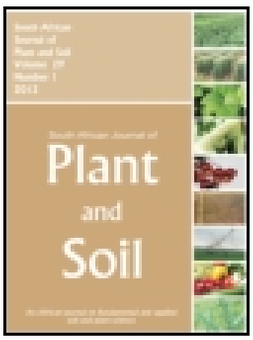

South African Journal of Plant and Soil

ISSN: 0257-1862 (Print) 2167-034X (Online) Journal homepage: http://www.tandfonline.com/loi/tjps20

Increasing crop diversity increased soil microbial activity, nitrogen-sourcing and crop nitrogen, but not soil microbial diversity

Zander S Venter , Samantha L Scott, Johann Strauss, Karin Jacobs & Heidi-Jayne Hawkins

To cite this article: Zander S Venter , Samantha L Scott, Johann Strauss, Karin Jacobs & Heidi-Jayne Hawkins (2017): Increasing crop diversity increased soil microbial activity, nitrogen-sourcing and crop nitrogen, but not soil microbial diversity, South African Journal of Plant and Soil, DOI: 10.1080/02571862.2017.1317852

To link to this article: http://dx.doi.org/10.1080/02571862.2017.1317852

Published online: 08 Jun 2017.

South African Journal of Plant and Soil 2017: 1–8
Printed in South Africa — All rights reserved

SOUTH AFRICAN JOURNAL OF
PLANT AND SOIL
ISSN 0257-1862 EISSN 2167-034X
http://dx.doi.org/10.1080/02571862.2017.1317852

Increasing crop diversity increased soil microbial activity, nitrogen-sourcing and crop nitrogen, but not soil microbial diversity

Zander S Venter[1]*, Samantha L Scott[2], Johann Strauss[3], Karin Jacobs[4] and Heidi-Jayne Hawkins[5,6]

[1] *Department of Animal Sciences, Stellenbosch University, Stellenbosch, South Africa*
[2] *Plant Conservation Unit, University of Cape Town, Cape Town, South Africa*
[3] *Directorate-Plant Sciences, Western Cape Department of Agriculture, Elsenburg, South Africa*
[4] *Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa*
[5] *Conservation South Africa, Centre for Biodiversity Conservation, Kirstenbosch National Botanical Gardens, Cape Town, South Africa*
[6] *Department of Biological Sciences, University of Cape Town, Cape Town, South Africa*
* *Corresponding author, email: zander.v@hotmail.com*

The relationships between crop diversity, soil microbial diversity and agroecosystem functioning are not well understood. Soils under wheat monoculture, wheat–medic, and wheat–medic plus clover rotations from a 19-year-old wheat rotation trial in South Africa were measured for soil microbial functional and genetic diversity using community-level physiological profiling and automated rRNA intergenic spacer analysis. A ^{15}N–^{13}C dual isotope mixing model determined the nitrogen (N) sourcing when wheat was supplied with N from fertiliser and sheep dung (monoculture), or fertiliser, diazotrophy from one or two legumes, and sheep dung (wheat in rotation). Historical wheat yields and foliar [N] were 28% and 106% higher in wheat–legume rotations compared with wheat monoculture. Increasing crop diversity was related to increased soil microbial activity, but not increased microbial richness or diversity, which depended more on known abiotic drivers of microbial community structure. The $\delta^{15}N$ mixing models revealed an increasing dependence on legume-derived N with increasing legume species in rotation. This suggests that enhanced N cycling and yield in crop–legume rotations is not a result of microbial diversity *per se*, but rather increased microbial activity when keystone legume species and their associated N_2-fixing symbionts were present.

Keywords: agroecosystem, diazotrophy, manure, microbes, stable isotopes, wheat–legume rotation

Introduction

Increasing aboveground crop diversity through crop rotations is an important component of conservation agriculture, which has been suggested as a sustainable alternative to conventional monocultures (Hobbs et al. 2008). The yield increase associated with crop rotations has been attributed to ecosystem services such as soil fertility, water-use efficiency, maintenance of soil structure and disruption of pest cycles (Smith et al. 2008). Microorganisms mediate these ecosystem services including climate mitigation via many biogeochemical reactions in soils (de Vries et al. 2013). However, the drivers in the relationship between crop rotation and diversity, soil microbial diversity and agroecosystem function or fertility, are poorly understood.

It has been posited that aboveground biodiversity drives belowground biodiversity (Hooper et al. 2000). Within agroecosystems, the diversity of root exudates and plant litter build-up from residual crops in rotation is predicted to lead to greater resource availability and niche differentiation, and hence abundance and diversity of microorganisms, respectively (Kent and Triplett 2002; Costa et al. 2006). In a meta-analysis of 20 studies, Venter et al. (2016) found that soils under crop rotations produced higher average microbial richness (+15.1%) and diversity scores (+3.4%) than soils under crop monocultures. However, the link between this effect and components of agroecosystem function, such as soil fertility and crop yield, was not investigated in many of the studies.

The understanding of agroecosystem biodiversity–function relationships can be enhanced through examining the functional relationships between specific system components. Plant and soil nitrogen (N) ratios ($\delta^{15}N$) can give an indication of mechanisms that underlie N cycling within ecosystems (Handley and Raven 1992; Craine et al. 2015). Specifically, mixing models using $\delta^{15}N$ isotopic signatures can be used to assess the contributions of different N sources (e.g. diazotrophy, fertiliser or animal manure) to a N sink (e.g. cash crop). The ecological interpretations of simple mixing models can be limited due to the N isotope fractionations, N gains, losses and pool mixing that affect the $\delta^{15}N$ signatures in ecosystems (Robinson 2001). However, agroecosystems tend to be relatively simplified systems with few available potential N sources, and mixing models have worked well to assess sources of foliar N (Pate and Unkovich 1999).

To gain clearer insight into microbial processes and assess the effect of agricultural practices in soil ecosystems it is essential to study functional and genetic diversity

simultaneously (Kent and Triplett 2002). Community-level physiological profiling (CLPP) gives an indication of the functional diversity within a soil sample by measuring the potential utilisation of 31 different naturally-occurring carbon (C) sources (Garland 1997). Automated rRNA intergenic spacer analysis (ARISA) gives an indication of the genetic diversity within a soil by measuring the relative abundance of rRNA intergenic spacer amplicons of varying lengths representing separate genetic subunits or operational taxonomic units (OTUs) (Ranjard et al. 2001).

