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

F Becker¹, C MacLaren¹,², CJ Brink³, K Jacobs³, MR le Roux¹, PA Swanepoel¹*  
¹Department of Agronomy; Stellenbosch University  
²Sustainable Agricultural Sciences, Rothamsted Research  
³Department of Microbiology, Stellenbosch University  
*Corresponding Author: pieterswanepoel@sun.ac.za; +27218084668

ORCID IDs  
CM: 0000-0002-6700-3754; KJ: 0000-0003-3972-5343; CB: 0000-0003-2607-6831; MR: 0000-0002-6473-237X PS: 0000-0002-6481-0673

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Core ideas

- Over-fertilization of N is common in canola in South Africa
- We evaluated N fertilization rates and application timings for canola
- Canola growth and yield were generally not affected by N rates or timing
- A low N rate favoured soil bacterial communities more likely to mineralize N
- A low N rate (60 to 90 kg ha⁻¹) split into two or three applications can sustain yield and have minimal effects on soil bacterial functioning

Abbreviations: ARISA = Automated Ribosomal Intergenic Spacer Analysis; CLPP = Community level physiological profiling: carbon source utilization; DAE = days after emergence; LAI = leaf area index; NMS = non-metric multidimensional scaling; OTU = operational taxonomic unit
Abstract

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

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

Canola (*Brassica napus*) was introduced into crop rotation systems of South Africa in 1994 to increase crop diversity (BFAP 2018). Canola production in South Africa is growing, and according to predictions made by the Bureau for Food and Agricultural Policy (BFAP 2018), will increase to ca. 200,000 tons by 2027. Canola has a higher N demand than most other cash crops (Ma and Herath, 2015), but dependence on inorganic N fertilizers to increase canola production is not sustainable and, therefore, attention should be shifted to retaining and fixing N on-farm through optimising soil biological activity. Moreover, due to the lack of robust guidelines tailored for the South African climate and soil conditions, N fertilization is often applied injudiciously, which may have negative impacts on the finely balanced interactions in the soil environment. There is a need to understand how much inorganic N is necessary to produce satisfactory canola yields in the region, and how different fertilization strategies affect soil biological function.
The relationship between soil microbial diversity, ecosystem functioning, associated services, and management practices (e.g. N fertilization) is under increasing scrutiny to elucidate the complexities that underpin the productivity of agroecosystems (Brussaard et al., 2007; Hartmann et al., 2015; Hartman et al., 2018). Increased biodiversity in the microbial community may enhance the functional capacity of the soil ecosystem (Bender et al., 2016). Thus, the use of agricultural management practices that can maintain soil functional diversity is advocated in order to build inherent resilience to environmental shocks. The same motive has driven the implementation of crop rotation systems in the Western Cape (Venter et al., 2017).

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

Our understanding of the effect of N fertilization of canola on soil bacterial communities is currently limited. The overall aim of this study was to evaluate different N fertilization rates and N distribution for canola production in South Africa, but it specifically seeks to determine the effect of fertilizer N application on the composition and diversity of soil bacterial communities. The relationships between different N fertilizer application strategies, the soil bacterial
community, and canola growth and yield were assessed. The effects of both the quantity of N fertilizer and whether it was all applied at once or distributed throughout the season (at planting and as a top-dressing) were investigated.

Materials and methods

Trial location

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

Experimental design

The trials followed a crossed full factorial design, with treatments receiving either 60 kg ha\(^{-1}\) or 150 kg ha\(^{-1}\) of N, of which 20 kg ha\(^{-1}\) was applied at planting and the remainder distributed in either one, two or three applications later in the season (at 30, 60 and 90 days after emergence; DAE). A null control was also included, which received no N at any point in the season, so the
trial included seven treatments in total (Table 2). These were laid out in a randomized complete
block design, with four replicates at each of the three farms. Plots were 2.75 x 5 m. Half of each
plot was intended for destructive measurements (sampling of plants), while the other half was used
for yield determination. Measures of canola plant production and soil bacterial community
composition and function were taken in each plot at various time points throughout the season
(Table 3). The methods for each of these are detailed in the following sections.

