Long-term agrichemical use leads to alterations in bacterial community diversity

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ABSTRACT

Bacterial communities are key drivers of soil fertility and agriculture productivity. Understanding how soil bacterial communities change in response to different conditions is an important aspect in the development of sustainable agriculture. There is a desire to reduce the current reliance on high inputs of chemicals and fertilisers in agriculture, but limited data are available on how this might impact soil bacterial communities. This study investigated the bacterial communities in a spring barley monoculture site subjected to two different input regimes for over 12 years: a conventional chemical/fertiliser regime, and a reduced input regime. A culture independent approach was performed to compare the bacterial communities through 16S rRNA gene PCR-DGGE. PCO analysis revealed that the rhizosphere has a strong structuring effect on the bacterial community. Moreover, high inputs of agrichemicals lead to an increase of phosphorus level in the soil and a concomitant reduction of the bacterial diversity. These results may help to evaluate the environmental risks associated with agrichemical usage.

Keywords: microbial diversity; fertilisers; rhizosphere; sustainable agriculture; DGGE

Pressure for high crop yields has resulted in a strong dependence on intensive application of chemical fertilisers and biocides with concomitant risks of environmental pollution and ecological perturbations (Morrissey et al. 2004). For instance, problems with excessive nitrogen fertiliser include production of greenhouse gases like N_2O (Velthof et al. 1997) and release of nitrates into surrounding water bodies (Tomer and Burkart 2003) and phosphorus fertiliser runoff to water bodies can cause algal blooms and fish kills (Carpenter et al. 1998). Consequently, the demand for decreasing these negative effects has created a growing need to evaluate how different chemical inputs impact on the ecology of soil systems.

Bacteria make up the major part of the soil microbial community and play significant ecological roles in all soil processes including nutrient cycling (Nannipieri 2003). Since alterations of the composition of the microbial community can be linked with functional changes in soil ecology, the soil bacterial community is an ideal indicator for the ecological status of soil and can thus help to evaluate how environmental and anthropogenic factors influence the soil ecosystem (Morris et al. 2002). Although previous work has revealed that

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Table 1. Managements of fertilisers and biocides

	Fertiliser amendment (kg/ha)		Biocides amendment (L/ha)	
	N	P	herbicide	fungicide
Reduced (low) input	105	0	Bandit 2.8	Fortress Duo 0.40 (during tillering); Allegro 0.40 (after tillering)
Conventional (high) input	137.5	144	Bandit 5.6	Allegro 0.75 (during and after tillering)

either chemical inputs (Kleineidam et al. 2010) or plants (Berg and Smalla 2009) can affect soil microbial communities, few studies have evaluated the combined influence of agricultural chemical inputs and plant on bacterial community structure (Garcia-Teijeiro et al. 2009, Doi et al. 2011). Furthermore, these studies have investigated agro-ecosystems subjected to relatively short-term inputs (from 6-week to 4-month periods).

The objective of this study was to evaluate the complex influences of agrichemical input regimes and plant effects on microbial diversity, using a long-term agricultural site that has been running since 1994 (Browne et al. 2009). The experimental monoculture system and its long period of stable chemical input levels make it a suitable model for studying the influences of these treatments on the bacterial community structure.

MATERIAL AND METHODS

Soil source and sampling. The experimental site, managed by Teagasc, is located at Knockbeg, County Carlow, Ireland (52°51'N, 6°56'W). Details concerning the agrichemical inputs and the geochemical properties of the soil have been described previously (Browne et al. 2009, Chhabra et al. 2012). The experiment design is a comparison between four replicate plots subjected to reduced (low) agricultural chemical inputs versus four subjected to conventional (high) inputs. Compared to the high input regime, the low input plots have received one quarter less nitrogen fertiliser, no phosphorus fertiliser, and a half reduction in biocides (Table 1). The crop is a continuous monoculture of spring barley (*Hordeum vulgare* L., cv. Regina). In April 2007, 10 randomly chosen plants per plot were removed with adhering soil from four replicate plots of two distinct agro-ecosystems (low or high inputs). Soil loosely adhering to the roots of each plant was removed and defined as the rhizosphere. The 10 samples from each plot were then combined to yield one rhizosphere sample per plot. Similarly, 10 randomly selected cores of bare soil, with the same size as rhizosphere sample were taken and combined to give the bulk soil samples. The soils were sealed in sterile plastic bags immediately after collection, and stored at –20°C until used. Soil geochemical characteristics were measured by Chhabra et al. (2012) and are described in Table 2.