To the authors' knowledge, no study has used a combination of microbial analyses and $\delta^{15}N$ techniques to look at the effect of crop rotation on soil microbial populations and N cycling. This study investigates the links between agroecosystem biodiversity and function in a long-term crop rotation trial in the Swartland Local Municipality of South Africa. To do this, the present study assessed (1) the impact of wheat–legume crop diversity on soil microbial activity, richness and diversity using CLPP and ARISA methods, and (2) the associated effect of the above- and belowground biodiversity on agroecosystem function (as soil fertility, wheat yield and N sourcing from inorganic N fertiliser, sheep manure and diazotrophic legumes).

Materials and methods

Experimental site

The study was conducted on the soils and crops from a 19-year, long-term crop rotation trial initiated in 1996 at Langgewens Experimental Farm near Mooreesburg in the Western Cape of South Africa (18.700° E, 33.283° S). Soil and crop sampling was performed during the winter growing season from August to November 2014. This important wheat-producing region experiences a Mediterranean climate with wet, cool winters and hot, dry summers. Long-term (n = 40 years) mean daily minimum and maximum temperatures range between 10.7 °C and 22.3 °C between August and November (P Lombard, Western Cape Department of Agriculture, pers. comm., 2014). Long-term average annual precipitation is 394.8 mm y^{-1}, with 473 mm falling during the year sampling took place (2014). Soils are of the Mispah and Glenrosa soil forms, consisting of shallow (200–400 mm) sandy loam with a 45% stone content in the A horizon. A high susceptibility to water-logging was the reason to 'ridge-and-furrow' the trial site prior to the start of the trial. Given that this took place more than 19 years ago, it was assumed that any effects on the soil microbial load along the soil depth gradient would have been nullified.

Experimental layout

The long-term trial had been set up and run uninterrupted for 19 years as an unbalanced randomised block design with a split-plot arrangement, consisting of 2 ha plots, each replicated twice. In the growing season of 2014, five of these two-year rotation treatments were compared (Table 1): continuous wheat (WW) (hereafter referred to as 'monoculture'), and two combinations of wheat/legume-pasture rotation systems (hereafter referred to as 'rotation'). The wheat cultivar used was *Triticum aestivum* 'SST 027' and the annual legumes comprised medic (*Medicago trunculata* 'Parabinga' and 'Sephi') and clover (*Trifolium repens* 'Balansa' and 'Roos'). The rotations consisted of wheat followed by medic (Wm) and wheat followed by a mixed pasture of medic and clover (Wmc). In any one year, the rotation treatments always included the next rotation cycle, i.e. medic followed by wheat (mW) and medic/clover mix followed by wheat (mcW). The inclusion of both alternate rotation years within the long-term trial was designed to account for climate variability. Based on the assumption of soil homogeneity within sampling plots, each plot was divided into three subplots, creating three sampling units per replicate.

Crop management

Conventional conservation farming practices were used in the planting, protection and harvesting of the crop. Wheat was planted using a no-till planter that disturbs approximately 20% of the soil surface. Pasture crops originally planted in 1996 were regenerated from soil-stored seed banks in each subsequent year, with renewed plantings as needed. Each successive wheat crop was planted in the dried-out pasture residue. Thus, soil disturbance occurred every year in the monoculture plots and every second year in the rotation plots. Soil macro- and trace elements were maintained each year at equal levels across treatments using P and N levels of 35 mg kg^{-1} and 50 mg kg^{-1} as benchmarks, respectively (Non-Affiliated Soil Analysis Work Committee 1990). The NPK fertiliser (Kynoch (Pty) Ltd, South Africa) was band-placed with planting and broadcast as a topdressing after the emergence of the crop. Weeds were controlled post-germination with broad spectrum herbicides including 750 g kg^{-1} triasulfuron and 360 g L^{-1} glyphosate before planting of the wheat crop. In 2008, the monoculture plots were burned post-harvest in an attempt to reduce the herbicide-resistant ryegrass infestation. Sheep were stocked on the rotation and wheat monoculture plots following harvest each year at a stocking density of four ewes per hectare for a few weeks, providing additional N inputs as dung.

Sampling procedure

Yield records for the 2014 season and the historical yield data for the last 10 years on the experimental plots was obtained from Langgewens Experimental Farm. This was measured in tonnes of grain per hectare each year after harvest with a combine harvester at the point when the water content of wheat grain was less than 13%. Remotely sensed normalised difference vegetation index (NDVI) was used as an additional measure of in-season wheat production.

Table 1: Experimental trial treatment codes and rotation combinations. Data collection took place during the first year of the rotation cycle (2014)

Treatment code	Rotation treatment	
	Year 1 (2014)	Year 2
WW	Wheat	Wheat
Wm	Wheat	Medic
mW	Medic	Wheat
Wmc	Wheat	Medic/clover
mcW	Medic/clover	Wheat

The NDVI is well-correlated with grain yield in winter wheat agroecosystems and has been used as a measure of plant vigour, which responds to environmental variables such as soil moisture, nutrient status and soil salinity (Raun et al. 2001). Two cloud-free Landsat 8 8-day NDVI composite orthorectified scenes were acquired of the experimental farm during the active wheat growth phase (13 and 29 August 2014) from the Google Earth Engine Explorer (https://earthengine.google.com/). Pixels that intercepted a diagonal transect line through each plot were used to compute a mean NDVI reflectance value based on the two time points. Transects avoided the edge effect in each field; however, similar results were obtained when averaging pixels across a whole field/treatment, including edges.

