Compost Science & Utilization

ISSN: 1065-657X (Print) 2326-2397 (Online) Journal homepage: http://www.tandfonline.com/loi/ucsu20

Study of Total Bacteria and Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea in Response to Irrigation with Sewage Sludge Compost Tea in Agricultural Soil

M. Vela-Cano, M. Gómez-Brandón, C. Pesciaroli, H. Insam & J. González-López

To cite this article: M. Vela-Cano, M. Gómez-Brandón, C. Pesciaroli, H. Insam & J. González-López (2018): Study of Total Bacteria and Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea in Response to Irrigation with Sewage Sludge Compost Tea in Agricultural Soil, Compost Science & Utilization, DOI: 10.1080/1065657X.2018.1432429

To link to this article: https://doi.org/10.1080/1065657X.2018.1432429

COMPOST SCIENCE & UTILIZATION
https://doi.org/10.1080/1065657X.2018.1432429

Taylor & Francis
Taylor & Francis Group

Study of Total Bacteria and Ammonia-Oxidizing Bacteria and Ammonia-Oxidizing Archaea in Response to Irrigation with Sewage Sludge Compost Tea in Agricultural Soil

M. Vela-Cano[a], M. Gómez-Brandón[b], C. Pesciaroli[a], H. Insam[b], and J. González-López[a]

[a]Institute of Water Research, University of Granada, Granada, Spain; [b]Institut für Mikrobiology, Universität Innsbruck, Innsbruck, Austria

ABSTRACT

Organic amendments have been shown to improve the quality of agricultural soils. Thus, the use of sewage sludge compost tea as a fertilizer can be considered a worthy and environmentally friendly alternative as it also offers the option of recycling sludge at the same time. However, an in-depth knowledge of how the addition of this product affects soil microbial diversity is still necessary. As such, the main objective of this study was to evaluate, at a microcosm level, the effects of irrigation with sewage sludge compost tea in an olive grove soil, focusing on the changes in the total bacteria, ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA). For this purpose plastic pots were filled with olive grove soil, watered with different amounts of water and/or compost tea, and incubated at 21 and 35°C for a period of 90 days. Denaturing gradient gel electrophoresis (DGGE) fingerprinting, real-time PCR, and 454-pyrosequencing analysis were performed. Our results suggested that the addition of sewage sludge compost tea (liquid fertilizer) slightly increased the soil biological diversity during the incubation time which suggests that sewage sludge compost tea did not have any negative effects in the soil microbiota. Accordingly and regardless of the incubation temperature, significant changes in the soil community structure were not observed throughout the experiment, suggesting that the treated soils maintained their microbiological stability.

Introduction

Over the last decades, concern about environmental preservation has increased and as such, the correct management of wastewater has become an important matter. However, despite this large amounts of sewage sludge are produced as a consequence of the depuration of water. In order to solve this problem several ways of managing the sludge produced have been tried. Composting is one of the most environmental friendly and widely used options to recycle, dispose of, and reuse this product (Jiang et al. 2015). Along these lines, sewage sludge compost and the resulting compost tea (CT), a water extract from compost (Ksheem et al. 2015; Pant et al. 2012) can be used as organic amendments in agricultural soils which could lead to an increase in soil productivity, as well as to promote cost-effective nutrient management (Wang et al. 2015). It has been shown that the addition of organic amendments improves the quality of degraded and poor soils by maintaining soil organic matter; acting as a resource of nutrients such as nitrogen, phosphorus, and sulfur for crops (Habai et al. 2016); and sustaining soil fertility, thereby ultimately increasing agricultural production (Tejada, Hernandez, and Garcia 2009). Furthermore, it may enhance plant growth and reduce the need for mineral fertilizers, which in turn reduces the costs for farmers (Mohanty et al. 2011). In addition, it is important to highlight that organic amendments generally improve the physical, chemical, and biological properties of soils (Diacono and Montemurro 2010). The growth and activity of soil microbes are stimulated by soil organic matter, leading to an efficient mineralization of crop nutrients (Tejada, Hernandez, and Garcia 2009). In particular, CT applied to soil

CONTACT M. Vela-Cano mvc@ugr.es Institute of Water Research, Edificio Fray Luis de León, C/Ramón y Cajal, 4, 18071, Granada, Spain.
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/ucsu.
Supplemental data for this article can be accessed on the publisher's website.

affects the rhizosphere of the plant by carrying nutrients and microorganisms (Bess 2000).

However, nowadays there is still a lack of in-depth general knowledge about the effects of the addition of CT in soils. In this sense, the study of soil bacterial diversity and in particular of those bacterial populations involved in the nitrogen cycle in response to the application of CT into soils is essential for a better understanding of the effect of this fertilizer. For this purpose, fingerprinting methodologies such as temperature/denaturing gradient gel electrophoresis (TGGE and DGGE), have been used in several studies (Egert et al. 2004) to provide further knowledge to this area. Nevertheless, these techniques are not capable of revealing populations whose abundance is excessively low (Marzorati et al. 2008). In order to dig deeper into the bacterial community structure and population dynamics, quantitative polymerase chain reaction (PCR) assay and 454-pyrosequencing have also been used (Poulsen et al. 2013; Wan, Wang, and Xie 2014; Youssef et al. 2009). While quantitative PCR is able to estimate an accurate quantification of a specific microbial group 454-pyrosequencing gives information about the presence of any kind of bacteria.

The aim of this work was to evaluate the influence of the application of different dosages of sewage sludge CT on the total bacteria, ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA) community structure in an olive grove soil using a microcosm system. Due to the extremely high temperatures reached in Mediterranean areas during the summer months, we also checked the effect of two incubation temperatures on the abovementioned microbial groups in response to the addition of CT.

Materials and Methods

Soil and Compost Tea

The soil used in the present study was collected from an olive grove soil located in Jaén (Southern Spain). It is a Hypocalcic Luvic Calcisol, containing 44.1% silt, 39.1% clay, 16.2% sand, and 0.6% gravel. The chemical composition of the soil was as follows: 0.946% organic matter; pH 7.9; total nitrogen 0.09 %; total phosphorus 0.0085 %; and the content of heavy metals: 115.66 mg Cu kg^{-1}, 15.12 mg Pb kg^{-1}, 176.02 mg Zn kg^{-1}, and 0.4 mg Cd kg^{-1} (Pérez-Lomas et al. 2010). The soil was air-dried and sieved (<2 mm) in order to remove gravel and plant residues.

CT was made according to Vela-Cano et al. (2014). Briefly: CT was produced by mixing 1.5 kg of sewage sludge compost and 100 L of distilled water in a bioreactor (fig. S1). The mixture was continuously aerated using an aeration pump in order to maintain aerobic conditions throughout the process. The CT obtained after 5 days of incubation had an organic matter percentage of 0.016%, pH of 7.7, and electrical conductivity of 506 $\mu s\ cm^{-1}$. The amount of N, P, and K was 0.005, 0.008, and 0.018%, respectively. The heavy metals content was 0.6 mg Cu L^{-1}, 0.08 mg Pb L^{-1}, 0.7 mg Zn L^{-1}, and 0.01 mg Cd L^{-1}. Finally, the number of cultivable heterotrophic bacteria was 1.1 10^7 CFU mL^{-1} (Vela-Cano et al. 2014).

