots. A log transformation was used for the response variables of soil
262 mineral N, canola biomass, and LAI so that the data fit the assumptions of linear regression of
263 normality and homoscedastic variance in the model residuals.

264 To assess bacterial community composition, NMDS ordination based on the Bray-Curtis
265 dissimilarity measure was used for both OTUs and carbon sources utilized. PERMANOVA was

266 used to test for significant differences in composition between different amounts and distributions
267 of N with farm as a grouping variable, to detect effects of these variables within potentially
268 different bacterial communities on different farms. It is currently not possible with available
269 software to nest grouping variables in PERMANOVA, otherwise replicate could have been used
270 as a grouping variable too. Data analysis was undertaken in R, version 3.4.3 (R Core Team, 2017).
271

272 **Results**

273 ***Soil mineral nitrogen and canola production***

274 As the season progressed, soil mineral N tended to become higher in plots that received greater
275 amounts of N applied in a more even distribution (Table 4, Figure 1). Applying 60 kg ha^{-1} of N
276 across three applications resulted in more available N later in the season than applying 150 kg ha^{-1}
277 at 30 DAE in a single dose (Figure 1).

278 Canola biomass and LAI responded slightly to the amount of N applied and its distribution
279 (Table 4), but were generally not different between treatments (Figure 1). When N fertilizer was
280 distributed in three applications compared to one or two applications, biomass and LAI tended to
281 be lower at 60 and 90 DAE for 60 kg ha^{-1} treatments, but higher in the 150 kg ha^{-1} treatments at
282 60 and 90 DAE. It is possible that treatment 60(3) did not receive sufficient N for full growth prior
283 to 90 DAE, whereas a more even distribution of fertilizer was advantageous where more N was
284 applied in treatment 150(3). However, these small differences in plant growth did not result in any
285 significant differences in either canola harvest index or plant tissue N content at physiological
286 maturity between the treatments, and only the control differed ($P < 0.05$) from the other treatments
287 (Table 5, Figure 2). In terms of random effects, variability between replicates tended to be very
288 low, while variability within plots of the same treatment at the same site (the residual error term)
289 was often similar or larger than the variability between farms (Tables 3 and 4), indicating relatively
290 high within-site and within-replicate variability that could not be explained by the treatments.

291 The lack of substantial differences in canola growth and yield indicates that uptake of N
292 by canola does not explain the reduced soil mineral N observed later in the season in treatments
293 where all N fertilizer was applied early (Figure 1). It can therefore be assumed that the N is lost
294 from the system (perhaps to leaching, bacterial immobilisation or weeds) and not incorporated into
295 crop plant matter.

296

297 ***Bacterial community composition, diversity and function***

298 No significant differences were observed in OTU richness, Shannon diversity or the number of
299 *nifH* gene copies between treatments, and again variability within treatments and replicates (the
300 residual) was higher than variability between replicates and farms (Table 6). However, the non-
301 metric multidimensional scaling (NMS) ordination (Figure 3) and PERMANOVA of the OTUs
302 found in each plot indicate that N amount does have a significant impact on bacterial community
303 composition (Table 7). In the ordination, plots that received 150 kg ha^{-1} N are shifted higher along
304 the first axis of the NMS compared to control plots or plots receiving 60 kg ha^{-1} N at the same
305 farm (Figure 4). This suggests that increasing N levels favours a different group of bacteria to
306 those found under low N levels.

307

308 The NMS ordination and PERMANOVA for carbon source utilization suggest that the amount of
309 N fertilizer applied may alter microbial function (Table 7, Figure 4). Plots that received more N
310 tend to be shifted higher along axes 1 and 2 of the NMS, which is associated with higher use of
311 carbohydrates, phosphorylated compounds and carboxylic acids and lower use of amino acids,
312 amines, esters and polymers. Amines and amino acids always contain N, while some esters and
313 polymers do, so it is possible that lower mineral N availability favours bacteria that can break
314 down N-containing carbon sources and thus extract N as well as C from those sources, to meet
315 their N needs. In particular, Figure 4(a) shows a greater association of plots receiving 150 kg N