Soil DNA extraction. Soil crude DNA was extracted using the method described by Carrigg et al. (2007) based on bead beating and incubation with lysozyme at 37°C for 30 min. However, a modified CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 1.0 mol/L sodium chloride and 120 mmol sodium phosphate buffer at pH 8.0) was used. The crude DNA was further purified by gel extraction using GELASE^{TM} (Epicentre, Madison, USA) to remove humic acids prior to polymerase chain reaction (PCR).

16S ribosomal DNA amplification and denaturing gradient gel electrophoresis (DGGE). The V3 region of the 16S rRNA gene was amplified using bacterial universal primers 338f/GC and 518r (Muyzer et al. 1993). PCR was performed in a 50 µL volume amplification reaction containing 20 pmol of each primer, 25 µL of GoTaq Green Master Mix (Promega, Madison, USA) and 200 ng of DNA. The thermocycling reaction consisted of an initial denaturation step at 95°C (3 min), followed by

Table 2. Soil chemical characteristics

	Soil pH	Soil mineral elements (mg/kg)					
		P	K	Mg	Cu	Mn	Zn
Conventional input	6.70 (0.83)	16.1 (1.9)	105.0 (14.1)	121.8 (7.9)	7.10 (0.21)	659.5 (65.8)	6.90 (1.14)
Reduced input	6.30 (0.13)	7.3 (1.1)	122.0 (7.9)	139.5 (9.3)	6.70 (0.41)	559.9 (133.3)	5.80 (0.43)

Standard deviation is presented in parentheses. Measured by Chhabra et al. (2012)

30 cycles [95°C (30 s), 55°C (30 s), 72°C (40 s)], and a final extension step at 72°C (5 min). DGGE was performed in a D-Code electrophoresis system (BioRad Laboratories, Hercules, USA). Polyacrylamide gels were prepared with a denaturant gradient of urea and formamide from 40% to 60% and run at 60°C, 68V for 15 h. After electrophoresis, the gel was stained with SYBR-Gold (Invitrogen, USA, Carlsbad) for 30 min, followed by a de-staining step in distilled water for 5 min. Gel images were captured by a GelDocIt^{TM} Imaging System (UVP, Upland, USA).

Sequencing of representative DGGE ribotypes. Each gel band (ribotype) present in at least two soil samples was excised from the DGGE gel and eluted overnight in 20 µL of TE buffer at 4°C. Eluted DNA was used as template for PCR amplification using the same primers and conditions described above. Platinum High Fidelity *Taq* DNA polymerase (Invitrogen, Carlsbad, USA) was used instead of the GoTaq GreenTaq Master Mix for sequence fidelity. PCR products were ligated into the pCR 2.1-TOPO vector using TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA) and were sequenced by Eurofins MWG Operon Inc. (Ebersberg, Germany). Taxonomic information of ribotype sequences were obtained from Genbank database using BLASTn. All sequences generated in this study have been deposited in the Genbank database under accession numbers JF682373–JF682386.

Statistical analysis. The DGGE gels were scanned and the density of each ribotype was determined using the peak area quantification of QuantityOne^{TM} (BioRad Laboratories, Hercules, USA) (Yang et al. 2001). Statistical analysis was carried out based on the presence and quantity of DGGE ribotype profiles using the multi-variate statistical software package (Kovach 2007). A community dendrogram was constructed based on the Jaccard's coefficient and clustered by the nearest neighbour-joining method using the unweighted pair group method with arithmetic mean (UPGMA) (Figure 1). Principal coordinates (PCO) analysis was performed by calculating the Eigen value percentages of the Euclidean distances between samples, and scattered the PCO case scores of the samples to a two-dimensional graph. Community diversity in different treatments was estimated using the Shannon-Wiener index with 95% confidence limits. The distribution patterns of ribotypes were viewed in a Venn diagram constructed by VENNY (Oliveros 2007).