**Trial management**

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

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

**Soil nitrogen content analysis**

Soil cores (ø 45 mm) were taken to a depth of 150 mm. Three sub-samples were taken from each plot and combined to form a single composite sample per plot, then air-dried at room temperature, and sieved with a 1 mm sieve. Samples were taken 30, 60, 90 DAE and at when the canola reached physiological maturity (approx. 150 DAE). Soil samples were analysed for ammonium and nitrate content using the indophenol-blue (Keeney et al., 1982) and salicylic acid methods (Cataldo et al., 1975) respectively. Total soil mineral N (kg ha$^{-1}$) was calculated as ammonium plus nitrate, which is the N readily available to plants. To convert the total mineral N concentration (mg kg$^{-1}$) to stock (kg ha$^{-1}$) a bulk density of 1400 kg m$^{-3}$ was used, which is the average bulk density for the region reported by (de Clercq et al., 2013).

**Soil bacterial community analysis**

Soil cores (ø 45 mm) were taken to a depth of 150 mm using a stainless steel pipe and a hammer. The pipe was washed and sterilized with 70 % ethanol between sampling of different plots. Samples were collected at canola physiological maturity only.

**Automated Ribosomal Intergenic Spacer Analysis (ARISA)**

The bacterial community composition within each sample was determined with Automated Ribosomal Intergenic Spacer Analysis (ARISA) (Ranjard et al., 2001). DNA was extracted from 0.25 g of soil using the Zymo research soil microbe DNA MicroPrep™ kit (Zymo research USA). Extracted and purified DNA was separated on a 1 % agarose gel stained with ethidium bromide to visualize under ultraviolet light.
The polymerase chain reaction (PCR) reactions were performed on the purified DNA using ITSReub (5'-GTCGTAACAAGGTAGCCGTA-3') and FAM (carboxy-fluorescein) labelled ITSF (5'-GCCAAGGCATCCACC-3') primer set for the 16S rRNA intergenic spacer region to determine bacterial diversity using ARISA (Cardinale et al. 2004, Slabbert et al., 2010b). PCR reactions were done using a 2720 Thermal Cycler (Applied Biosystems, USA). The reaction mixture contained 0.5 µl purified genomic DNA, 500 nM of each primer, 4.1 µl PCR grade water (nuclease free) and 5 µl KapaTaq readymix (Kapa Biosystems, South Africa) for a total volume of 10 µl. The PCR consisted of an initial denaturing step of 5 minutes at 94°C, followed by 40 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 extension at 72°C for 7 minutes and then cooled and held at 4°C. All the samples were done in triplicate and pooled to compensate for PCR bias.

The PCR products of the pooled samples were run on an ABI 3010xl Genetic Analyser to obtain an electropherogram of different fragment lengths and fluorescent intensities. Bacterial ARISA samples were run along the LIZ 1200 size standard which is designed for sizing DNA fragments in the 100 – 1200 base pair (bp) range. Fluorescence intensities were converted to electropherograms using the Genemapper 5 software. The peaks on the electropherogram represent different fragments of different sizes, termed operational taxonomic units (OTUs), and the heights of the peaks indicate relative abundance of the fragments. The lengths were calculated by plotting a best fit curve using the size standard and extrapolating the fragment size from the sample. Only fragment sizes between 100 and 1000 base pairs and peak heights above 150 fluorescent units were used for analysis as OTU’s. A bin size of 3 bp was used to minimize inaccuracies of the ARISA profile (Brown et al., 2005; Slabbert et al., 2010b).

Community level physiological profiling (CLPP): carbon source utilization
The CLPP was done by determining the carbon source utilization of the soil bacterial community. Soil samples were diluted in distilled nuclease free water and inoculated, in triplicate, into Biolog EcoPlates™ (Biolog Inc., USA). The plates contain 31 different C sources in different wells and a control well containing no C source. Plates were incubated at 28°C. Utilization of the C sources by microbial populations reduce the tetrazolium dye inside the plate wells that cause a colour change. This colour change was measured twice daily over a period of 5 to 10 days with a spectrophotometer at 590 nm to determine the average well colour development (AWCD).

**Nitrogen fixation capacity: number of nifH gene copies**

The *nifH* PCR product from a *Burkholderia* strain was used for preparation of the standards as well as a positive control. The PCR product was purified using GeneJET PCR Purification Kit (Thermo Scientific). The PCR product size of 380 bp was verified by electrophoresis on a 1 % agarose gel. The purified PCR products were quantified using a μLITE (Biodrop, Cambridge, UK) and the *nifH* gene copy number was determined using the fragment length, molecular weight and Avogadro’s number. The known concentration of the PCR product was used to prepare a standard curve, in triplicate, to measure *nifH* gene copy numbers. The *nifH* gene copy numbers of the soil samples were quantified by using quantitative PCR (qPCR) using the *nifH* F1 and *nifH* 438r primers (Boulygina et al., 2002; De Meyer et al., 2011). The qPCR assays were performed using the LightCycler 96 (Roche) with a SYBR Green 1 fluoroprobe as the protocol suggested (Brink et al., 2019).