316 ha^{-1} with non-N-containing carbon sources, and Figure 4(b) suggests a higher affinity for polymers
317 of control plots and plots receiving 60 kg N ha^{-1}.
318
319 **Discussion**
320 ***Optimising fertilization strategy for canola growth***
321 This study provided no evidence that increasing N fertilizer from 60 kg ha^{-1} to 150 kg ha^{-1} has any
322 benefits for canola production in the winter rainfall region of South Africa's Western Cape. There
323 were no detectable differences in yield or harvest index, and only minor differences in plant
324 biomass and LAI (Figures 1 and 2). This finding concurs with other recent and ongoing trials in
325 the region, and may be a result of a regional switch to conservation agriculture practices over the
326 last twenty years. Conservation agriculture practices including reduced tillage, maintenance of
327 crop residues, and crop rotation can increase soil organic matter and thus increase rates of N
328 mineralization (Plaza et al., 2013; McDaniel et al., 2014), so it is possible these practices may have
329 reduced canola N requirements over time. Current fertilizer guidelines for canola in the Western
330 Cape were based on conventional tillage systems. These were determined the from target yield
331 potential of canola for specific regions (a result of in-season rainfall), taking soil texture and crop
332 rotation into consideration (Fertasa, 2016). However, preliminary work to re-evaluate fertilizer
333 guidelines for conservation agriculture systems indicates that total seasonal N rates above 50 to 75
334 kg ha^{-1} would not be recommended due to the risk of leaching and low N use efficiencies of canola
335 (du Toit, 2018).
336 Splitting the fertilizer into two or three applications throughout the season was
337 advantageous compared to applying all fertilizer by 30 DAE, in terms of reducing N loss to the
338 environment. It did not result in yield differences in our study, but other studies have observed a
339 higher canola yield quality (increased oil percentage) when N availability is higher later in the
340 season (du Toit, 2018; Swanepoel et al., 2019). The reduced levels of N observed later in the
341 season under a single application was not compensated for by an increase in crop growth in these

342 treatments, indicating that the N has moved elsewhere. Some N may have been immobilized by
343 microbial activity, but leaching is also likely as N in the form of nitrate is readily soluble in water
344 and thus carried away through rainwater infiltration or surface water run-off. This can have
345 detrimental consequences in particular for surrounding aquatic ecosystems, with fertilizer run-off
346 a major contributor to eutrophication and algal blooms in both freshwater and marine environments
347 (Seitzinger and Phillips, 2017). Excess N fertilizer can also cause problems for crop production by
348 promoting weeds. Weeds increase as nutrient availability increases (MacLaren et al., 2019), and
349 so a high dose of N applied early in the season when crops are too small to capture it can be
350 expected to promote the establishment of weeds that will become competitive with crops later in
351 the season as resource availability diminishes.
352
353 ***Effects of N fertilizer on soil bacterial community composition and function***
354 The results of this study suggest that applying more N fertilizer alters the soil-bacteria community
355 composition, which can affect soil carbon and N cycling (Buchkowski et al. 2015). Increased N
356 appeared to shift the community toward a group of bacteria that are less reliant on N-containing
357 carbon sources (amines and amino acids; Figure 4). Fierer et al., (2012) also observed that the
358 bacterial communities become less reliant on organic forms of N as N fertilization increased,
359 suggesting a shift from oligotrophic to copiotrophic communities. Bacteria typically require a 25:1
360 carbon:nitrogen ratio, and so where sufficient mineral N is available to meet this need, bacteria
361 can decompose carbon-rich organic molecules more readily. In contrast, if mineral N availability
362 is low, then bacteria are limited in the amount of organic matter they can decompose by the need
363 to acquire organic N. Such conditions also promote N release through mineralization (Mengel,
364 1996).
365 Abundant N, therefore, could increase the capacity of the bacterial community to
366 metabolize soil carbon and release it into the atmosphere. This outcome would not be beneficial

367 to either farmers (loss of soil organic matter) or the environment (carbon emissions) (Hasselquist
368 et al., 2012). It is not yet clear whether N fertilizer universally increases carbon emissions, as other
369 studies have observed that the addition of N in some instances negatively affects soil respiration,
370 leading to an overall increase in carbon sequestration (Janssens et al., 2010). N fertilizer can reduce
371 both microbial biomass as well as activity, particularly in bacteria (Demoling et al., 2008), and
372 mainly in the presence of recalcitrant organic matter, while positive effects are observed when N
373 is added to easily degradable organic material (Fog, 1988). This study measured neither *in situ*
374 bacterial biomass nor activity and so further research will be necessary to determine if high N rates
375 do release carbon from South African soils.