Soils were sampled before harvest at the end of October 2014 using a rocky soil auger (40 mm ∅) to a depth of 150 mm and 70 mm for nutrient and microbial analyses, respectively. For the nutrient analysis, three soil cores were collected randomly per subplot to a depth of 150 mm around the tagged plants and were pooled into one plastic bag sample. The soil cores provided an indication of soil nutrients accessible to wheat roots. The samples were subsequently air-dried for 3 d and subsequently analysed for macro- and trace elements using inductively coupled plasma–mass spectrometry as previously described where NH_4^+-N was analysed using routine Kjeldahl digestion (Crooke and Simpson 1971). For microbial analysis, six soil cores were collected per subplot to a depth of 70 mm around the tagged plants from within wheat rows and pooled together into separate plastic bags. These soil samples were taken adjacent to the soil cores used for nutrient analysis, but to a shallower depth as soil microbes were known to be stratified in this region (K Jacobs, pers. comm., 2014). Soil was immediately sieved through a 2-mm sieve, collected onto ice and stored at 4 °C. Soils were extracted for DNA within 2 d of collection and pre-incubated for CLPP profiling within one week of collection.

Six wheat plants were randomly harvested per subplot from the monoculture and wheat-phase rotation plots (WW, Wm and Wmc) for nutrient and isotopic analysis. In addition, 200 g legume plants and 500 g sheep dung were harvested randomly over each subplot. Samples were dried in an oven at 70 °C. Wheat plants were ground and analysed for macro- and trace elements using inductively coupled plasma optical emission (ICP 6000 series, Thermo Electron Corporation, Franklin, MA, USA) after dry-ashing. A 1 kg sample of the fertiliser was obtained directly from the supplier (Kynoch) for isotopic analysis later.

Community-level physiological profile

Substrate utilisation patterns of culturable soil microbial (bacterial) communities, as a measure of functional diversity, were assessed using Biolog-EcoPlates™ (Biolog Inc., Hayward, CA, USA) according to a procedure adapted from Garland and Mills (1991). A pre-incubation was performed to allow microbial utilisation of residual soluble organic C present in the soil. To do this, 5 g dry-weight soil samples were moistened to 40% water-holding capacity and incubated for 6 d at 25 °C. Samples were covered in Parafilm® to allow for CO_2 and O_2, but not H_2O, exchange. After incubation, samples were shaken with 40 mL of 0.8% NaCl buffer solution for 30 min on an orbital shaker. After sediment had settled, 5 mL supernatant was removed and softly centrifuged for 30 s at 300 g to form a pellet. Thereafter, 125 µL aliquots of the supernatant were used to inoculate each well of the Biolog-EcoPlates™. Six EcoPlates were used per treatment (one per subplot), which contained internal triplicates of 31 different C sources suitable for soil microbes (Choi and Dobbs 1999). Plates were stored on an orbital shaker in a dark room at 25 °C. The utilisation of the carbon sources (indicated by reduction of tetrazolium dye) was recorded on a Bio-Rad Micro Plate Reader at 590 nm at 24, 48, 72 and 96 h after inoculation. Absorbance values were corrected by subtracting the control well from the actual reading. Any negative results after correction were recorded as zero.

Total microbial activity was expressed as average well colour development (AWCD), calculated as $\sum OD_i/31$, where OD_i is the ratio of the corrected optical density value of each well. Microbial diversity was determined using measures of richness and evenness by comparing plates that most approximate an AWCD of 0.75 (Garland 1997). The threshold for a positive test was determined as any value, after background correction, exceeding 0.25. Richness (S) was determined as the count of positive testing absorbance values. Biodiversity or evenness was determined using the Shannon–Weaver index (H) as $H = \sum p_i(\ln p_i)$ (Shannon and Weaver 1969), where p_i is the ratio of the corrected absorbance value of each well to the sum of the absorbance value of all wells at a wavelength of 590 nm.

Automated ribosomal intergenic spacer analysis

Total microbial DNA was extracted from soils within one week of field sampling. The bacterial ARISA, as a measure of genetic diversity, involved the extraction of total community DNA from soil samples, polymerase chain reaction (PCR) amplification using fluorescence-tagged oligonucleotide primers targeting the intergenic spacer region transcribed from between the small (16S) and large (23S) subunits of rRNA, laser detection of fluorescent DNA fragments and analysis of banding patterns. Total DNA was extracted from 0.35 g moist soil using the ZR Soil Microbe DNA kit (Zymo Research, Irvine, CA, USA) following the manufacturer's instructions, and stored at −18 °C until further analysis.

The PCR reactions were carried out using the bacteria-specific primers ITSReub and FAM (carboxy-fluorescein) labelled ITSF (Cardinale et al. 2004). For each treatment, consisting of six samples, the reaction mixture contained 40 µL of 2× KapaTaq Readymix (KapaBiosystems, Cape Town, South Africa) master mix, 24 µL ultrapure water (Milli-Q®), and 4 µL of each primer. Amplification was performed on a GeneAmp® PCR System 9700 machine using the following cycling parameters: 5 min at 94 °C, 30 cycles of 30 s at 94 °C, 45 s at 56 °C, 1 min 10 s at 72 °C, and a final 5 min at 72 °C. This was repeated three times for each sample set so as to create triplicate results, which were then pooled into one Eppendorf sample tube. PCR samples were separated on a 1% agarose gel, stained with ethidium bromide and visualised using ultraviolet light. The amplicons from each bacteria-specific PCR were run on an ABI 3010xl Genetic Analyser to obtain an

electropherogram of the different fragment lengths and fluorescent intensities. ARISA samples were run with the ROX 1.1 size standard, which varied from 20 to 900 bp (Slabbert et al. 2010). The ARISA data were analysed using Genemapper 4.1 software, which converted fluorescence data to an electropherogram representing fragments of different sizes. Only fragment sizes larger than 0.5% of the total fluorescence, ranging from 120 to 1 000 bp in length, were considered for analysis. A bin size of 3 bp for fragments below 700 bp and 5 bp for fragments above 700 bp was employed to minimise the inaccuracies in the ARISA profiles (Ranjard et al. 2001; Slabbert et al. 2010). Shannon–Weaver and richness scores were calculated using data OTUs as determined from the ARISA data.