Experimental Design: Microcosm Setup

Plastic pots (6.0 cm diameter × 35 cm length), previously washed with HCl (0.1 N) in order to eliminate any kind of contamination, were filled with 230 g of soil (dry weight). To prevent light entering and the possible growth of plants, each pot was covered with a dish. A second dish was placed at the bottom of each pot to collect the leachate.

All of the microcosms were incubated at 21 and 35°C in an incubation chamber to check the effect of the temperature for an incubation period of 90 days. Four samples from each microcosm were taken at 4, 8, 30, and 90 days.

Sixty-nine mL of distilled water (DW) or CT were added in the first irrigation. Then, during the experimental period, microcosms incubated at 21°C were irrigated once every 10 days while the microcosms incubated at 35°C were irrigated once every 5 days, in order to maintain soil field capacity (30 g g^{-1}), with 26 ± 3 mL of DW or CT respectively. Microcosm irrigation was performed with DW and/or CT according to the four treatments shown in table 1.

DNA Extraction and PCR-DGGE Analysis of Microbial Community

Total DNA was extracted in triplicate from 0.5 g of each sample (fresh weight) using the FastDNA® SPIN Kit for Soil and the FastPrep® 24-Instrument (MP Biomedicals, Germany) according to the manufacturer's protocol. After the extraction, a mixture was elaborated mixing 20 μL of each repetition. PCR was performed for the amplification of total bacteria, aerobic AOB, and AOA. PCR was

Table 1. Overview of the irrigations with distilled water (DW) and compost tea (CT) used for the different treatments included in the microcosm experiment: Control (C), Low Dosage (LD), Medium Dosage (MD) and High Dosage (HD).

	Days of incubation																		
	0	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
C	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW
LD	CT	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW	DW
MD	CT	DW	DW	CT	DW	DW	DW	DW	DW	DW	DW	DW	CT	DW	DW	DW	DW	DW	DW
HD	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT	CT

performed with 1 μL DNA in a total volume of 25 μL containing 200 nM of each primer (fort otal bacteria CG-P_1: CGCCCGCCGCGCGCGGCG GGCGGGGCGGGGGCACGGGGGGCCTACGG-GAGGCAGCAG and P_2: ATTACCGCGGCTGC TGG (Muyzer, De Waal, and Uitierlinden 1993), for AOB, CTO189F A+B and CTO189C used ina ratio 2:1 and CTO654r were used (Kowalchuk et al. 1997) and for AOA Arch-amoAf and Arch-amoAR (Francis, Beman, and Kuypers 2005) (table 2)).

For the amplification, 1X reaction buffer [16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl {pH 8.8}, 1.5 mM $MgCl_2$, 0.01% Tween 20] (Biotherm^{TM}, GeneCraft, Germany), 0.5 mM $MgCl_2$ (Biotools, Spain), 0.2 mM deoxyribonucleotide triphosphate (dNTPs; Biotherm^{TM}, GeneCraft, Germany), 0.4 mg mL^{-1} bovine serum albumin (BSA; Sigma, Austria), 0.025 U Bio- Therm^{TM} DNA polymerase (Biotherm^{TM}, GeneCraft, Germany), and sterile water were added to each sample. Negative controls containing all the components except DNA templates were also included. Amplification was performed in a Flex Cycler (Analytik Jena, Germany) following the conditions as described by Muyzer, De Waal, and Uitierlinden (1993), Kowalchuk et al. (1997), and Francis, Beman, and Kuypers (2005) for total bacteria, AOB, and AOA, respectively. Proper sizes of amplification products were verified by electrophoresis in 2% agarose gels, and PCR product concentration was determined with PicoGreen dsDNA quantification reagent (Invitrogen, Carlsbad, USA). Fluorescence was measured using an Anthos Zenyth 3100 multimode reader (Anthos Labtec, Austria) and the Software for Anthos Multimode Detectors (Version 2.0.0.13).

The DGGE of bacterial and archaeal communities was performed by loading 60 ng of PCR products in 8.5 and 7% (w/v) polyacrylamide gels for AOB and AOA, respectively containing a specific denaturing gradient for each microbial group, 40%–70% for total bacteria, 40%–65% for AOB, and 25%–50% for AOA (100% denaturant corresponds to 7 M urea and 40% [v/v] formamide; A 100 bp DNA ladder (Genecraft®, Germany) served as marker. Gels were run in 1 X TAE (20 mM Tris-HCl, 10 mM acetate, 0.5 mM Na_2EDTA) at 60°C for 16 h at 100 V by using an INGENYphorU System (Ingeny International BV, The Netherlands). Gels were stained with silver nitrate using the Hoefer Automated Gel Stainer (Amersham Pharmacia Biotech, Germany), air-dried, and scanned for subsequent image analysis.

Fingerprinting Analysis

The band patterns generated by DGGE were normalized, compared, and clustered using the Gel Compar II software, version 5.102 (Applied Maths, Belgium). For the proper normalization of band patterns, a common sample of the MBMBR system was used as an internal marker in all of the gels. DGGE profiles were compared and clustered using the Dice coefficient. A 1% band position tolerance was applied for band assignment (Gómez-Silván et al. 2014). Dendrograms relating band pattern similarities were automatically calculated with unweighted pair group method with arithmetic mean algorithms (UPGMA). Significance of UPGMA clustering was estimated by calculating the cophenetic correlation coefficients (Sokal and Rohlf 1962).

Based on the DGGE analysis, the following theoretical indices were calculated: range weighted richness (Rr), functional organization (Fo), and Shannon–Wiener (H') (Calderón et al. 2012; Marzorati et al. 2008).

Quantitative Real-Time PCR

Quantitative real-time PCR analysis was chosen to determine the 16S rRNA gene copy number of AOA, AOB, and total bacteria. This analysis was performed with the 1 X Sensimix^{TM} SYBR® Hi-rox (Bioline, USA) based on the DNA-intercalating dye SYBR Green I. The Rotorgene 6200 cycler (Corbett research – now

Table 2. Sequences of the primers used in the qPCR reaction.