376 In cases where N fertilizers do reduce carbon emissions by suppressing bacterial activity,
377 then a negative effect on other functions can also be expected. This includes N mineralization, an
378 important pathway by which N is released from soil organic matter and made available to plants.
379 High N levels tend to reduce mineralization in general, whether or not they suppress bacterial
380 activity, as microbes tend to take up more mineral N when it is in high quantities, thus delaying
381 the mineralization of N from organic sources (Fog, 1988; Zhou et al., 2012).

382 Overall, a bacterial community that is more adapted to decomposing N-containing carbon
383 sources in the presence of low mineral N would therefore be expected to release more N through
384 mineralization per unit of carbon respired. This quality could promote sustainability through
385 maximising nitrogen availability to crops via mineralization, while minimising carbon lost from
386 soil to the atmosphere. Our study suggests that such communities can be promoted by restricting
387 the amount of N fertilizer. If the amount of N supplied to crops through mineralization rather than
388 fertilization can be increased, this could also reduce the large carbon footprint associated with
389 synthetic N fertilizers (Lal, 2004).

390 Despite the observed shift in carbon source utilization in this study, there was no effect of
391 fertilizer amount or distribution on either N fixation potential (number of nifH gene copies) or

392 bacterial community diversity. This contrasts with other studies, which have shown that N
393 fertilization can reduce the abundance of rhizobia (Ledgard, 2001) and free-living diazotrophs (Orr
394 et al., 2011; Compton et al., 2004). N fertilization has also been observed to overall community
395 diversity (Ramirez et al., 2010, Coolon et al., 2013; Wang et al., 2015) and activity (Kennedy et
396 al., 2004, Demoling et al., 2008). This negative effect of N on microbial activity is mainly found
397 in the presence of recalcitrant organic matter, while a positive effect is observed when N is added
398 to easily degradable organic material (Fog, 1988). That N fertilizer tends to raise soil pH is at least
399 partially responsible for such trends (Kennedy et al., 2004; Lauber et al., 2009; Wang et al., 2015).
400 Such effects were not observed in this study, possibly as a result of the history of the trial sites.
401 Perhaps local diversity and N-fixing bacteria were already depleted after decades of intensive
402 cropping. Bacterial diversity and community composition may also depend on other soil qualities
403 (Williams et al., 2013) and it is not known whether these were limiting at any or all of the sites in
404 the present study. The effect of adding N to a microbial system remains difficult to model and
405 explain (Hasselquist et al., 2012; Janssens et al., 2010), with different studies often producing
406 apparently contradictory results (e.g. Williams et al., 2013; van der Bom et al., 2018). This
407 emphasizes the importance of continuing studies to elucidate the complex relationships between
408 farm management, microbial communities, and carbon and nutrient cycling. However, increased
409 awareness of the effect of inorganic N on microbial biomass and activity will inform better
410 management practices.
411

412 ***Conclusion***

413 This study suggests that applying less N fertilizer more often over a cropping season is optimal for
414 both crop production and environmental protection, and may help to sustain the capacity of the
415 soil bacterial community to contribute to both. Applying 150 kg ha^{-1} of N fertilizer compared to
416 60 kg ha^{-1} in this study did not increase canola yield, and splitting the fertilizer into three

417 applications throughout the season reduced N losses, which may have been due to leaching and/or
418 bacterial immobilisation. This increased the soil mineral N available later in the season, where it
419 may contribute to higher yield quality. Furthermore, applying a large amount of N fertilizer
420 appeared to shift the community toward taxonomic groups that are more prone to immobilize soil
421 N and release soil carbon.

422 Applying a high level of N fertilizer is thus a 'lose-lose-lose' situation for farmers, their
423 soil ecosystems, and the environment. Fortunately, farmers in the Western Cape tend to apply 70-
424 90 kg ha^{-1} and some do split it over two or three applications in the season. This study confirms
425 the wisdom of such practices: canola does not use additional N, and excess N may affect the
426 functioning of agricultural soil and threaten natural ecosystems. Ongoing trials in the region will
427 assess the response of canola to a greater range of fertilization strategies (du Toit, 2018) to allow
428 the optimal amount and distribution of N fertilizer for canola to be refined.