A standard curve was generated for every qPCR run ranging from 1x10^10 to 1x10^0 gene copies μL⁻¹. Each run also included a positive control as well as a negative control. The same soil DNA samples used for ARISA was used for qPCR and was run in duplicate. The reaction volume contained 3 μL nuclease free water, 2 μL nifH F1 (100 nM) and nifH 438r (100 nM) primers, 10 μL SYBR Green I Master Mix (2x) and 5 μL sample DNA as described by manufacturers.
specifications. The thermal cycle used for qPCR consisted of 95°C for 5 minutes followed by 40
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
40 cycles to verify specificity of amplicons. This analysis identified the number of nifH gene
copies per 5 μL of DNA, which was converted to the copy number per gram soil based on 100 μL
DNA per 0.25 g soil.

Canola growth and yield determination

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

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

\[
\text{Harvest index (\%)} = \frac{\text{Dry mass of harvest component}}{\text{Total biomass at harvest}} \times 100
\]

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

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

\[- \sum_i p_i \ln p_i\]

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

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

To assess bacterial community composition, NMDS ordination based on the Bray-Curtis dissimilarity measure was used for both OTUs and carbon sources utilized. PERMANOVA was
used to test for significant differences in composition between different amounts and distributions of N with farm as a grouping variable, to detect effects of these variables within potentially different bacterial communities on different farms. It is currently not possible with available software to nest grouping variables in PERMANOVA, otherwise replicate could have been used as a grouping variable too. Data analysis was undertaken in R, version 3.4.3 (R Core Team, 2017).

Results

Soil mineral nitrogen and canola production

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

Canola biomass and LAI responded slightly to the amount of N applied and its distribution (Table 4), but were generally not different between treatments (Figure 1). When N fertilizer was distributed in three applications compared to one or two applications, biomass and LAI tended to be lower at 60 and 90 DAE for 60 kg ha\(^{-1}\) treatments, but higher in the 150 kg ha\(^{-1}\) treatments at 60 and 90 DAE. It is possible that treatment 60(3) did not receive sufficient N for full growth prior to 90 DAE, whereas a more even distribution of fertilizer was advantageous where more N was applied in treatment 150(3). However, these small differences in plant growth did not result in any significant differences in either canola harvest index or plant tissue N content at physiological maturity between the treatments, and only the control differed (P <0.05) from the other treatments (Table 5, Figure 2). In terms of random effects, variability between replicates tended to be very low, while variability within plots of the same treatment at the same site (the residual error term) was often similar or larger than the variability between farms (Tables 3 and 4), indicating relatively high within-site and within-replicate variability that could not be explained by the treatments.
The lack of substantial differences in canola growth and yield indicates that uptake of N by canola does not explain the reduced soil mineral N observed later in the season in treatments where all N fertilizer was applied early (Figure 1). It can therefore be assumed that the N is lost from the system (perhaps to leaching, bacterial immobilisation or weeds) and not incorporated into crop plant matter.

**Bacterial community composition, diversity and function**

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

The NMS ordination and PERMANOVA for carbon source utilization suggest that the amount of N fertilizer applied may alter microbial function (Table 7, Figure 4). Plots that received more N tend to be shifted higher along axes 1 and 2 of the NMS, which is associated with higher use of carbohydrates, phosphorylated compounds and carboxylic acids and lower use of amino acids, amines, esters and polymers. Amines and amino acids always contain N, while some esters and polymers do, so it is possible that lower mineral N availability favours bacteria that can break down N-containing carbon sources and thus extract N as well as C from those sources, to meet their N needs. In particular, Figure 4(a) shows a greater association of plots receiving 150 kg N
ha\(^{-1}\) with non-N-containing carbon sources, and Figure 4(b) suggests a higher affinity for polymers of control plots and plots receiving 60 kg N ha\(^{-1}\).