Primer	Sequence (5→3)	Bacterial group	Reference
1055F	ATGGCTGTCGTCAGCT	Total Bacteria	Ferris et al. (1996)
1392R	ACGGGCGGTGTGTAC		
CTO189F A+B	CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA GRA AAG CAG GGG ATC G	AOB	Kowalchuk et al. (1997)
CTO189C*	CGC CCG CCG CGC GGC GGG CGG GGC GGG GGC ACG GGG GGA GGA AAG TAG GGG ATC G		
CTO654r	CTAGCYTTGTAGTTTCAAACGC		
Arch-amoAF	STAATGGTCTGGCTTAGACG	AOA	Francis et al. (2005)
Arch-amoAR	GCGGCCATCCATCTGTATGT		

Qiagen, Germany) was used in combination with the Rotor-Gene Series Software 1.7. Standard construction was performed with end-point PCR using the following sample/pure cultures as a template: DNA of *Nitrosomonas europaea DSMZ 21879* for total bacteria and AOB; and plasmid containing an *amoA* sequence – AOA. The R^2 was 0.99 in all of the cases and the efficiency was 0.92, 0.84, and 0.93 for total bacteria, AOB, and AOA, respectively. Stock concentration [gene copies μL^{-1}] was determined via PicoGreen measurement and freshly prepared. Ten-fold dilutions were used for standard curve construction. Quantitative PCR was performed in 20-μL assays with each reaction mix containing 1X Sensimix™ SYBR® Hi-rox (Bioline, USA), forward and reverse primers (primer sequences and references are listed in table 2), 0.4 mg mL^{-1} BSA, distilled water (RNase/DNase free, Gibco™, UK), and 2 μL of either 1:10 diluted DNA-extract or ten-fold diluted standard DNA. To check for product specificity and potential primer dimer formation runs were completed with a melting analysis starting from 60 to 99°C with temperature increments. All the qPCR conditions are detailed in table 3.

Analysis of Bacterial Community by 454-Pyrosequencing

A total of 16 samples corresponding to those collected at days 8 and 90 of incubation of each treatment were selected for the bacterial diversity analy sis by 454-pyrosequencing of partial bacterial 16S rRNA genes. 454-pyrosequencing was conducted by Research and Testing Laboratory (Lubbock, USA) on a GS FLX + platform (Roche, Branford, USA) using a modification of the primers 28F/519R targeting the V1–V3 hypervariable regions of bacterial 16S rRNA genes (28F: 5′-GAGTTTGATCNTGGCTCAG-3′, 519R: 5′-GTNTTACNGCGGCKGCTG-3′). Reads were trimmed and denoising was performed using the USEARCH algorithm. Chimera checking was carried out on the selected operational taxonomic unit (OTU) using the UCHIME chimera detection software executed in de novo mode. The resulting sequences were then clustered into OTUs using the UPARSE algorithm. The taxonomic affiliation was assigned to each OTU using the RDP Classifier with a minimum confidence of 80%.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed using the software package Statgraphics 3.0 Plus version (STSC Inc., Rockville, MD, USA) in order to identify the effect of the different irrigation treatments on the studied microbial groups. A significance level of 95% ($p < 0.05$) was selected.

Results and Discussion

DGGE Analysis of the Total Bacteria, AOA and AOB Community Structure

The DGGE profiles of total bacteria in the microcosm setup are shown in figure 1. The dendrogram based on

Table 3. qPCR conditions for the studied groups.

Total bacteria		AOB and AOA	
MasterMix	Cycling conditions	MasterMix	Cycling conditions
Distilled water → 5.6 mL	94 °C → 10 minutes	Distilled water → 7.3 mL	95 °C → 10 minutes
SYBR Green → 10 mL	95 °C → 20 seconds	SYBR Green → 10 mL	95 °C → 40 seconds
Primer Forward → 0.8 mL	58 °C → 15 seconds	Primer Forward → 0.2 mL	55 °C → 30 seconds
Primer Reward → 0.8 mL	72 °C → 30 seconds	Primer Reward → 0.2 mL	72 °C → 25 seconds
BSA → 0.8 mL	40 cycles	BSA → 0.5 mL	40 cycles
DNA → 2 mL		DNA → 2 mL	

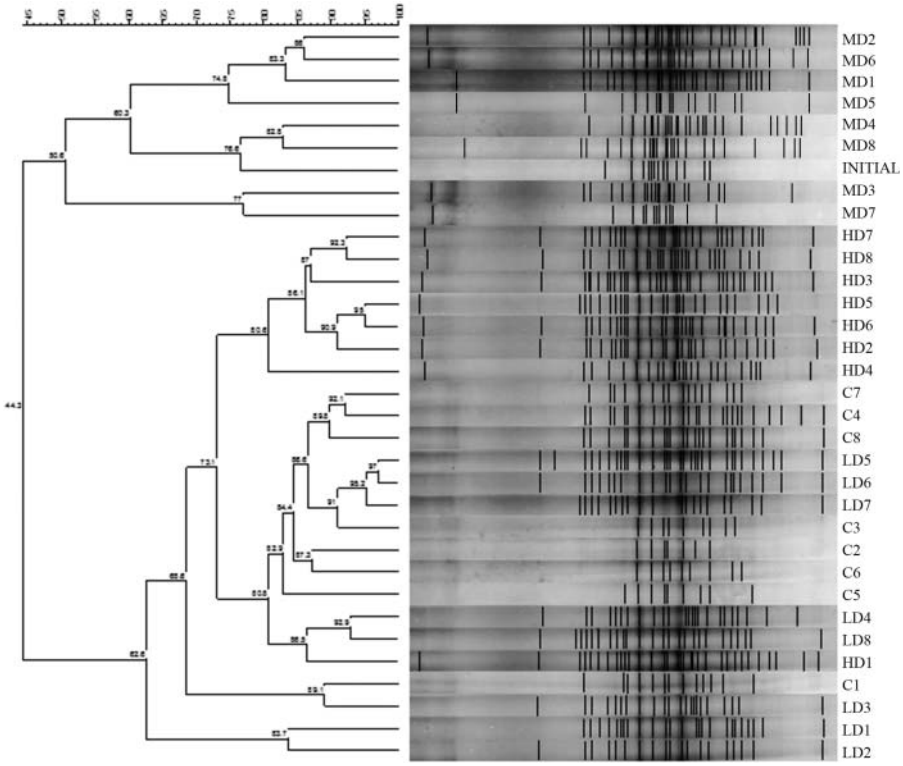

Figure 1. The dendrogram of total bacteria generated by UPGMA clustering (Dice correlation coefficient) of 16S rRNA-based DGGE patterns from microcosms irrigated with water and/or compost tea and incubated at different times and temperatures. The scale bar indicates the percentage of similarity. Samples were as follows: control (C), low dosage (LD), medium dosage (MD), and high dosage (HD). The numbers from 1 to 4 represent day of sampling (4, 8, 30, and 90) at 21°C, while the numbers from 5 to 8 represent the same day of sampling at 35°C.

the Dice coefficient analysis showed that total bacterial communities clustered in two main groups (44% similarity). One cluster is composed by the samples collected from the pots irrigated with a medium dosage (MD) of CT and the initial soil (dry soil before irrigation). In the other cluster, the samples that received a low dosage (LD) of CT and the control samples clustered together, while samples watered only with CT (HD) grouped together in a subcluster that presented more than 80% of similarity. In the 33 soil samples studied, the image analysis performed with Gel Compar II allowed the identification of 47 different banding positions (classes of bands, data not shown) representing the different species present in each sample. Only two of the 47 bands were found in all of the samples. The average number of bands detected in the control samples were 13, while the number was 23, 23, and 26 in LD, MD, and HD, respectively. Of note was that the number of bands in soil irrigated with CT was higher than in the soil irrigated with DW. This fact indicates that the number of microbial species seemed to highly increase after the addition of CT.