429

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440

441

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598 **FIGURES**

599 **Figure 1:** Soil mineral nitrogen (N) and canola biomass and leaf area index (LAI) in each
600 treatment at 60, 90 and 120 days after emergence (DAE). LAI was not measured at 120 DAE.
601 Labels along the x-axis refer to the different treatments: 'Cont' = Control, and other labels
602 indicate the total N rate in kg ha^{-1} (60 or 150) and the number of applications in which this was
603 applied (1, 2 or 3). Soil samples were taken prior to fertilization. Error bars indicate the standard
604 error of the mean. Lowercase letters above the bars indicate pairwise differences significant at
605 P=0.05, based on contrasts estimated from the ANOVAs in Table 4. Treatments that differ do not
606 share the same letter. Shading denotes the control treatment (pale), the 60 kg N ha^{-1} treatments
607 (medium) and the 120 kg N ha^{-1} treatments (dark). Note the log scales on the y-axes.

608

609 **Figure 2:** Canola yield in response to N fertilizer rates and timing. Labels along the x-axis refer
610 to the different treatments: 'Cont' = Control, and other labels indicate the total N rate in kg ha^{-1}
611 (60 or 150) and the number of applications in which this was applied (1, 2 or 3). Lowercase letters
612 indicate pairwise differences significant at P=0.05, based on contrasts estimated from the
613 ANOVAs in Table 5. Treatments that differ do not share the same letter. Shading denotes the
614 control treatment (pale), the 60 kg N ha^{-1} treatments (medium) and the 120 kg N ha^{-1} treatments
615 (dark).

616

617 **Figure 3:** The NMS ordination of the OTUs of bacterial DNA extracted from soil in each
618 treatment at the end of the season. The amount of N applied to each plot is denoted by shading,
619 while the number of N applications is not indicated as the community did not differ in relation to
620 application number.

621

622 **Figure 4:** Axes 1 and 2 (a) and 1 and 3 (b) of the NMS ordination of carbon source utilization by
623 bacterial communities from soil in each treatment at the end of the season. The amount of N
624 applied to each plot is denoted by shading (see legend), while the number of N applications nor
625 farm are indicated as these did not affect carbon utilization. The blue letters indicate the mean
626 centroid of each N amount treatment (H = high; 150 kg N ha^{-1}, L = low; 60 kg N ha^{-1} and N =
627 none; control), and the blue circle indicates the standard error of that mean. The boxed labels refer
628 to the carbon sources most used by sites in that part of the ordination. Labels further away from
629 the centre of the plot indicate a greater change in the use of that carbon source in that direction.
630 a. acids = amino acids; c. acids = carboxylic acids; carbs = carbohydrates; phos.cpds =
631 phosphorylated compounds
632

633 **Table 1:** Soil chemical and physical characteristics of the research sites, Langgewens Research
634 Farm, Altona and Roodebloem. Samples were taken prior to onset of the trial and were taken to a
635 depth of 150 mm.

	Langgewens	Altona	Roodebloem
pH(KCl)	5.8	6.3	5.8
Calcium (mg kg^{-1})§	944	1572	1250
Magnesium (mg kg^{-1})§	160	191	204
Potassium (mg kg^{-1})§	230	209	463
Phosphorus (mg kg^{-1})§	111	73	116
Sulphur (mg kg^{-1}) ¶	32	25	8.8
Mineral Nitrogen (mg kg^{-1})*	880	3040	2080
Organic Carbon (%)	0.94	1.11	1.58
Textural class	Sandy loam	Sandy loam	Sandy loam
Sand (%)	63	57	71
Slit (%)	30	18	14
Clay (%)	7	25	15

636 §Citric acid extraction (Non-affiliated Soil Analysis Work Committee, 1990)
637 ¶Calcium phosphate extraction (Non-affiliated Soil Analysis Work Committee, 1990)
638 *Indophenol-blue and salicylic acid methods (Cataldo et al., 1975; Keeney et al., 1982)
639

640 **Table 2:** Nitrogen fertilization rates and distribution for canola production at planting, 30 days
641 after emergence (DAE), 60 DAE and 90 DAE. Treatment 0 is the control, which received no
642 nitrogen (N) fertilization throughout the season.

Treatment		Amount of N applied at each time point (kg ha^{-1})			
Rate (kg N ha^{-1})*	Number of applications**	At planting	30 DAE	60 DAE	90 DAE
0	**0**	0	0	0	0
60	**1**	20	40	0	0
60	**2**	20	20	20	0
60	**3**	20	13.3	13.3	13.3
150	**1**	20	130	0	0
150	**2**	20	65	65	0
150	**3**	20	43.3	43.3	43.3

643 * Refers to the total amount applied over the whole season
644 **Refers to the number of applications after planting, as all treatments (except the control)
645 received 20 kg ha^{-1} N at planting, following common practice in the region.
646