Discussion

**Optimising fertilization strategy for canola growth**

This study provided no evidence that increasing N fertilizer from 60 kg ha\(^{-1}\) to 150 kg ha\(^{-1}\) has any benefits for canola production in the winter rainfall region of South Africa’s Western Cape. There were no detectable differences in yield or harvest index, and only minor differences in plant biomass and LAI (Figures 1 and 2). This finding concurs with other recent and ongoing trials in the region, and may be a result of a regional switch to conservation agriculture practices over the last twenty years. Conservation agriculture practices including reduced tillage, maintenance of crop residues, and crop rotation can increase soil organic matter and thus increase rates of N mineralization (Plaza et al., 2013; McDaniel et al., 2014), so it is possible these practices may have reduced canola N requirements over time. Current fertilizer guidelines for canola in the Western Cape were based on conventional tillage systems. These were determined the from target yield potential of canola for specific regions (a result of in-season rainfall), taking soil texture and crop rotation into consideration (Fertasa, 2016). However, preliminary work to re-evaluate fertilizer guidelines for conservation agriculture systems indicates that total seasonal N rates above 50 to 75 kg ha\(^{-1}\) would not be recommended due to the risk of leaching and low N use efficiencies of canola (du Toit, 2018).

Splitting the fertilizer into two or three applications throughout the season was advantageous compared to applying all fertilizer by 30 DAE, in terms of reducing N loss to the environment. It did not result in yield differences in our study, but other studies have observed a higher canola yield quality (increased oil percentage) when N availability is higher later in the season (du Toit, 2018; Swanepoel et al., 2019). The reduced levels of N observed later in the season under a single application was not compensated for by an increase in crop growth in these
treatments, indicating that the N has moved elsewhere. Some N may have been immobilized by microbial activity, but leaching is also likely as N in the form of nitrate is readily soluble in water and thus carried away through rainwater infiltration or surface water run-off. This can have detrimental consequences in particular for surrounding aquatic ecosystems, with fertilizer run-off a major contributor to eutrophication and algal blooms in both freshwater and marine environments (Seitzinger and Phillips, 2017). Excess N fertilizer can also cause problems for crop production by promoting weeds. Weeds increase as nutrient availability increases (MacLaren et al., 2019), and so a high dose of N applied early in the season when crops are too small to capture it can be expected to promote the establishment of weeds that will become competitive with crops later in the season as resource availability diminishes.

Effects of N fertilizer on soil bacterial community composition and function

The results of this study suggest that applying more N fertilizer alters the soil-bacteria community composition, which can affect soil carbon and N cycling (Buchkowski et al. 2015). Increased N appeared to shift the community toward a group of bacteria that are less reliant on N-containing carbon sources (amines and amino acids; Figure 4). Fierer et al., (2012) also observed that the bacterial communities become less reliant on organic forms of N as N fertilization increased, suggesting a shift from oligotrophic to copiotrophic communities. Bacteria typically require a 25:1 carbon:nitrogen ratio, and so where sufficient mineral N is available to meet this need, bacteria can decompose carbon-rich organic molecules more readily. In contrast, if mineral N availability is low, then bacteria are limited in the amount of organic matter they can decompose by the need to acquire organic N. Such conditions also promote N release through mineralization (Mengel, 1996).

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

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

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

Despite the observed shift in carbon source utilization in this study, there was no effect of fertilizer amount or distribution on either N fixation potential (number of nifH gene copies) or
bacterial community diversity. This contrasts with other studies, which have shown that N fertilization can reduce the abundance of rhizobia (Ledgard, 2001) and free-living diazotrophs (Orr et al., 2011; Compton et al., 2004). N fertilization has also been observed to overall community diversity (Ramirez et al., 2010, Coolon et al., 2013; Wang et al., 2015) and activity (Kennedy et al., 2004, Demeling et al., 2008). This negative effect of N on microbial activity is mainly found in the presence of recalcitrant organic matter, while a positive effect is observed when N is added to easily degradable organic material (Fog, 1988). That N fertilizer tends to raise soil pH is at least partially responsible for such trends (Kennedy et al., 2004; Lauber et al., 2009; Wang et al., 2015). Such effects were not observed in this study, possibly as a result of the history of the trial sites.

Perhaps local diversity and N-fixing bacteria were already depleted after decades of intensive cropping. Bacterial diversity and community composition may also depend on other soil qualities (Williams et al., 2013) and it is not known whether these were limiting at any or all of the sites in the present study. The effect of adding N to a microbial system remains difficult to model and explain (Hasselquist et al., 2012; Janssens et al., 2010), with different studies often producing apparently contradictory results (e.g. Williams et al., 2013; van der Bom et al., 2018). This emphasizes the importance of continuing studies to elucidate the complex relationships between farm management, microbial communities, and carbon and nutrient cycling. However, increased awareness of the effect of inorganic N on microbial biomass and activity will inform better management practices.