DGGE clustering of AOB communities (figure 2) did not show a very clear differentiation among the treatments applied. However, the influence of the use of sewage sludge CT as a fertilizer was more notable when the number of bands was analyzed. The average number of bands detected in each treatment was 7, 8, 7, and 10 for C, LD, MD, and HD, respectively. This result suggested a slight influence of the addition of a high dosage of CT on this microbial group, as observed for total bacteria. Nevertheless, it is obvious that such an effect on the increase in the number of species was not as significant as that observed on the total bacterial populations.

Finally, DGGE fingerprints from AOA communities evidenced clear differences between those pots irrigated with CT and those with only DW (figure 3). The dendrogram based on the Dice coefficient analysis showed that AOA communities clustered in two main groups (48% similarity). The main cluster was composed by samples from the pots irrigated with the different dosages of CT (low, medium, and high), which were further separated into two subclusters, one of them composed by pots irrigated only with CT and the other one from microcosms irrigated only with DW. Considering the number of bands, 41 different

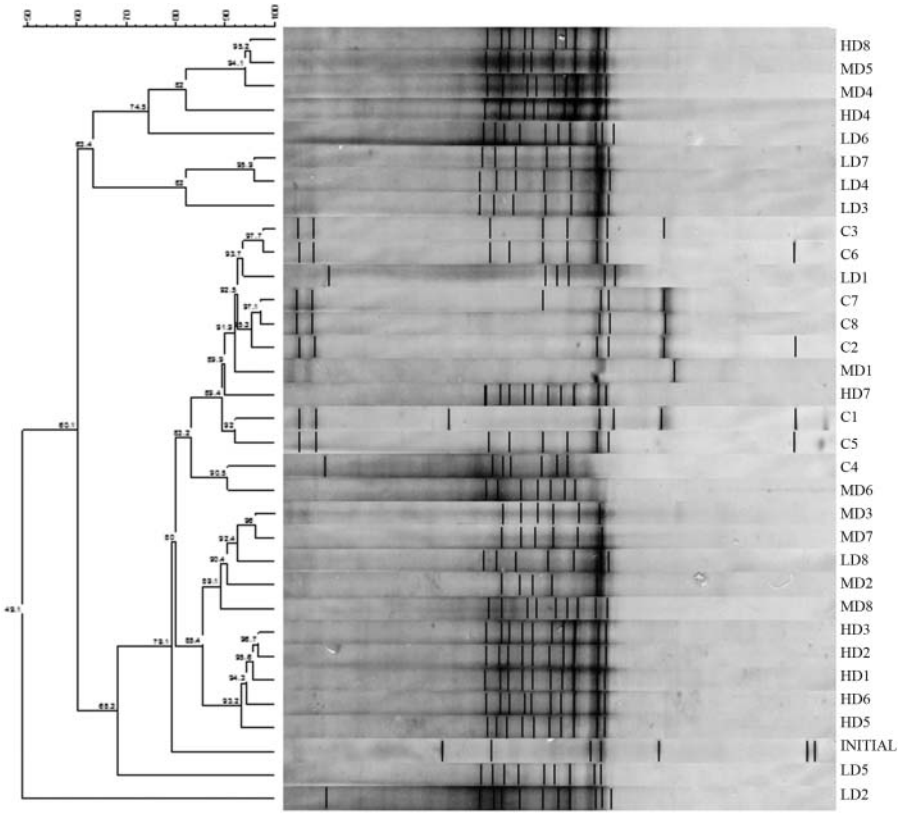

Figure 2. The dendrogram of AOB communities generated by UPGMA clustering (Dice correlation coefficient) of 16S rRNA-based DGGE patterns from microcosms irrigated with water and/or compost tea and incubated at different times and temperatures. The scale bar indicates the percentage of similarity. Samples were as follows: control (C), low dosage (LD), medium dosage (MD), and high dosage (HD). The numbers from 1 to 4 represent day of sampling (4, 8, 30, and 90) at 21°C, while the numbers from 5 to 8 represent the same day of sampling at 35°C.

banding positions were identified. In this case, the highest average number of bands was also detected in the HD samples (22) while in the control samples there were 19 bands.

All in all, it indicates that the use of the sewage sludge CT (particularly at a high dosage) as a fertilizer influenced the diversity of the bacterial and archaeal communities in the studied soil. Our results are in accordance with those obtained by other authors (Ding et al. 2013; Emmyrafedziawati, Krsek, and Wellington 2009; Wallis et al. 2010; Wenhui et al. 2007) who had been studying new ways of improving soil quality and analyzing the influence of the different methods on soil biodiversity. In these studies, it was found that the tillage and/or the addition of the organic and inorganic fertilizers positively affected soil biodiversity.

Richness and Diversity Indices

Richness and diversity indices, that represent the measure of the population diversity of total bacteria, AOB, and AOA communities in the pots are shown in figure 4. The values of the Rr index, which describes the diversity richness of a given community (Marzorati et al. 2008), significantly varied between the four treatments used (figure 4). The HD pots were characterized by the highest bacterial diversity, while the lower diversity was found in the control pots which only received DW. This is in agreement with those findings obtained by DGGE analysis, where the lowest number of bands was also registered in the control samples. It was also evident that the total bacterial populations were the most affected by the irrigation with CT, as demonstrated by the high level of variation among the different treatments.

The Fo index represents the distribution of individuals over different species in microbial communities, estimating their degree of specialization (Marzorati et al. 2008). In our case, the average values of Fo of the studied microbial groups in the microcosms were 0.50 for total bacteria, 0.44 for AOB, and 0.46 for AOA. The values obtained were quite low, reaching almost