647

648

649 **Table 3:** Summary of variables measured in this trial and the time point(s) at which they were
650 measured. DAE = Days after emergence

Response variables	**60 DAE**	**90 DAE**	**Physiological maturity/harvest (approximately 150 DAE)**
Soil N and canola growth measurements	Soil mineral N Biomass LAI	Soil mineral N Biomass LAI	Soil mineral N Biomass production Yield Harvest index Nitrogen (crude protein)
Bacterial community measurements			OTUs (ARISA) Carbon utilization nifH gene copies

651

652

653 **Table 4:** ANOVA F-statistics and P-values for the fixed effects in the mixed models of soil mineral
654 N and canola leaf area index and biomass, and standard deviations for each random effect and the
655 residual. Significant P-values <0.05 for fixed effects are emphasized in bold. Where cells are
656 blank, data was not collected for that response at that time point. DAE=days after emergence.

Time point	Effect type	Log soil mineral N			Log leaf area index			Log biomass		
60 DAE	**Fixed effects**	F statistic	*df*	P value	F statistic	*df*	P value	F statistic	*df*	P value
	Control vs treated	1.888	66	0.174	4.518	65	**0.037**	4.064	65	**0.048**
	N amount	0.488	66	0.487	10.344	65	**0.002**	11.721	65	**0.001**
	N distribution	2.561	66	0.085	1.654	65	0.199	1.89	65	0.159
	Interaction*	0.847	66	0.433	4.964	65	**0.01**	6.34	65	**0.003**
	Random effects	Standard deviation			Standard deviation			Standard deviation		
	Replicate	0.078			0.000			0.000		
	Farm	0.285			0.456			0.706		
	Residual	0.356			0.268			0.442		
90 DAE	**Fixed effects**	F statistic	*df*	P value	F statistic	*df*	P value	F statistic	*df*	P value
	Control vs treated	26.101	66	**<0.001**	7.391	65	**0.009**	3.954	65	**0.051**
	N amount	3.732	66	0.058	5.772	65	**0.019**	10.213	65	**0.002**
	N distribution	6.884	66	**0.002**	1.626	65	0.205	1.481	65	0.235
	Interaction*	0.535	66	0.588	1.253	65	0.293	3.204	65	**0.047**
	Random effects	Standard deviation			Standard deviation			Standard deviation		
	Replicate	0.046			0.104			0.000		
	Farm	0.412			0.249			0.202		
	Residual	0.359			0.400			0.551		
Physio-logical maturity	**Fixed effects**	F statistic	*df*	P value	F statistic	*df*	P value	F statistic	*df*	P value
	Control vs treated	37.97	66	**<0.001**				2.425	65	0.124
	N amount	8.541	66	**0.005**				1.885	65	0.175
	N distribution	14.867	66	**<0.001**				0.338	65	0.714
	Interaction*	4.713	66	**0.012**				0.977	65	0.382
	Random effects	Standard deviation			Standard deviation			Standard deviation		
	Replicate	0.155						0.059		
	Farm	0.469						0.262		
	Residual	0.334						0.366		

657 *The N amount x N distribution interaction.

658 **Table 5:** ANOVA F-statistics and P-values for the fixed effects in the mixed models of canola
659 yield, harvest index and tissue N content, and standard deviations for each random effect and the
660 residual. Significant P-values (< 0.05) for fixed effects are emphasized in bold. Where cells are
661 blank, data was not collected for that response at that time point. DAE=days after emergence.

	Yield			**Harvest index**			**Tissue N content**		
Fixed effects	F statistic	*df*	P value	F statistic	*df*	P value	F statistic	*df*	P value
Control vs treated	20.575	65	**<0.001**	0.017	65	0.896	1.251	64	0.267
N amount	1.058	65	0.308	1.634	65	0.206	1.537	64	0.22
N distribution	0.013	65	0.987	0.557	65	0.576	0.243	64	0.785
Interaction*	0.032	65	0.969	1.315	65	0.275	0.138	64	0.871
Random effects	Standard deviation			Standard deviation			Standard deviation		
Replicate	0.000			0.000			0.064		
Farm	0.481			0.094			0.187		
Residual	0.956			0.085			0.247		

662 *The N amount x N distribution interaction.
663

664 **Table 6:** ANOVA F-statistics and P-values for the amount and distribution of nitrogen (N) in the
665 mixed models of bacterial richness, Shannon diversity and copies of the nifH gene in the soil when
666 the canola reached physiological maturity. Significant P-values <0.05 are emphasized in bold.