Isotope ratio mass spectrometry

Dried and ground leaf, dung and fertiliser material was weighed at 1 mg into duplicate tin caps for Dumas combustion at 1 020 °C prior to analysis of percentage N, as well as natural abundance of ^{15}N on a Flash HT Plus integrated via a ConFlo IV system with a Delta V Plus Isotope Ratio mass spectrometer (Thermo Scientific, Bremen, Germany). Nitrogen isotope values were corrected against an in-house standard (Merck Gel $\delta^{13}C$ = −20.57‰; $\delta^{15}N$ = +6.80‰), run after every 12 unknowns in a sequence. A blank was also run after every 12 unknowns to confirm that there was no sample-to-sample memory effect from incomplete combustion. Isotope ratios were expressed as $\delta^{15}N$ relative to the standard, following the equation:

$$\delta^{15}\mathrm{N}\,(‰) = \left(\frac{R_{\mathrm{sample}}}{R_{\mathrm{standard}}} - 1\right) \times 1000$$

where R is the ratio of $^{15}N/^{14}N$. The R_{standard} is calibrated against air, the $\delta^{15}N$ of which was arbitrarily set to zero.

Statistical analyses

Relationships between rotation treatments and measured response variables were analysed using a linear mixed effects model in R 3.2.3 (R Core Team 2015) using the lme4 package (Bates et al. 2015). Whole-farm trials commonly make use of one or two large areas per treatment because replication at smaller scales is meaningless, i.e. is pseudoreplication (Millar and Anderson 2004). In order to account for the necessity of subplot replication within the two available plots per treatment, the rotation treatment was used as the fixed effect and subplot number as the random effect. The *p*-values were determined using likelihood ratio tests of the full model with the treatment effect, against the model without the treatment effect. *Post-hoc* comparisons between treatments were performed using the Tukey honest significant difference (HSD) test. Regression analyses were performed on richness and diversity scores, as well as CLPP and ARISA results. Correlation matrices were constructed using crop diversity, microbial diversity, richness and activity scores, plant and soil nutrient levels, and yield data. To assess the proportional contribution of different N sources to wheat plant N, the $\delta^{15}N$ and $\delta^{13}C$ values were entered into a dual-isotope, three-source mixing model (SIMM, Isoerror, v 1.04), which takes into account the signature variability of both the sources and the mixture (Phillips and Gregg 2001).

Results

Wheat yield and NDVI

There was no effect of rotation treatment on historical wheat yield at harvest (χ^2 (2) = 5.428, p = 0.066; Figure 1a), although Wm produced a 28% higher average yield than WW, which is of economic significance (J Strauss, pers. comm., 2014). Early growing season NDVI was higher in rotation treatments than the wheat monoculture (χ^2 (2) = 13.721, $p < 0.001$; Figure 1b).

Microbial activity and functional diversity (CLPP)

The soil microbial activity, as measured by utilisation of C substrates (AWCD), differed significantly between treatments (χ^2 = 15.772, p = 0.008; Figure 2). *Post-hoc* Tukey HSD tests revealed higher activity values in Wmc compared with WW (p = 0.004) and Wm (p = 0.027).

Increasing crop diversity through wheat–legume rotations did not significantly affect the microbial community

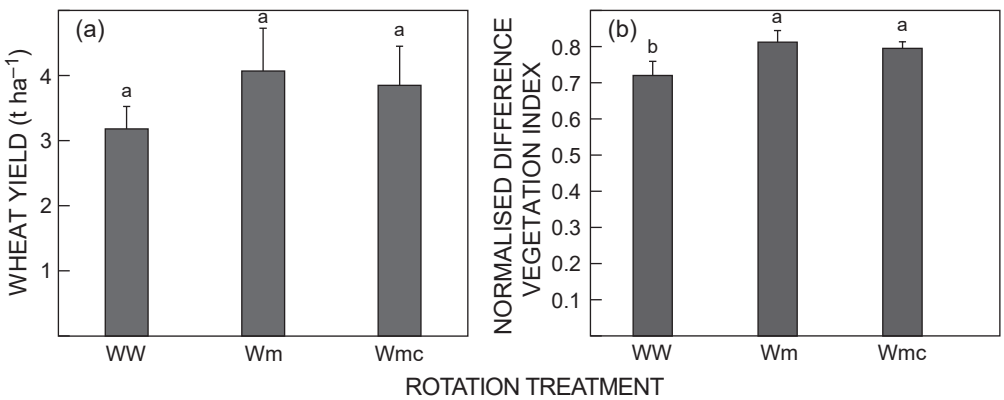

Figure 1: Average total wheat grain yield for the past 10 years (a), and average normalised difference vegetation index for two time points of active wheat growth during the 2014 season (b). Whiskers indicate the SE. Different letters above the bars indicate significant differences in the least squared means of treatments from a mixed-effects linear model at the $P < 0.05$ significance level after a *post-hoc* Tukey HSD test. WW = wheat/wheat, Wm = wheat/medic, Wmc = wheat/medic and clover

functional richness (χ^2 (5) = 5.005, p = 0.415) or diversity (χ^2 (5) = 2.957, p = 0.707) as measured by CLPP.

Genetic diversity (ARISA)

As indicated by CLPP, ARISA indicated that there was no significant difference between rotation treatments in microbial genetic richness (χ^2 (5) = 3.842, p = 0.572) and diversity (χ^2 (5) = 5.377, p = 0.3717). The WW monoculture tended to produce the lowest average genetic richness (S = 41.5) and diversity (H = 3.24) scores, whereas the reference site produced the highest (S = 46.3, H = 3.57).

Nutrient cycling

Soil N and C did not differ between treatments (χ^2 (5) = 5.167, p = 0.395; χ^2 (5) = 8.272, p = 0.142, Table 2), and there was no correlation between soil N and microbial diversity (p = 0.874) or yield (p = 0.162). Microbial activity was negatively correlated with other soil nutrients including sodium (Na; r = −0.524) and potassium (K; r = −0.53), yet positively correlated with phosphorus (P; r = 0.433). Furthermore, functional microbial diversity was negatively correlated to sodium (r = −0.499).

Post-hoc Tukey HSD tests revealed that wheat plant N increased by 106% in the Wmc treatment relative to that of WW (p = 0.069). Wmc produced significant increases in wheat plant magnesium (Mg; $p < 0.05$) levels relative to those of WW. No differences were found in grain nutrient levels (Table 2).