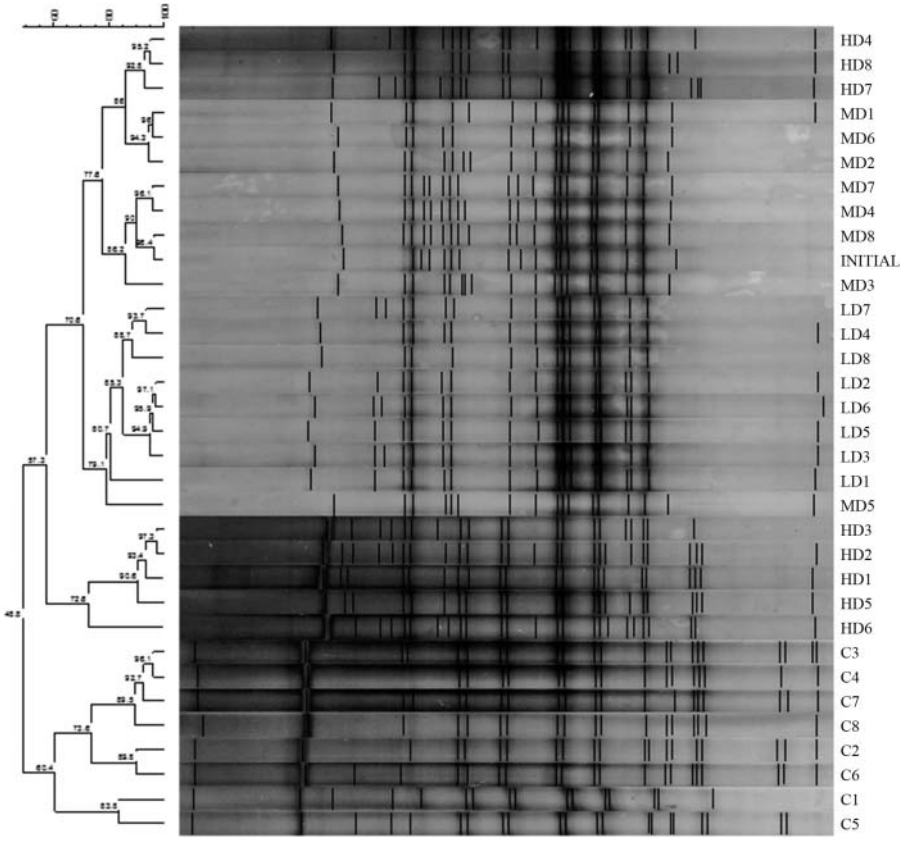

Figure 3. The dendrogram of AOA communities generated by UPGMA clustering (Dice correlation coefficient) of Arch-amoA-based DGGE patterns from microcosms irrigated with water and/or compost tea and incubated at different times and temperatures. The scale bar indicates the percentage of similarity. Samples were as follows: control (C), low dosage (LD), medium dosage (MD), and high dosage (HD). The numbers from 1 to 4 represent day of sampling (4, 8, 30, and 90) at 21°C, while the numbers from 5 to 8 represent the same day of sampling at 35°C.

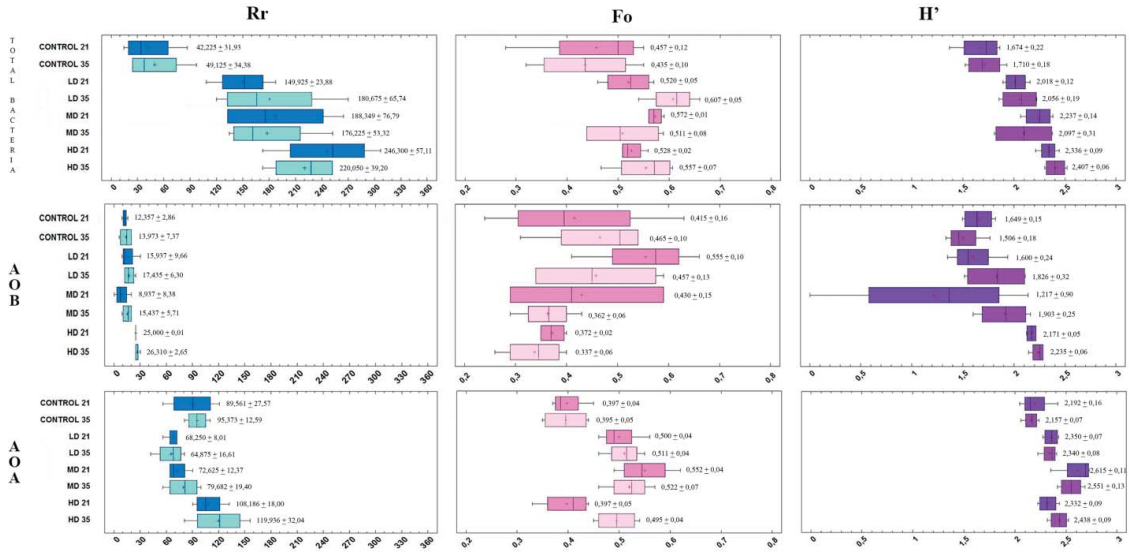

Figure 4. Box-and-Whisker plots of the ecological indices obtained for the soil total bacteria, ammonia-oxidizing bacteria (AOB), and ammonia-oxidizing archaea (AOA) communities: range weighted richness index (Rr), functional organization (Fo), and Shannon–Wiener index, calculated for microcosms irrigated with distilled water and/or compost tea (control (C), low dosage (LD), medium dosage (MD), and high dosage (HD)) and incubated at 21°C or 35°C.

Table 4. Gene copy number g^{-1} soil of total bacteria, ammonia-oxidizing bacteria (AOB) and archaea (AOA) in the different CT treatments.