RESULTS AND DISCUSSION

After 12 years of continuous fertilisations, the soil available phosphorus content increased from 7.5 mg/kg to 16.1 mg/kg between reduced input and conventional input soils, while the level of other mineral elements and soil pH were not significantly affected (Table 2). The relative influence of spring barley monoculture and fertiliser input on the soil bacterial community structure was assessed by a culture-independent approach performed on 16 distinct samples. According to the DGGE profiles and following principal coordinates (PCO) analysis the most stringent separation of the bacterial community occurred between samples collected from the rhizosphere and the bulk soil, regardless of the input regime (H or L) (Figure 2a). Indeed bacterial community associated with the rhizosphere and with the bulk soil only shared 41% similarity to each other (Figure 1). This result highlights the over-riding selective influence of the rhizosphere over the chemical inputs and is in accordance with a multitude of studies, which have reported that the plant shapes the structure of the soil microbial communities (Berg and Smalla 2009). Indeed root exudation could affect the

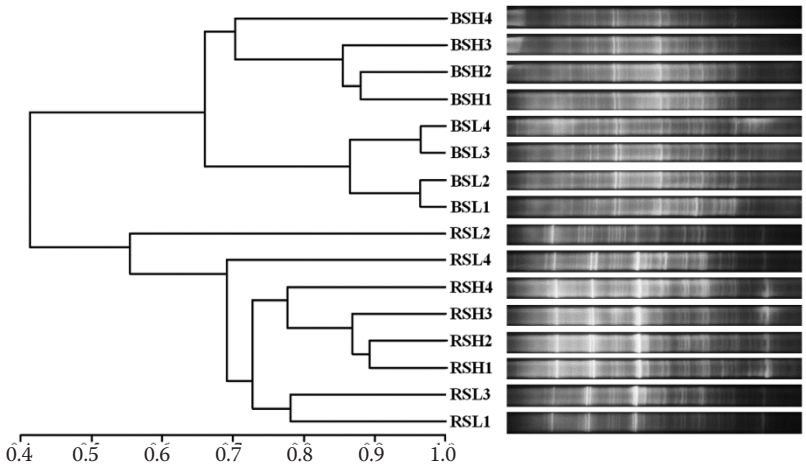

Figure 1. Comparison of bacterial community structure in rhizosphere and bulk soil from high and low input treatments. Denaturing gradient gel electrophoresis (DGGE) gels and clustering analysis illustrating the difference in banding profiles obtained from the different samples. Labelling is as follows: RS – rhizosphere soil; BS – bulk soil; L – low input; H – high input. The number denotes the sample (plot) number for each agricultural input regime