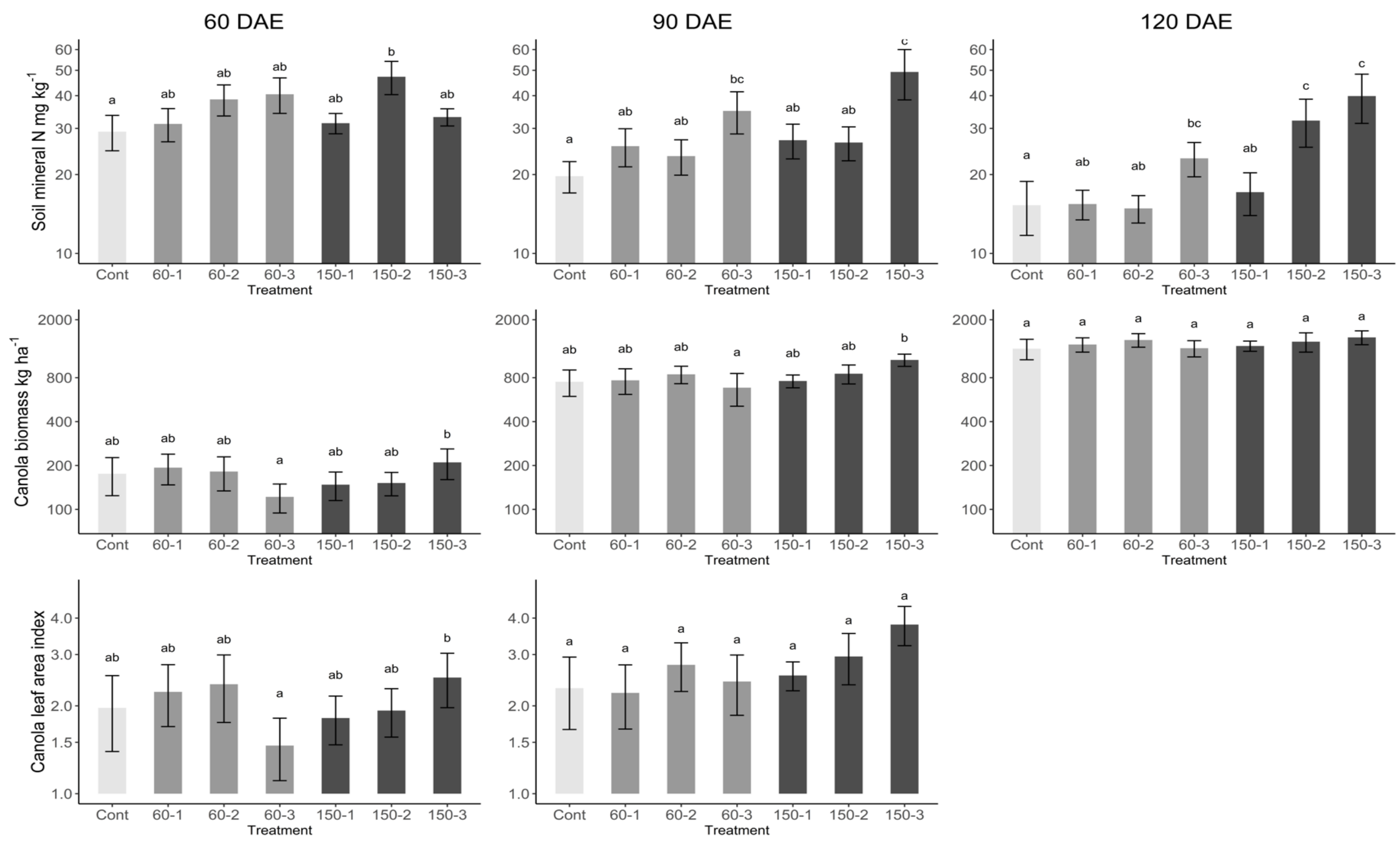
	OTU Shannon diversity			**OTU richness**			**nifH**		
Fixed effects	F statistic	*df*	P value	F statistic	*df*	P value	F statistic	*df*	P value
Control vs treated	1.598	58	0.211	1.513	58	0.224	0.002	75	0.969
N amount	0.162	58	0.689	0.224	58	0.638	0.714	75	0.401
N distribution	1.459	58	0.241	2.579	58	0.084	2.324	75	0.106
Interaction*	1.748	58	0.183	3.22	58	**0.047**	1.513	75	0.228
Random effects	Standard deviation			Standard deviation			Standard deviation		
Replicate	0.000			0.00			0.0		
Farm	0.796			12.35			629.4		
Residual	1.091			14.98			1059.0		

667 *The N amount x N distribution interaction.
668

669 **Table 7:** PERMANOVA F-statistics and P-values for the dissimilarity matrices of bacterial DNA
670 and carbon source functional composition. The PERMANOVAs were calculated on the same
671 Bray-Curtis dissimilarity matrices used for the NMDS ordinations (Figures 4 and 5). Significant
672 P-values <0.05 are emphasized in bold.