		4 Days		8 Days		30 Days		90 Days	
		21 °C	35 °C	21 °C	35 °C	21 °C	35 °C	21 °C	35 °C
Total Bacteria	C	$1.47{\cdot}10^{9}$	$1.15{\cdot}10^{9}$	$1.95{\cdot}10^{9}$	$1.17{\cdot}10^{9}$	$1.65{\cdot}10^{9}$	$1.19{\cdot}10^{9}$	$1.12{\cdot}10^{9}$	$1.05{\cdot}10^{9}$
	LD	$1.24{\cdot}10^{9}$	$1.19{\cdot}10^{9}$	$1.66{\cdot}10^{9}$	$1.00{\cdot}10^{9}$	$1.02{\cdot}10^{9}$	$1.46{\cdot}10^{9}$	$1.82{\cdot}10^{9}$	$1.99{\cdot}10^{9}$
	MD	$1.38{\cdot}10^{9}$	$1.12{\cdot}10^{9}$	$1.68{\cdot}10^{9}$	$1.23{\cdot}10^{9}$	$1.91{\cdot}10^{9}$	$2.24{\cdot}10^{9}$	$2.08{\cdot}10^{9}$	$1.21{\cdot}10^{9}$
	HD	$1.77{\cdot}10^{9}$	$1.18{\cdot}10^{9}$	$1.71{\cdot}10^{9}$	$1.55{\cdot}10^{9}$	$1.88{\cdot}10^{9}$	$2.16{\cdot}10^{9}$	$2.85{\cdot}10^{9}$	$2.60{\cdot}10^{9}$
AOB	C	$6.71{\cdot}10^{6}$	$8.87{\cdot}10^{6}$	$2.49{\cdot}10^{6}$	$1.11{\cdot}10^{6}$	$4.27{\cdot}10^{6}$	$3.04{\cdot}10^{6}$	$2.60{\cdot}10^{6}$	$2.38{\cdot}10^{6}$
	LD	$7.49{\cdot}10^{6}$	$3.51{\cdot}10^{6}$	$1.88{\cdot}10^{6}$	$1.74{\cdot}10^{6}$	$2.76{\cdot}10^{6}$	$4.31{\cdot}10^{6}$	$3.54{\cdot}10^{6}$	$2.65{\cdot}10^{6}$
	MD	$8.30{\cdot}10^{6}$	$9.30{\cdot}10^{6}$	$2.54{\cdot}10^{6}$	$1.01{\cdot}10^{6}$	$4.06{\cdot}10^{6}$	$3.30{\cdot}10^{6}$	$6.18{\cdot}10^{6}$	$1.51{\cdot}10^{6}$
	HD	$8.12{\cdot}10^{6}$	$5.72{\cdot}10^{6}$	$2.45{\cdot}10^{6}$	$1.85{\cdot}10^{6}$	$4.99{\cdot}10^{6}$	$3.15{\cdot}10^{6}$	$3.13{\cdot}10^{6}$	$1.31{\cdot}10^{6}$
AOA	C	$1.44{\cdot}10^{6}$	$1.96{\cdot}10^{6}$	$1.52{\cdot}10^{6}$	$2.91{\cdot}10^{6}$	$1.49{\cdot}10^{6}$	$1.0^{6}{\cdot}10^{6}$	$1.29{\cdot}10^{6}$	$1.24{\cdot}10^{6}$
	LD	$1.35{\cdot}10^{6}$	$1.33{\cdot}10^{6}$	$7.33{\cdot}10^{6}$	$1.80{\cdot}10^{6}$	$1.24{\cdot}10^{6}$	$1.89{\cdot}10^{6}$	$1.48{\cdot}10^{6}$	$2.17{\cdot}10^{6}$
	MD	$1.26{\cdot}10^{6}$	$1.54{\cdot}10^{6}$	$6.81{\cdot}10^{6}$	$1.65{\cdot}10^{6}$	$2.11{\cdot}10^{6}$	$2.10{\cdot}10^{6}$	$2.26{\cdot}10^{6}$	$1.94{\cdot}10^{6}$
	HD	$2.85{\cdot}10^{6}$	$3.20{\cdot}10^{6}$	$6.59{\cdot}10^{6}$	$2.22{\cdot}10^{6}$	$2.41{\cdot}10^{6}$	$1.65{\cdot}10^{6}$	$1.77{\cdot}10^{6}$	$1.37{\cdot}10^{6}$

No significant differences were detected among the four treatments.

the complete evenness. This meant that the soil microcosms were composed of homogeneous microbial communities, with high flexibility, thus capable of reacting to sudden stressful conditions.

The H′ index takes into account both the community species richness and its functional organization. The average values of H′ obtained for total bacteria and AOB communities were significantly lower in pots watered only with DW than those pots irrigated with CT. However, AOA values were similar (2.1–2.6) in all of the treatments, indicating a medium richness and functional organization of this microbial group.

Overall, the results obtained confirm the positive influence of CT on the studied soil biodiversity. In fact, numerous studies have frequently reported that microbial diversity is affected by fertilization regimes (Esperschütz et al. 2007; Zhang, Shao, and Ye 2012). Moreover, a higher number of individuals and a higher diversity of microbial communities were found in organically fertilized systems rather than in inorganically fertilized ones, which is consistent with our results.

Abundance of Bacteria, Ammonia-Oxidizing Bacteria (AOB), and Ammonia-Oxidizing Archaea (AOA)

Quantitative PCR is a molecular biology technique that is very useful in tracking changes in the abundance of genes, which permits to link these changes with variations in ambient operating conditions (Smith and Osborn 2008). For this reason, total bacteria and two of the microbial groups involved in the soil N cycle (AOB and AOA), were studied by real-time PCR.

The addition of CT to the olive grove soil influenced the abundance of the bacterial and archaeal communities, according to the copy numbers determined by qPCR. Results obtained are shown in table 4.

Data in table 4 showed a great homogeneity in the number of DNA copies·g^{-1} soil with regard to the studied microbial groups. In fact, no significant changes were detected in soil bacterial abundance when comparing microcosms watered with CT versus those irrigated with only DW (control pots). In this context, the temperature did not seem to have a significant effect on any of the studied microbial groups, since the obtained values were very similar in all of the cases. These results are in accordance with the data obtained by Ding et al. (2016) who analyzed the effect of the addition of inorganic fertilizer and manure amendment on the structure of bacterial and archaeal communities in soil from China. Wessen et al. (2010), who added organic fertilizers into soil, found a minimal variation in the number of DNA copies g^{-1} soil (6.9×10^{9} to 1.9×10^{10}) between treatments. Furthermore, in the study carried out by Tian et al. (2015) it was observed that nitrogen fertilization had no significant impact on the soil used. This trend was also observed in our study. On the other hand, the ratio of AOA/AOB values revealed the numerical predominance of AOB over AOA which differs from the findings obtained by these authors.

Study of the Bacterial Community by 454-Pyrosequencing

Due to the strong stability of the bacterial community structure observed in the DGGE fingerprinting and real-time PCR analysis, samples from pots incubated during 8 and 90 days of each treatment were

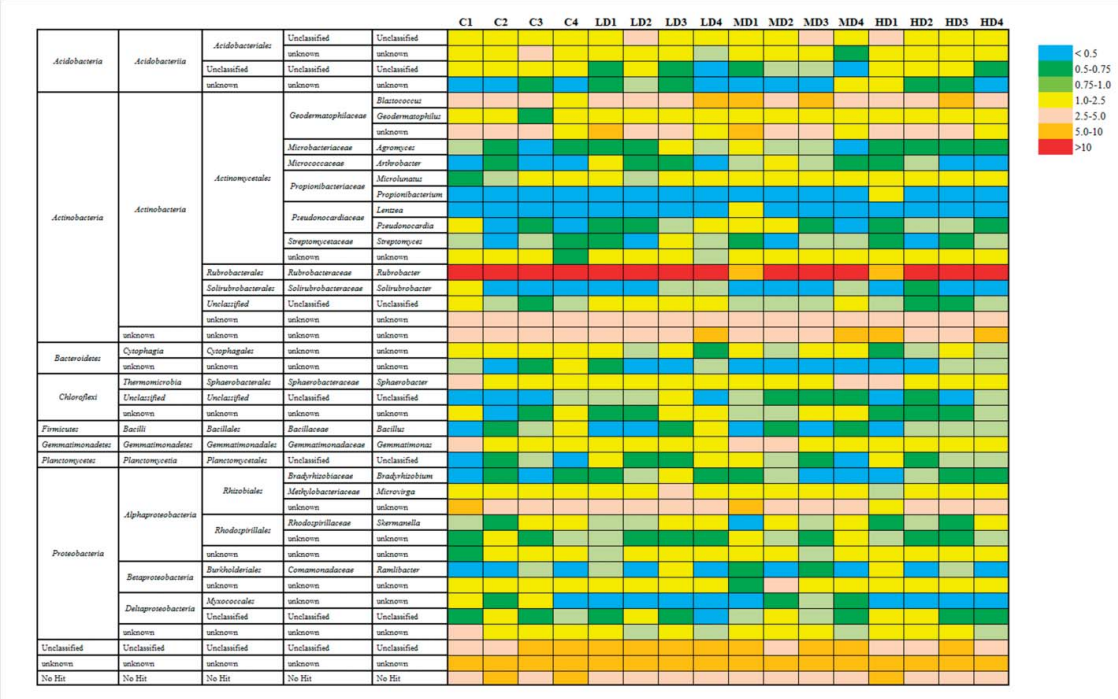

Figure 5. Heat map of the relative abundances of all of the bacterial phyla with regard to the four different treatments. Samples were as follows: control (C), low dosage (LD), medium dosage (MD), and high dosage (HD). The numbers 1 and 2 represent day of sampling 8 and 90, respectively at 21°C, while numbers from 3 and 4 represent the same day of sampling at 35°C.

selected to study the diversity of the bacterial community by 454-pyrosequencing.