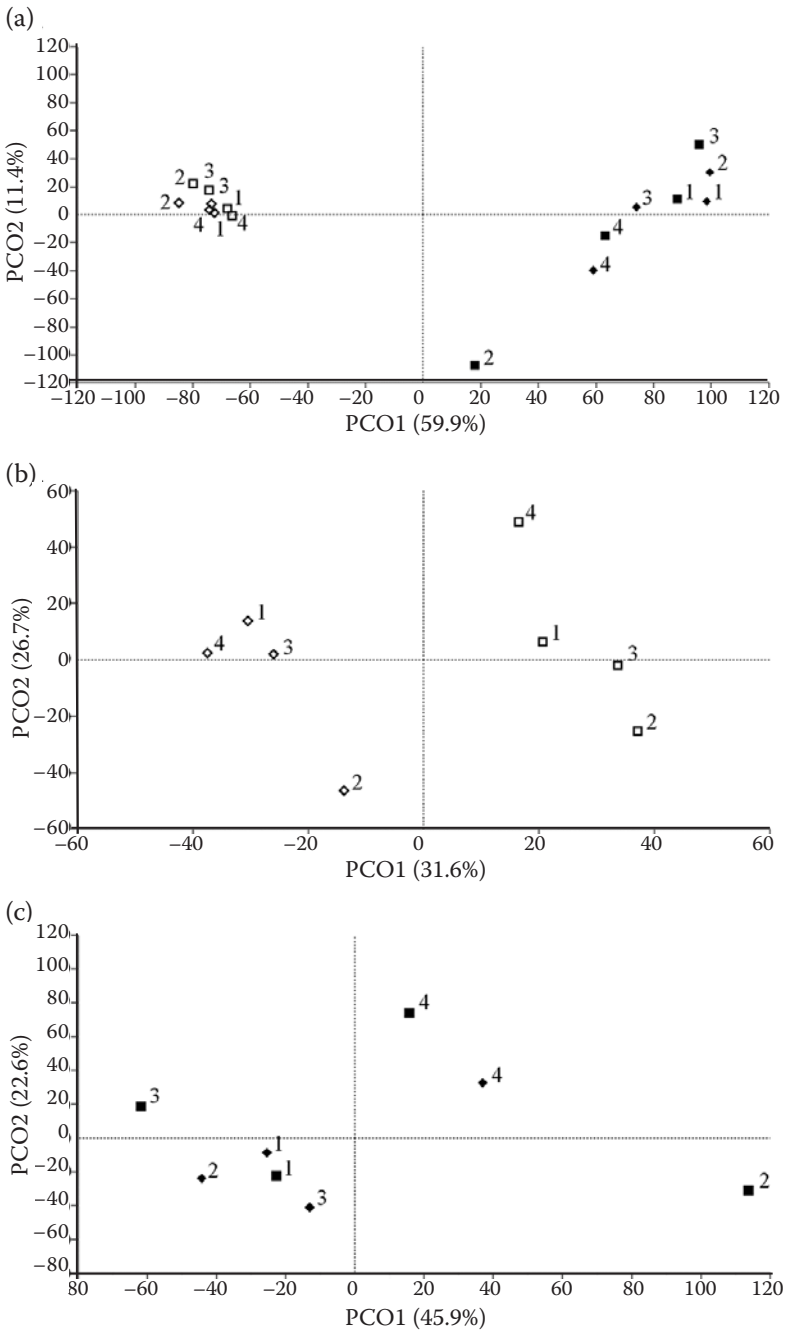

Figure 2. Principal coordinate (PCO) analysis of similarity matching data produced from denaturing gradient gel electrophoresis (DGGE) profiles. (a) rhizosphere versus bulk soil; (b) bulk soil low versus high inputs; (c) rhizosphere soil low versus high input. The scale bar represents the variance percentage. Labelling is as follows: rhizosphere soil samples are indicated with solid symbols, bulk soil samples with open symbols; high input plots are indicated with diamonds, low input plots with squares; replicate samples are numbered 1–4

bacterial community diversity through excretion of nutritional, bioactive, or signal substances that can modulate specific microorganisms (Bais et al. 2006). The stronger impact of the rhizosphere on the soil microbial community in comparison to agrichemical input levels seems to be general rather than specific to the type of fertiliser applied. For example, it has already been reported that *nifH* nitrogen-fixing bacterial communities are shaped primarily by the sorghum rhizosphere, while the effect associated with nitrogen fertiliser amendment level is auxiliary (Coelho et al. 2009). PCO analysis also shows a clear separation between bulk soil samples derived from the low and high input agro-ecosystem (Figure 2b). In the bulk soil, the low agrichemical input, which contains no phosphorus fertiliser, results in a lower inorganic phosphate level in the soil (Table 2). This low phosphate environment could potentially provide a competitive advantage to phosphorus-solubiliser microorganisms and consequently modify the bacterial community structure. Indeed, a number of studies have identified microorganisms possessing strong phosphorus solubilisation abilities in low-P soils (Khan et al. 2009, Saha and Biswas 2009). However, according to PCO analysis, the bacterial communities associated with the rhizosphere amended with low and high inputs are similar (Figure 2c). This could be due to an increase of the phosphorus solubilisation in the rhizosphere, which