	Bacterial DNA		**Community function**	
	PERMANOVA F	P Value	PERMANOVA F	P Value
Control vs treated	1.167	0.079	1.8	0.124
N amount	1.552	**0.007**	2.564	**0.051**
N distribution	0.875	0.316	1.248	0.272
Interaction*	1.185	**0.031**	1.468	0.169

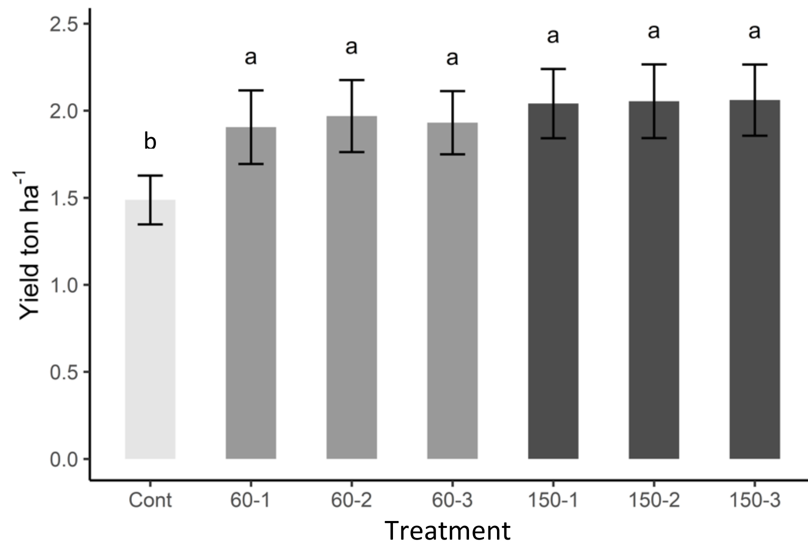
673 *The N amount x N distribution interaction.