Sixteen bacterial phyla were identified across the entire data set (fig. S2), five of which were denominated "minor phyla," constituting less than 1% of the total sequences in all of the samples.

Actinobacteria and *Proteobacteria* were predominant in all of the microcosms, followed by *Acidobacteria, Chloroflexi, Gemmatimonadetes, Bacteroidetes, Planctomices, and Firmicutes*. Ding et al. (2016) and Wan, Wang, and Xie (2014) found the same major groups in their soils. The phyla *Actinobacteria* and *Proteobacteria* occupied 61%–67% of the microbial sequences obtained from the treated soils. *Acidobacteria* and *Chloroflexi* were the next most abundant phyla, accounting for 3.3%–8.2% and 3.1%–5.6% of the total reads, respectively. The phyla *Gemmatimonadetes* (1.7%–3.8%), *Bacteroidetes* (1.5%–3.1%), *Planctomycetes* (0.7%–1.7%), and *Firmicutes* (0.3%–1.4%) represented more than 1% in at least one of the samples. Finally, *Cyanobacteria, Nitrospirae, Synergistetes, Verrucomicrobia*, and *Aquificae* were all less than 1% of the total reads. The results obtained by other authors, using pyrosequencing techniques, showed similar major phyla composition in agricultural soils (Ding et al. 2016; Poulsen et al. 2013; Tian et al. 2015; Wan, Wang, and Xie 2014; Zhao et al. 2013). Both, the phylum *Chloroflexi* and the phylum *Bacteroidetes* were present in all of the soil samples, however, the relative abundance of *Chloroflexi* was usually higher than *Bacteroidetes* which is consistent with the findings reported by Roesch et al. (2007) and Janssen (2006) using 16S rRNA gene clone libraries or pyrosequencing.

As previously described by other authors (Dolfing et al. 2004; Marschner, Kandeler, and Marschner 2003), in general our data did not reveal significant changes in the relative abundance of the different bacterial phyla among the studied treatments (figure 5), which can probably be due to the short period of time studied. Additionally, it is of note that the genus *Rubrobacter* was the most abundant in all of the treatments followed by *Blastococcus*. On the other hand, bacterial genera with the lowest number of individuals were *Propionibacterium* and *Lentzea*, all of them belonging to the phylum *Actinobacteria*.

Conclusions

The application of sewage sludge CT in an olive grove soil as a liquid fertilizer increased the soil diversity of total bacteria, AOB, and AOA during the studied incubation time. However, the pyrosequencing analysis revealed no significant changes in the distribution

and relative abundance of the bacterial phyla among the four different treatments. These findings seem to confirm that toxic elements, such as heavy metals present in our CT did not damage the soil microbiota as its use did not entail an ecological risk even when applied at a higher dosage.

References

Bess, V. H. 2000. Understanding compost tea. *BioCycle* 41: 71–72.

Calderón, K., B. Rodelas, N. Cabirol, J. González-López, and A. Noyola. 2011. Analysis of microbial communities developed on the fouling layers of a membrane-coupled anaerobic bioreactor applied to wastewater treatment. *Bioresource Technology* 102:4618–27. doi:10.1016/j.biortech.2011.01.007.

Calderón, K., J. Martín-Pascual, J. M. Poyatos, and J. González-López. 2012. Comparative analysis of the bacterial diversity in a lab-scale moving bed biofilm reactor (MBBR) applied to treat urban wastewater under different operational conditions. *Bioresource Technology* 121:119–126.

Diacono, M., and F. Montemurro. 2010. Long-term effects of organic amendments on soil fertility. *Agronomy for Sustainable Development* 30:401–22. doi:10.1051/agro/2009040.

Ding, G. C., Y. M. Piceno, H. Heuer, N. Weinert, and A. B. Dohrmann. 2013. Changes of soil bacterial diversity as a consequence of agricultural land use in a semi-arid ecosystem. *PLoS ONE* 8 (3):e59497. doi:10.1371/journal.pone.0059497.

Ding, J., X. Jiang, M. Ma, B. Zhou, D. Guan, B. Zhao, J. Zhou, F. Cao, L. Li, and J. Li. 2016. Effect of 35 years inorganic fertilizer and manure amendment on structure of bacterial and archaeal communities in black soil of northeast China. *Applied Soil Ecology* 105:187–95. doi:10.1016/j.apsoil.2016.04.010.

Dolfing, J., A. Vos, J. Bloem, P. A. I. Ehlert, N. B. Naumova, and P. J. Kuikman. 2004. Microbial diversity in archived soils. *Science* 306:813. doi:10.1126/science.306.5697.813a.

Egert, M., S. Marhan, B. Wagner, S. Scheu, and M. W. Friedrich. 2004. Molecular profiling of 16S rRNA genes reveals diet-related differences of microbial communities in soil, gut, and casts of *Lumbricus terrestris L.* (*Oligochaeta: Lumbricidae*). *FEMS Microbiology Ecology* 48:187–97. doi:10.1016/j.femsec.2004.01.007.

Emmyrafedziawati, A. K. R., M. Krsek, and E. M. H. Wellington. 2009. Microbial diversity of various agricultural soil sites using denaturing gradient gel electrophoresis (DGGE) community profiling. *Journal of Tropical Agriculture and Food Science* 37 (2):233–9.

Esperschütz, J., A. Gattinger, P. Mader, M. Schloter, and A. Fließbach. 2007. Response of soil microbial biomass and community structures to conventional and organic farming systems under identical crop rotations. *FEMS Microbiology Ecology* 61:26–37. doi:10.1111/j.1574-6941.2007.00318.x.

Ferris, M. J., and G. Muyzer. 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Applied and Environmental Microbiology* 62 (2):340–6.

Francis, C. A., J. M. Beman, and M. Kuypers. 2005. New processes and players in the nitrogen cycle: the microbial ecology of anaerobic and archaeal ammonia oxidation. *The ISME Journal* 1:19–27. doi:10.1038/ismej.2007.8.