Synthetic fertiliser produced the most depleted δ^{15}N signature (−0.9‰) across the range of N sources and foliar mixtures, with sheep dung producing the most enriched signature (3.8‰) (Figure 3). Foliar δ^{15}N signatures of the legumes, clover and medic, grouped closer to 0‰, whereas wheat plant signatures grouped closer to the dung

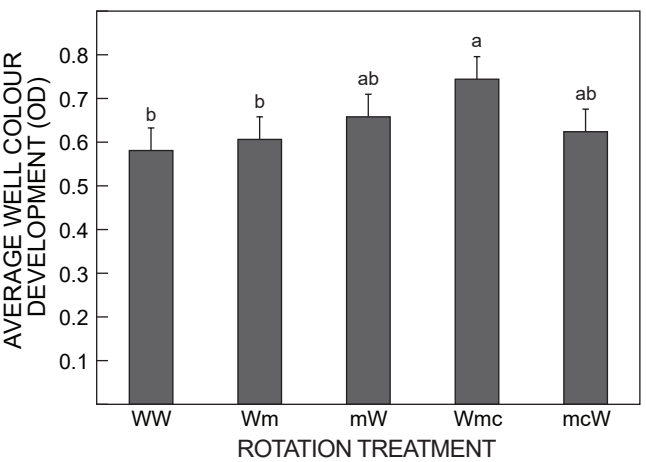

Figure 2: Average well colour development, measured as optical density (OD), from community-level physiological profiles obtained by Biolog-EcoPlate™ inoculation with treatment soil samples. Data shows means ± SE (n = 2) and least squared means of treatments from a mixed-effects linear model. Different letters above bars indicate a significant difference ($P < 0.05$) after a *post-hoc* Tukey HSD test. WW = wheat/wheat, Wm = wheat/medic, mW = medic/wheat, Wmc = wheat/medic and clover, mcW = medic and clover/wheat

Table 2: Soil, wheat shoot and grain average nutrient levels for rotation treatments at Langgewens Experimental Farm, Western Cape, South Africa. Values are the mean ± SE. WW = wheat/wheat, Wm = wheat/medic, mW = medic/wheat, Wmc = wheat/medic and clover, mcW = medic and clover/wheat

Rotation	NH_4^+-N (%)	CV (%)	P (mg kg^{-1})	CV (%)	K (mg kg^{-1})	CV (%)	Mg (cmol kg^{-1})	CV (%)	Na (mg kg^{-1})	CV (%)	Ca (cmol kg^{-1})	CV (%)	pH	CV (%)	C (%)	CV (%)
Soil																
WW	0.11 ± 0.01^{a}	9.09	80.33 ± 3.89^{a}	4.84	143.97 ± 16.96^{ab}	4.84	0.95 ± 0.16^{a}	16.84	18.50 ± 1.41^{b}	7.62	5.84 ± 0.75^{ab}	12.84	6.00 ± 0.20^{ab}	3.33	1.41 ± 0.11^{a}	7.80
Wm	0.13 ± 0.01^{a}	7.69	92.17 ± 3.31^{a}	3.59	110.67 ± 11.44^{b}	10.34	1.11 ± 0.07^{a}	6.31	22.83 ± 3.17^{b}	13.89	9.22 ± 1.93^{a}	20.93	6.43 ± 0.12^{a}	1.87	1.47 ± 0.10^{a}	6.80
mW	0.11 ± 0.01^{a}	9.09	76.33 ± 8.85^{a}	11.59	119.33 ± 10.98^{b}	9.20	1.09 ± 0.16^{a}	14.68	22.00 ± 1.13^{b}	5.14	5.59 ± 0.56^{ab}	10.02	6.18 ± 0.11^{a}	1.78	1.26 ± 0.14^{a}	11.11
Wmc	0.13 ± 0.01^{a}	7.69	92.33 ± 8.22^{a}	8.90	95.50 ± 17.64^{b}	18.47	1.25 ± 0.32^{a}	25.60	17.33 ± 0.92^{b}	5.31	6.17 ± 0.92^{ab}	14.91	6.17 ± 0.20^{a}	3.24	1.54 ± 0.17^{a}	11.04
mcW	0.11 ± 0.01^{a}	9.09	94.83 ± 11.8^{a}	12.44	155.83 ± 33.49^{ab}	2.24	0.64 ± 0.07^{a}	10.94	23.17 ± 0.83^{b}	3.58	4.71 ± 0.24^{b}	5.10	5.88 ± 0.14^{ab}	2.38	1.28 ± 0.11^{a}	8.59
p-value	0.395		0.373		0.374		0.146		0.055*		0.038**		0.125		0.142	
Shoot																
WW	0.43 ± 0.02^{b}	4.65	0.06^{b}	0.00	1.73 ± 0.06^{a}	3.47	0.07^{b}	0.00	240.38 ± 14.61^{b}	6.08	0.21 ± 0.01^{a}	4.76	–		–	
Wm	0.58 ± 0.04^{b}	6.90	0.07^{b}	0.00	2.17 ± 0.15^{a}	6.91	0.09 ± 0.01^{ab}	11.11	376.19 ± 21.16^{a}	5.63	0.27 ± 0.01^{a}	3.70	–		–	
Wmc	1.18 ± 0.22^{a}	18.60	0.20 ± 0.05^{a}	25.00	1.34 ± 0.35^{a}	26.12	0.10 ± 0.01^{a}	0.00	242.14 ± 37.03^{ab}	2.90	0.18 ± 0.03^{a}	16.67	–		–	
p-value	0.069*		0.106		0.149		0.014**		0.054*		0.199					
Grain																
WW	1.47 ± 0.07^{a}	4.76	0.3 ± 0.01^{a}	0.03	0.66 ± 0.04^{a}	6.06	0.11^{a}	0.00	155.18 ± 8.57^{a}	5.53	0.08 ± 0.01^{a}	12.50	–		–	
Wm	1.66 ± 0.05^{a}	3.01	0.34 ± 0.01^{a}	2.94	0.71 ± 0.05^{a}	7.04	0.12^{a}	0.00	145.55 ± 10.25^{a}	7.04	0.09^{a}	0.00	–		–	
Wmc	1.19 ± 0.22^{a}	18.49	0.21 ± 0.06^{a}	28.57	1.19 ± 0.21^{a}	17.65	0.10 ± 0.01^{a}	10.00	265.56 ± 67.06^{a}	25.26	0.18 ± 0.04^{a}	22.22	–		–	
p-value	0.301		0.262		0.149		0.178		0.281		0.175					

* $p < 0.1$, ** $p < 0.05$

signature. The δ^{15}N mixing models revealed an increasing dependency on legume-derived N by wheat plants with increasing number of legumes in rotation (Figure 4).