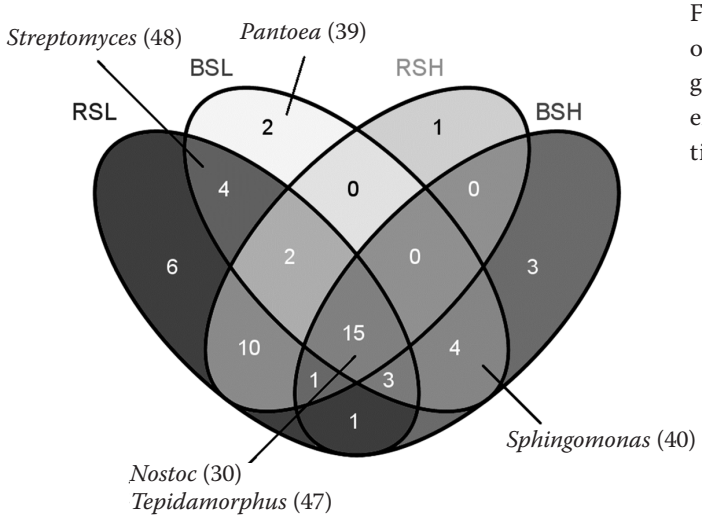

Figure 3. Venn diagram showing distribution of 52 ribotypes from the denaturing gradient gel electrophoresis (DGGE) profiles under the experimental conditions. Abbreviation explanations – see Figure 1

might support the growth of soil microorganisms with poor phosphorus solubilisation abilities. The intensification of the phosphorus solubilisation in the rhizosphere could be due to the plant itself (Kirk 1999, Gunes et al. 2007) or to the local acidification of the pH in this environment (Uroz et al. 2009). This observation suggested that the rhizosphere effect could have a buffering effect against the influence of agrichemical input levels.

The diversity of the microbial community within each agro-ecosystem was also estimated by the Shannon-Wiener index. This estimator indicates that the bacterial diversity is significantly increased in the rhizosphere (3.4682 ± 0.0006) and bulk soil (3.2868 ± 0.0004) samples derived from the reduced inputs plots in comparison to rhizosphere (3.1779 ± 0.0005) and bulk soil (3.1029 ± 0.0008) samples associated with high input plots. The difference of available phosphorus between reduced and conventional inputs could explain this shift. Indeed a decrease of the microbial diversity in soils subjected to phosphorus fertiliser input has already been reported in previous studies (Richardson and Simpson 2011). This could be explained, in part, by a lower relative abundance and diversity of the phosphorus solubilising bacterial community in soil amended with phosphorus fertiliser. However, the increase in bacterial diversity in soils samples

Table 3. Distribution of the total 52 ribotypes from the denaturing gradient gel electrophoresis (DGGE) profiles under the experimental conditions

Ribotype distribution patterns	Number of ribotypes	Rhizosphere soil		Bulk soil	
		low input	high input	low input	high input
Universal	15	+	+	+	+
By plant influences	10	+	+	–	–
	4	–	–	+	+
By input levels	4	+	–	+	–
	0	–	+	–	+
By a combination of plant influences and input levels	6	+	–	–	–
	1	–	+	–	–
	2	–	–	+	–
	3	–	–	–	+
	3	+	–	+	+
	2	+	+	+	–
	1	+	+	–	+
Other	1	+	–	–	+

Ribotypes were sorted based on their presence (+) or absence (–) under different agricultural input regime

derived from rhizosphere environments is more difficult to explain. Indeed the rhizosphere usually selects some specific bacterial populations and therefore decreases the bacterial diversity in comparison to bulk soils (Normander and Prosser 2000, Kleineidam et al. 2010). This effect observed in this study could be partially explained by possible introduction of endophytic bacteria associated to the barley seeds (Coombs and Franco 2003).

A total of 52 separate ribotypes were determined by DGGE profiles, each representing different taxonomic groups in the bacterial communities (Figure 1). The distribution of ribotypes in the different input regimes was compared by a Venn diagram (Figure 3, Table 3). A total of 15 abundant ribotypes, which correspond in part to *Nostoc* and *Tepidamorphus* spp., were present in each soil sample (Figure 3). Consistent with the rhizosphere effect observed previously, 18 and 9 ribotypes were specifically detected in the rhizosphere or in the bulk soil, respectively. Finally, 12 ribotypes were specifically associated with low input soils, among which the most abundant belongs to *Streptomyces* spp. While unequivocal conclusions cannot be drawn without a more extensive study, these observations seem to indicate that chemical inputs may decrease bacterial diversity through elimination of certain bacterial taxa such as some actinobacterial genii.

This study demonstrates that in a monoculture agricultural system with long-term stable chemical regimes, the crop plays a major influence on the bacterial community structure. However, there is also a clear effect of agricultural chemicals, namely a reduction in number of certain taxonomic groups. From this study, it is not possible to identify the particular components of the chemical input responsible for this effect but this deserves further investigation.

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