Gómez-Silvan, C., R. Vílchez-Vargas, J. Arévalo, M. A. Gómez, J. González-López, D. H. Pieper, and B. Rodelas. 2014. Quantitative response of nitrifying and denitrifying communities to environmental variables in a full-scale membrane bioreactor. *Bioresource Technology* 169:126–133.

Habai, R., V. Nwakaego, P. Deusdedit, I. Odeh, A. Singh, D. Buchan, and S. De Neve. 2016. Nitrogen mineralization dynamics of different valuable organic amendments commonly used in agriculture. *Applied Soil Ecology* 101:185–93. doi:10.1016/j.apsoil.2016.01.006.

Janssen, P. 2006. Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology* 72:1719–28. doi:10.1128/AEM.72.3.1719-1728.2006.

Jiang, J., X. Liu, Y. Huang, and H. Huang. 2015. Inoculation with nitrogen turnover bacterial agent appropriately increasing nitrogen and promoting maturity in pig manure composting. *Waste Management* 39:78–85. doi:10.1016/j.wasman.2015.02.025.

Kowalchuk, G. A., J. R. Stephen, W. De Boer, J. I. Prosser, T. M. Embley, and J. W. Woldendorp. 1997. Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Applied and Environmental Microbiology* 63:1489–97.

Ksheem, A., J. Bennett, D. Antille, and S. Raine. 2015. Towards a method for optimized extraction of soluble nutrients from fresh and composted chicken manures. *Waste Management* 45:76–90. doi:10.1016/j.wasman.2015.02.011.

Marschner, P., E. Kandeler, and B. Marschner. 2003. Structure and function of the soil microbial community in a long-term fertilizer experiment. *Soil Biology and Biochemistry* 35:453–61. doi:10.1016/S0038-0717(02)00297-3.

Marzorati, M., L. Wittebolle, N. Boon, D. Daffonchio, and W. Verstraete. 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental Ecology* 10 (6):1571–81.

Mohantya, M., K. Reddy, M. E. Probert, R. C. Dalal, A. Subba, and N. W. Menzies. 2011. Modelling N mineralization from green manure and farmyard manure from a laboratory incubation study. *Ecological Modelling* 222:719–26. doi:10.1016/j.ecolmodel.2010.10.027.

Muyzer, G., E. De Waal, and A. G. Uitierlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59:695–700.

Pant, P., T. Radovich, N. Hue, and R. Paull. 2012. Biochemical properties of compost tea associated with compost quality

and effects on pak choi growth. *Scientia Horticulturae* 148:138–46. doi:10.1016/j.scienta.2012.09.019.

Pérez-Lomas, A. L., G. Delgado, J. Párraga, R. Delgado, G. Almendros, and V. Aranda. 2010. Evolution of organic matter fractions after application of co-compost of sewage sludge with pruning waste to four Mediterranean agricultural soils. A soil microcosm experiment. *Waste Management* 30:1957–65. doi:10.1016/j.wasman.2010.04.030.

Poulsen, P., W. Al-Soud, L. Bergmark, J. Magid, L. Hansen, and S. Sorensen. 2013. Effects of fertilization with urban and agricultural organic wastes in a field trial e Prokaryotic diversity investigated by pyrosequencing. *Soil Biology & Biochemistry* 57:784–93. doi:10.1016/j.soilbio.2011.12.023.

Roesch, L., R. Fulthorpe, A. Riva, G. Casella, A. Hadwin, A. Kent, S. Daroub, F. Camargo, W. Farmerie, and E. Triplett. 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. *The ISME Journal* 1:283–90. doi:10.1038/ismej.2007.53.

Smith, C., and A. Osborn. 2008. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiology Ecology* 67:6–20. doi:10.1111/j.1574-6941.2008.00629.x.

Sokal, R. R., and F. J. Rohlf. 1962. The comparison of dendrograms by objective methods. *Taxonomy* 11 (2):33–40. doi:10.2307/1217208.

Tejada, M., M. T. Hernandez, and C. Garcia. 2009. Soil restoration using composted plant residues: effects on soil properties. *Soil & Tillage Research* 102:109–17. doi:10.1016/j.still.2008.08.004.

Tian, W., L. Wang, Y. Li, K. Zhuang, G. Li, J. Zhang, X. Xiao, and Y. Xi. 2015. Responses of microbial activity, abundance, and community in wheat soil after three years of heavy fertilization with manure-based compost and inorganic nitrogen. *Agriculture, Ecosystems and Environment* 213:219–27. doi:10.1016/j.agee.2015.08.009.

Vela-Cano, M., A. Castellano-Hinojosa, A. F. Vivas, and M. V. Toledo. 2014. Effect of heavy metals on the growth of bacteria isolated from sewage sludge compost tea. *Advances in Microbiology* 4:644–55. doi:10.4236/aim.2014.410070.

Wallis, P. D., R. J. Haynes, C. H. Hunter, and C. D. Morris. 2010. Effect of land use and management on soil bacterial biodiversity as measured by PCR-DGGE. *Applied Soil Ecology* 46:147–50. doi:10.1016/j.apsoil.2010.06.006.

Wan, R., Z. Wang, and S. Xie. 2014. Dynamics of communities of bacteria and ammonia-oxidizing microorganisms in response to simazine attenuation in agricultural soil. *Science of the Total Environment* 472:502–8. doi:10.1016/j.scitotenv.2013.11.090.

Wang, X., H. Ciu, J. Shi, X. Zhao, Y. Zhao, and Z. Wei. 2015. Relationship between bacterial diversity and environmental parameters during composting of different raw materials. *Bioresource Technology* 198:395–402. doi:10.1016/j.biortech.2015.09.041.

Wenhui, Z., C. Zucong, Y. Lichu, and Z. He. 2007. Effects of the long-term application of inorganic fertilizers on microbial community diversity in rice-planting red soil as studied by using PCR-DGGE. *Acta Ecologica Sinica* 27 (10):4011−18. doi:10.1016/S1872-2032(07)60084-5.

Wessen, E., K. Nyberg, J. Jansson, and S. Hallin. 2010. Responses of bacterial and archaeal ammonia oxidizers to soil organic and fertilizer amendments under long-term management. *Applied Soil Ecology* 45:193–200. doi:10.1016/j.apsoil.2010.04.003.

Youssef, N., C. Sheik, L. Krumholz, F. Najar, B. Roe, and M. Elshahed. 2009. Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. *Applied and Environmental Microbiology* 75:5227–36. doi:10.1128/AEM.00592-09.

Zhang, T., M. Shao, and L. Ye. 2012. 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *The ISME Journal* 6:1137–47. doi:10.1038/ismej.2011.188.

Zhao, J., R. Zhang, C. Xue, W. Xun, L. Sun, Y. Xu, and Q. Shen. 2013. Pyrosequencing reveals contrasting soil bacterial diversity and community structure of two main winter wheat cropping systems in China. *Microbial Ecology* 67:443–53. doi:10.1007/s00248-013-0322-0.