Discussion

Increasing aboveground agroecosystem biodiversity via crop rotations can enhance ecosystem function through increased nutrient cycling and crop yields (Smith et al. 2008). In the present study, wheat–legume rotations resulted in increased shoot N concentrations ([N]) and plant vigour (NDVI), but not overall yield compared with wheat monoculture. While the yield difference was not significant, a net economic gain was indicated by an increased reliance on the legume–*Rhizobium* associations versus fertiliser as a source of N. Indeed, although the 28% yield increase found in this study is not statistically significant, it reflects the economically significant higher yields and economic gains per hectare from wheat–legume rotations that have encouraged the adoption of crop rotation within the Western Cape region (Hardy and Strauss 2011). As was expected, the increased N available in rotations, especially rotations with increased legume diversity, resulted in higher overall soil microbial activity. Equally interesting, this increase in function was not associated with an increase in soil microbial diversity. Thus, the expectation that increasing aboveground diversity will result in increased belowground diversity is an oversimplification. This is especially the case where the system is dominated by keystone species such as legumes, as will be discussed in more detail.

An increase in soil N resulting from legume rotations is thought to drive the associated yield increase (Smith et al. 2008). The results from the present study show no increase in the bulk soil [N]; however, N transfer between *Rhizobium* spp. and the host or adjacent plants may have occurred with little loss of N to the surrounding soil. The δ^{15}N mixing model supports the idea that wheat in rotation sourced N mainly from these legumes and their N_2-fixing microbes. Medic and clover signatures tended towards the atmospheric δ^{15}N signal (0‰), revealing their reliance on N_2-fixing *Rhizobium* bacteria (Handley 2002). As the number of legumes increased in rotation, so did the proportional reliance of wheat plants on legume N sources, which corresponded to an increase in foliar [N] by 106% with Wmc rotations compared with that of WW. Another, albeit smaller, source of N for the crop in rotation was dung. Dung signatures were significantly enriched compared with fertiliser and foliar δ^{15}N, probably due to fractionations and N losses through the animals' gut. It has been shown that plants fertilised with dung have higher δ^{15}N values (Szpak 2014). This was supported by the mixing model results, which revealed enriched foliar δ^{15}N values for wheat monoculture plants that derived most of their N from dung. Another possible contribution to the enriched WW δ^{15}N values may be due to the annual tillage of the soil before planting, compared with the biennial tillage of the Wm and Wmc soils for planting. This is because soil disturbance such as tillage has the immediate effect of enriching δ^{15}N in the system due to a flush of mineralisation of soil N (Handley and Raven 1992).

Crop rotation with legumes had a significant effect on microbial activity as measured by CLPP. The elevated levels of microbial activity in the Wmc treatment relative to the WW treatment may be due to increased C substrate quantity, quality and diversity associated with a diversity of crop residues. This aligns with the findings of a meta-analysis of 122 studies, which found that increasing crop diversity in rotation increased soil microbial biomass, C and N pools (McDaniel et al. 2014). Other studies have found functional links between the changes in quality and quantity of soil organic matter and microbial composition (Bird et al. 2011; Cusack et al. 2011). In addition, soil nutrients other than N

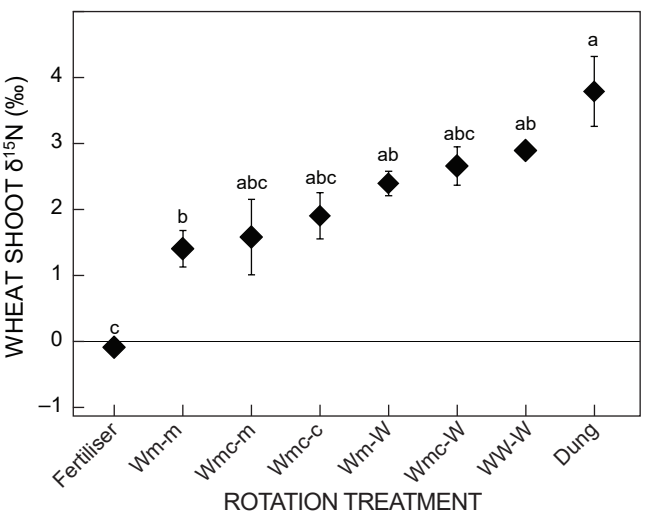

Figure 3: Mean nitrogen (δ^{15}N) isotopic signatures ± SE (n = 2) of different N sources and mixtures across rotation treatments. Different letters above bars indicate a significant difference in the least squared means of treatments from a mixed-effects linear model ($P < 0.05$) after a *post-hoc* Tukey HSD test. Wm = wheat/medic, Wmc = wheat/medic and clover, WW = wheat/wheat, -m = medic plant, -c = clover plant, -W = wheat plant

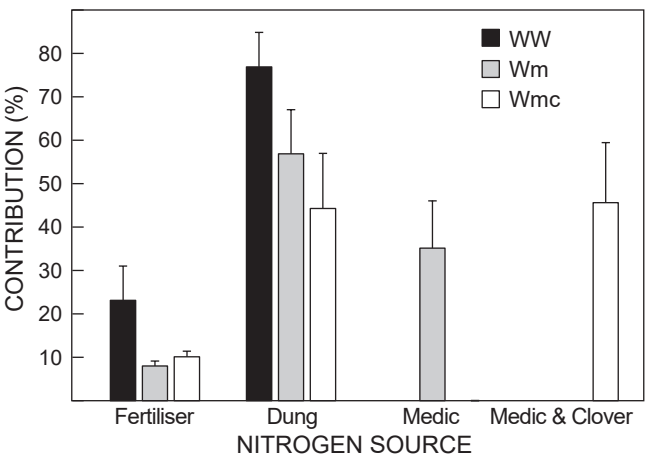

Figure 4: Percentage contribution of different N sources to wheat plants across rotation treatments using a dual-isotope, three-source δ^{15}N mixing model. Standard errors are indicated by whiskers (n = 2). WW = wheat/wheat, Wm = wheat/medic, Wmc = wheat/medic and clover

may be drivers of microbial activity. In the present study, microbial activity was negatively correlated with other soil nutrients including Na ($r = -0.524$) and K ($r = -0.53$), yet positively correlated with P ($r = 0.433$).