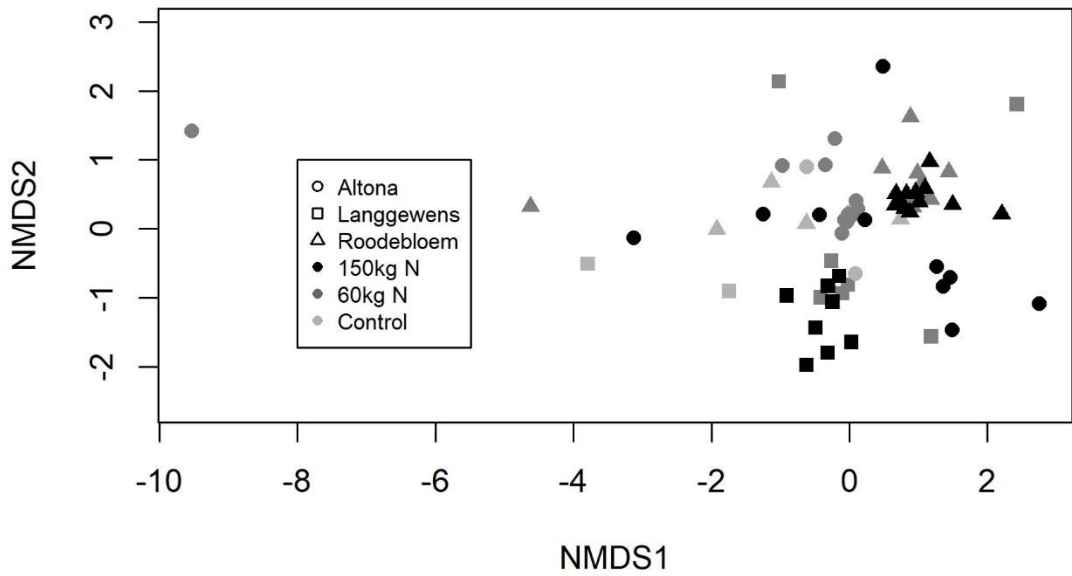
1

2 **Figure 1**

3



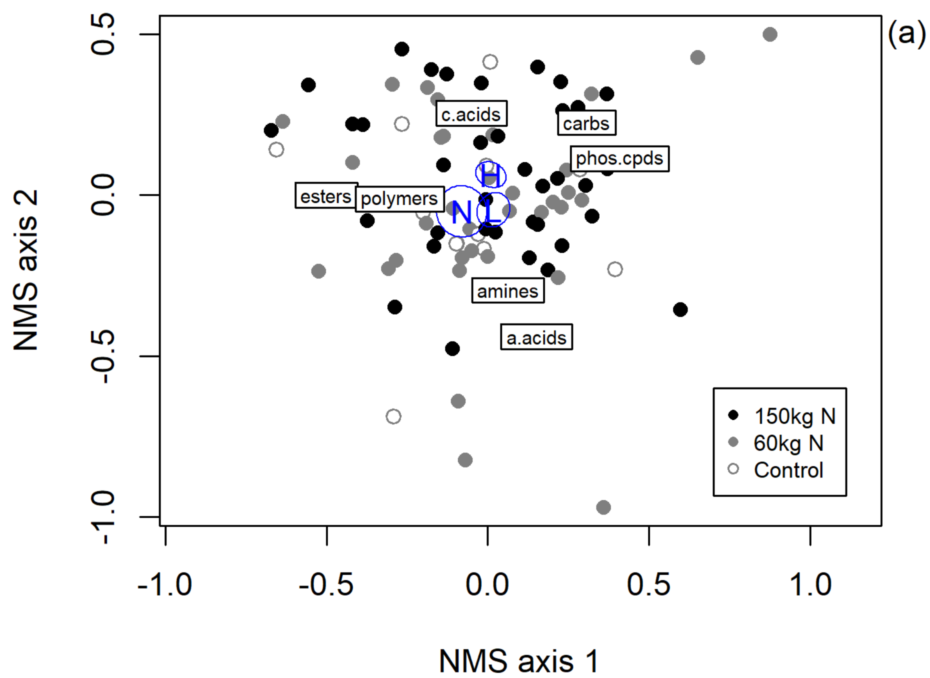
1
2 **Figure 2**



1

2 **Figure 3**

3



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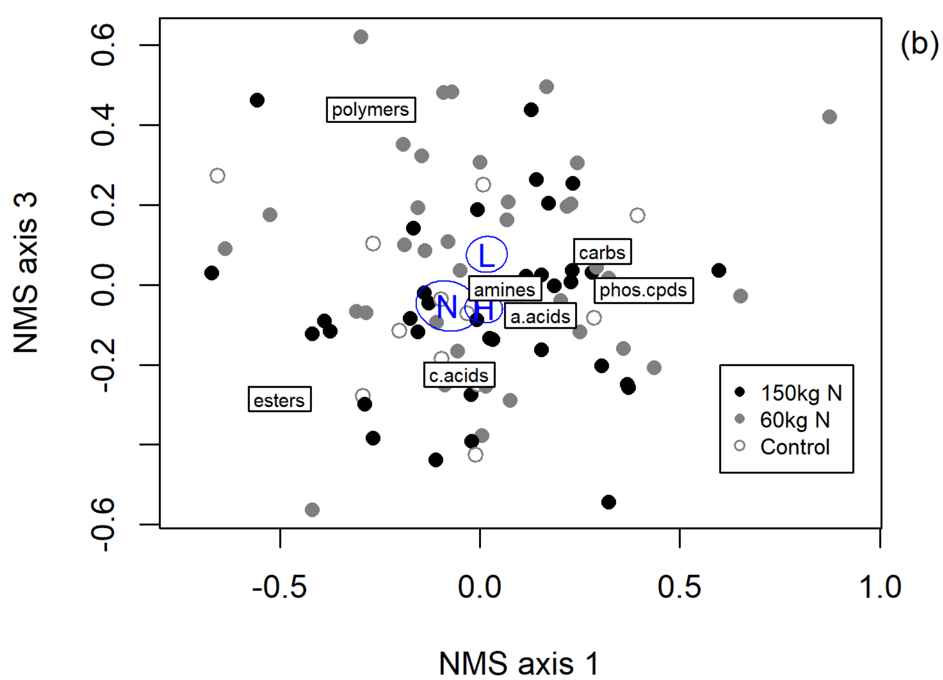

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3 **Figure 4**