**Conclusion**

This study suggests that applying less N fertilizer more often over a cropping season is optimal for both crop production and environmental protection, and may help to sustain the capacity of the soil bacterial community to contribute to both. Applying 150 kg ha\(^{-1}\) of N fertilizer compared to 60 kg ha\(^{-1}\) in this study did not increase canola yield, and splitting the fertilizer into three
applications throughout the season reduced N losses, which may have been due to leaching and/or bacterial immobilisation. This increased the soil mineral N available later in the season, where it may contribute to higher yield quality. Furthermore, applying a large amount of N fertilizer appeared to shift the community toward taxonomic groups that are more prone to immobilize soil N and release soil carbon.

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

Acknowledgements

This study formed part of the National Research Foundation of South Africa’s Research Career Advancement Fellowship of M le Roux (RCA13100150915). The field experiment was a component of a larger trial that was funded by the Protein Research Foundation. The principal investigator of the larger field trial, Prof GA Agenbag, is acknowledged for allowing us to work within the trial that was funded by the Protein Research Foundation. The Western Cape Department of Agriculture is thanked for availing Langgewens Research Farm, Overberg Agri for Roodebloem and Mr V Laubscher for Altona. The technical support team under leadership of Mr M la Grange is thanked for the assistance with fieldwork. Mr J Habig (Agritechnovation) is thanked for the analyses of the C-source utilisation.
References


du Toit, E. 2018 Developing nitrogen fertiliser management strategies for canola (Brassica napus L.) under conservation agriculture practices in the Western Cape. Dissertation, Stellenbosch University, South Africa.


**FIGURES**

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

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

**Figure 3:** The NMS ordination of the OTUs of bacterial DNA extracted from soil in each treatment at the end of the season. The amount of N applied to each plot is denoted by shading, while the number of N applications is not indicated as the community did not differ in relation to application number.
Figure 4: Axes 1 and 2 (a) and 1 and 3 (b) of the NMS ordination of carbon source utilization by bacterial communities from soil in each treatment at the end of the season. The amount of N applied to each plot is denoted by shading (see legend), while the number of N applications nor farm are indicated as these did not affect carbon utilization. The blue letters indicate the mean centroid of each N amount treatment (H = high; 150 kg N ha⁻¹, L = low; 60 kg N ha⁻¹ and N = none; control), and the blue circle indicates the standard error of that mean. The boxed labels refer to the carbon sources most used by sites in that part of the ordination. Labels further away from the centre of the plot indicate a greater change in the use of that carbon source in that direction. a. acids = amino acids; c. acids = carboxylic acids; carbs = carbohydrates; phos.cpds = phosphorylated compounds
Table 1: Soil chemical and physical characteristics of the research sites, Langgewens Research Farm, Altona and Roodebloem. Samples were taken prior to onset of the trial and were taken to a depth of 150 mm.

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

\(\$\) Citric acid extraction (Non-affiliated Soil Analysis Work Committee, 1990)

\(\¶\) Calcium phosphate extraction (Non-affiliated Soil Analysis Work Committee, 1990)

\(\*\) Indophenol-blue and salicylic acid methods (Cataldo et al., 1975; Keeney et al., 1982)
Table 2: Nitrogen fertilization rates and distribution for canola production at planting, 30 days after emergence (DAE), 60 DAE and 90 DAE. Treatment 0 is the control, which received no nitrogen (N) fertilization throughout the season.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amount of N applied at each time point (kg ha(^{-1}))</th>
<th>Number of applications**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rate (kg N ha(^{-1}))*</td>
<td>At planting</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>60</td>
<td>3</td>
<td>20</td>
</tr>
<tr>
<td>150</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>150</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>150</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

*Refers to the total amount applied over the whole season

**Refers to the number of applications after planting, as all treatments (except the control) received 20 kg ha\(^{-1}\) N at planting, following common practice in the region.