Crop rotations did not significantly alter the functional (CLPP) or genetic (ARISA) microbial diversity of the soil. Any differences in soil microbial diversity alone did not mediate an increase in plant production *per se*. Previous studies employing CLPP as a method have shown positive (Lupwayi et al. 1998; Murphy et al. 2011) or non-significant (Marais et al. 2012; Navarro-Noya et al. 2013) effects of crop rotations on functional microbial diversity. The same is the case with studies measuring genetic diversity with methods such as ARISA. These have found both lower (Yao et al. 2006; Guong et al. 2012) and higher (van Elsas et al. 2002; Mathimaran et al. 2007; Yin et al. 2010) microbial diversity in soils under crop rotations compared with monocultures. In these studies, other abiotic factors such as soil moisture and pH were found to be driving factors of microbial diversity.

The conflicting evidence in the literature and in the present study may be a result of the problems intrinsic to the microbial analysis methodology. The CLPP only measures the activity of a part of the total microbial community, mainly fast-growing bacteria, yeast and fungi, and may misrepresent the diversity of actual C sources in the soil (Stefanowicz 2006). ARISA is vulnerable to under-representing taxonomic diversity due to the overlapping size classes among unrelated populations (Ranjard et al. 2001). Future studies would do well to include next-generation-sequencing methods such as pyrosequencing to understand changes in the composition of specific functional species within the soil (Metzker 2010).

One of the challenges within microbiology is to understand the link between diversity and function (Torsvik and Øvreås 2002). Within agroecosystems, crop diversity and microbial diversity may be linked with functional traits such as soil fertility and yield. In the present study, there were no significant relationships between microbial diversity and crop yield or soil fertility. However, the correlation between legume presence and yield may provide support for the selection effect hypothesis where ecosystem function is a product of specific productive species, as opposed to the complementarity effect involving the facilitative interaction of a diversity of species (Hooper et al. 2000). The presence of N_2-fixing symbiotic bacteria (*Rhizobium* spp.) results in higher than expected agroecosystem function.

Conclusion

Increasing crop diversity through rotating wheat with legumes positively impacted cash-crop vigour, historical yield and foliar [N]. Increasing crop diversity was related to increased soil microbial activity, but not increased microbial richness or diversity, which depended more on known abiotic drivers of microbial community structure. Within this study, likely drivers included P availability, Na and K excess, and pH due to significant correlations with microbial activity and functional richness. Although soil microbial diversity may mediate the yield increase with crop rotation, the present study found no correlation between soil microbial activity and components of agroecosystem function (soil N, plant N and yield). Isotopic analyses showed that as the number of legumes increased in rotation, so did the proportional reliance of wheat plants on legume N sources. Thus, wheat–legume crop rotations may not rely on microbial diversity *per se* for the ecosystem services supporting increased yield. Rather, the role of specific functional groups in the yield increase of crop rotation systems requires further investigation.

Acknowledgements — The authors would like to acknowledge scholarships provided to ZSV by the following South African institutions: National Research Foundation, the Western Cape Department of Agriculture, the Oppenheimer Memorial Trust and Stellenbosch University, Departments of Agronomy and Microbiology. Collaboration between authors was facilitated by the Sustainable Agriculture in South Africa project (Netherlands Initiative for Capacity Development in Higher Education, Project ZAR 118).

ORCID

Zander Venter http://orcid.org/0000-0003-2638-7162

References

Bates D, Maechler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* 67: 1–48.

Bird JA, Herman DJ, Firestone MK. 2011. Rhizosphere priming of soil organic matter by bacterial groups in a grassland soil. *Soil Biology and Biochemistry* 43: 718–725.

Cardinale M, Brusetti L, Quatrini P, Borin S, Puglia AM, Rizzi A. 2004. Comparison of different primer sets for use in automated ribosomal intergenic spacer analysis of complex bacterial communities. *Applied Environmental Microbiology* 70: 6147–6156.

Choi K, Dobbs FC. 1999. Comparison of two kinds of Biolog microplates (GN and ECO) in their ability to distinguish among aquatic microbial communities. *Journal of Microbiological Methods* 36: 203–213.

Costa R, Götz M, Mrotzek N, Lottmann J, Berg G, Smalla K. 2006. Effects of site and plant species on rhizosphere community structure as revealed by molecular analysis of microbial guilds. *FEMS Microbiology Ecology* 56: 236–249.

Craine JM, Brookshire EN, Cramer MD, Hasselquist NJ, Koba K, Marin-Spiotta E. 2015. Ecological interpretations of nitrogen isotope ratios of terrestrial plants and soils. *Plant and Soil* 2015: 1–26.

Crooke WM, Simpson WE. 1971. Determination of ammonium in Kjeldahl digests of crops by an automated procedure. *Journal of the Science of Food and Agriculture* 22: 9–10.

Cusack DF, Silver WL, Torn MS, Burton SD, Firestone MK. 2011. Changes in microbial community characteristics and soil organic matter with nitrogen additions in two tropical forests. *Ecology* 92: 621–632.

de Vries FT, Thebault E, Liiri M, Birkhofer K, Tsiafouli MA, Bjornlund L. 2013. Soil food web properties explain ecosystem services across European land use systems. *Proceedings of the National Academy of Sciences of the USA* 110: 14296–14301.

Garland JL. 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microbiology Ecology* 24: 289–300.