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

<table>
<thead>
<tr>
<th>Response variables</th>
<th>60 DAE</th>
<th>90 DAE</th>
<th>Physiological maturity/harvest (approximately 150 DAE)</th>
</tr>
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<tbody>
<tr>
<td>Soil N and canola growth measurements</td>
<td>Soil mineral N Biomass LAI</td>
<td>Soil mineral N Biomass LAI</td>
<td>Soil mineral N Biomass production Yield Harvest index Nitrogen (crude protein)</td>
</tr>
<tr>
<td>Bacterial community measurements</td>
<td>OTUs (ARISA) Carbon utilization nifH gene copies</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4: ANOVA F-statistics and P-values for the fixed effects in the mixed models of soil mineral N and canola leaf area index and biomass, and standard deviations for each random effect and the residual. Significant P-values <0.05 for fixed effects are emphasized in bold. Where cells are blank, data was not collected for that response at that time point. DAE=days after emergence.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Effect type</th>
<th>Log soil mineral N</th>
<th>Log leaf area index</th>
<th>Log biomass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fixed effects</td>
<td>F statistic</td>
<td>df</td>
<td>P value</td>
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<tr>
<td>Control vs treated</td>
<td>1.888</td>
<td>66</td>
<td>0.174</td>
<td>4.518</td>
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<tr>
<td>N amount</td>
<td>0.488</td>
<td>66</td>
<td>0.487</td>
<td>10.344</td>
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<td>N distribution</td>
<td>2.561</td>
<td>66</td>
<td>0.085</td>
<td>1.654</td>
</tr>
<tr>
<td>Interaction*</td>
<td>0.847</td>
<td>66</td>
<td>0.433</td>
<td>4.964</td>
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<td>Random effects</td>
<td>Standard deviation</td>
<td>Standard deviation</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>Replicate</td>
<td>0.078</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Farm</td>
<td>0.285</td>
<td>0.456</td>
<td>0.706</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>0.356</td>
<td>0.268</td>
<td>0.442</td>
<td></td>
</tr>
<tr>
<td>Control vs treated</td>
<td>26.101</td>
<td>66</td>
<td><strong>&lt;0.001</strong></td>
<td>7.391</td>
</tr>
<tr>
<td>N amount</td>
<td>3.732</td>
<td>66</td>
<td>0.058</td>
<td>5.772</td>
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<tr>
<td>N distribution</td>
<td>6.884</td>
<td>66</td>
<td><strong>0.002</strong></td>
<td>1.626</td>
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<tr>
<td>Interaction*</td>
<td>0.535</td>
<td>66</td>
<td>0.588</td>
<td>1.253</td>
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<td>Standard deviation</td>
<td>Standard deviation</td>
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<tr>
<td>Replicate</td>
<td>0.046</td>
<td>0.104</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Farm</td>
<td>0.412</td>
<td>0.249</td>
<td>0.202</td>
<td></td>
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<td>Residual</td>
<td>0.359</td>
<td>0.400</td>
<td>0.551</td>
<td></td>
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<tr>
<td>Control vs treated</td>
<td>37.97</td>
<td>66</td>
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<td>2.425</td>
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<tr>
<td>N amount</td>
<td>8.541</td>
<td>66</td>
<td><strong>0.005</strong></td>
<td>1.885</td>
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<tr>
<td>N distribution</td>
<td>14.867</td>
<td>66</td>
<td><strong>&lt;0.001</strong></td>
<td>0.338</td>
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<td>Interaction*</td>
<td>4.713</td>
<td>66</td>
<td><strong>0.012</strong></td>
<td>0.977</td>
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<td>Replicate</td>
<td>0.155</td>
<td>0.059</td>
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<tr>
<td>Farm</td>
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<td>0.262</td>
<td></td>
<td></td>
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<tr>
<td>Residual</td>
<td>0.334</td>
<td>0.366</td>
<td></td>
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*The N amount x N distribution interaction.
Table 5: ANOVA F-statistics and P-values for the fixed effects in the mixed models of canola yield, harvest index and tissue N content, and standard deviations for each random effect and the residual. Significant P-values (< 0.05) for fixed effects are emphasized in bold. Where cells are blank, data was not collected for that response at that time point. DAE=days after emergence.

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

*The N amount x N distribution interaction.

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

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

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

<table>
<thead>
<tr>
<th></th>
<th>Bacterial DNA</th>
<th>Community function</th>
<th>Community function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PERMANOVA F</td>
<td>P Value</td>
<td>PERMANOVA F</td>
</tr>
<tr>
<td>Control vs treated</td>
<td>1.167</td>
<td>0.079</td>
<td>1.8</td>
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<tr>
<td>N amount</td>
<td>1.552</td>
<td><strong>0.007</strong></td>
<td>2.564</td>
</tr>
<tr>
<td>N distribution</td>
<td>0.875</td>
<td>0.316</td>
<td>1.248</td>
</tr>
<tr>
<td>Interaction*</td>
<td>1.185</td>
<td><strong>0.031</strong></td>
<td>1.468</td>
</tr>
</tbody>
</table>

*The N amount x N distribution interaction.
Figure 1
Figure 2
Figure 3
Figure 4