Garland JL, Mills AL. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied*

Environmental Microbiology 57: 2351–2359.
Guong VT, Rosling A, Alström S, Chai B, Högberg N. 2012. Different crop rotation systems as drivers of change in soil bacterial community structure and yield of rice, *Oryza sativa*. *Biology and Fertility of Soils* 48: 217–225.
Handley LL. 2002. Diazotrophy and δ^{15}N biology and environment. *Proceedings of the Royal Irish Academy* 102: 49–51.
Handley LL, Raven JA. 1992. The use of natural abundance of nitrogen isotopes in plant physiology and ecology. *Plant, Cell and Environment* 15: 965–985.
Hardy MB, Strauss JA. 2011. Gross margin analysis of crop and crop/pasture rotation systems that are being evaluated in the long-term crop rotation trial at the Langgewens Agricultural Research Farm – 2002 to 2010. Interim report. Cape Town: Western Cape Department of Agriculture.
Hobbs PR, Sayre K, Gupta R. 2008. The role of conservation agriculture in sustainable agriculture. *Philosophical Transactions of the Royal Society B: Biological Science* 363: 543–555.
Hooper DU, Bignell DE, Brown VK, Brussard L, Dangerfield JM, Wall DH, Wardle DA, Coleman DC, Giller KE, Lavelle P, van der Putten WH, de Ruiter PC, Rusek J, Silver WL, Tiedje JM, Wolters V. 2000. Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: patterns, mechanisms, and feedbacks. *BioScience* 50: 1049–1061.
Kent AD, Triplett EW. 2002. Microbial communities and their interactions in soil and rhizosphere ecosystems. *Annual Review of Microbiology* 56: 211–236.
Lupwayi N, Rice W, Clayton G. 1998. Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. *Soil Biology and Biochemistry* 30: 1733–1741.
Marais A, Hardy M, Booyse M, Botha A. 2012. Effects of monoculture, crop rotation, and soil moisture content on selected soil physicochemical and microbial parameters in wheat fields. *Applied and Environmental Soil Science* 2012: 1–13.
Mathimaran N, Ruh R, Jama B, Verchot L, Frossard E, Jansa J. 2007. Impact of agricultural management on arbuscular mycorrhizal fungal communities in Kenyan ferralsol. *Agriculture, Ecosystems and Environment* 119: 22–32.
McDaniel M, Tiemann L, Grandy A. 2014. Does agricultural crop diversity enhance soil microbial biomass and organic matter dynamics? A meta-analysis. *Ecological Applications* 24: 560–570.
Metzker ML 2010. Sequencing technologies – the next generation. *Nature Reviews Genetics* 11: 31–46.
Millar RB, Anderson MJ. 2004. Remedies for pseudoreplication. *Fish Research* 31: 397–407.
Murphy D, Cookson W, Braimbridge M, Marschner P, Jones D, Stockdale E. 2011. Relationships between soil organic matter and the soil microbial biomass (size, functional diversity, and community structure) in crop and pasture systems in a semi-arid environment. *Soil Research* 49: 582–594.
Navarro-Noya YE, Gómez-Acata S, Montoya-Ciriaco N, Rojas-Valdez A, Suárez-Arriaga MC, Valenzuela-Encinas C, Jimenez-Bueno N, Verhulst N, Govaerts B, Dendooven L. 2013. Relative impacts of tillage, residue management and crop-rotation on soil bacterial communities in a semi-arid agroecosystem. *Soil Biology and Biochemistry* 65: 86–95.
Non-Affiliated Soil Analysis Work Committee. 1990. *Handbook of standard soil testing methods for advisory purposes*. Pretoria: Soil Science Society of South Africa.
Pate JS, Unkovich MJ. 1999. Measuring symbiotic nitrogen fixation: case studies of natural and agricultural ecosystems in a Western Australian setting. In: Press MC, Scholes JDI (ed.), *Physiological plant ecology*. London: Blackwell Science. pp 153–173.
Phillips DL, Gregg JW. 2001. Uncertainty in source partitioning using stable isotopes. *Oecologia* 127: 171–9.
R Core Team. 2015. R: a language and environment for statistical computing. Vienna: R Foundation for Statistical Computing. Available at https://www.R-project.org/.
Ranjard L, Poly F, Lata JC, Mougel C, Thioulouse J, Nazaret S. 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Applied and Environmental Microbiology* 67: 4479–4487.
Raun WR, Solie JB, Johnson GV, Stone ML, Lukina EV, Thomason WE, Schepers JS. 2001. In-season prediction of potential grain yield in winter wheat using canopy reflectance. *Agronomy Journal* 93: 131–138.
Robinson D. 2001. δ^{15}N as an integrator of the nitrogen cycle. *Trends in Ecology and Evolution* 16: 153–162.
Shannon PO, Weaver W. 1969. *The mathematical theory of communication* (4th edn). Urbana: University of Illinois Press.
Slabbert E, Kongor RY, Esler KJ, Jacobs K. 2010. Microbial diversity and community structure in Fynbos soil. *Molecular Ecology* 19: 1031–1041.
Smith RG, Gross KL, Robertson GP. 2008. Effects of crop diversity on agroecosystem function: crop yield response. *Ecosystems* 11: 355–366.
Stefanowicz A. 2006. The Biolog plates technique as a tool in ecological studies of microbial communities. *Polish Journal of Environmental Studies* 15: 669–676.
Szpak P. 2014. Complexities of nitrogen isotope biogeochemistry in plant-soil systems: implications for the study of ancient agricultural and animal management practices. *Frontiers in Plant Science* 5: 1–19.
Torsvik V, Øvreås L. 2002. Microbial diversity and function in soil: from genes to ecosystems. *Current Opinion in Microbiology* 5: 240–245.
van Elsas JD, Garbeva P, Salles J. 2002. Effects of agronomical measures on the microbial diversity of soils as related to the suppression of soil-borne plant pathogens. *Biodegradation* 13: 29–40.
Venter ZS, Jacobs K, Hawkins HJ. 2016. The impact of crop rotation on microbial diversity; a meta-analysis. *Pedobiologia* 59: 215–233.
Yao H, Jiao X, Wu F. 2006. Effects of continuous cucumber cropping and alternative rotations under protected cultivation on soil microbial community diversity. *Plant and Soil* 284: 195–203.
Yin C, Jones KL, Peterson DE, Garrett KA, Hulbert SH, Paulitz TC. 2010. Members of soil bacterial communities sensitive to tillage and crop rotation. *Soil Biology and Biochemistry* 42: 2111–2118.

Received 30 September 2016, revised 27 February 2017, accepted 26 March 2017
Associate Editor: Alexander Valentine