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Rational application of treated sewage sludge with urea increases GHG mitigation opportunities in Mediterranean soils

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ABSTRACT

Mediterranean soils, which are carbonate-rich and organic matter-poor, are prone to erosion and important carbon losses due to seasonal changes associated with dry summers and wet winters. The use of thermophilic digested sewage sludge (TSS) in these agricultural systems, as a soil amendment, has been acknowledged as an interesting way to supply organic matter and nutrients. Data on the long-term evaluation of TSS applied to Mediterranean soils are scarce. Moreover, the effect of the application is unpredictable because of the intrinsic variation in the TSS. The scope of this study was to determine whether the continued application of TSS for 20 years leads to increased carbon sequestration in the soil without affecting emissions of greenhouse gases.

To conduct this evaluation, the doses applied since 1992 have been as follows: 40 t ha^{-1} and 80 t ha^{-1} every year, and 40 t ha^{-1} every 3 years, plus annual mineral N fertilization depending on the crop. A control without TSS or mineral fertilization and a treatment with only mineral N fertilizer were also evaluated. In this case, urea was used as the mineral treatment. The TSS doses were applied annually in October, while the mineral was split into one dose around January and another in March. The chemical parameters, greenhouse gas emissions, nitrate and ammonium concentrations of the soil were measured during the crop cycle. The bacterial community and enzymes in the soil were surveyed 15 days after the last annual application and at harvest. Fifteen days after fertilization with TSS and urea, nitrification and denitrification potentials were measured.

The 80 t ha^{-1} yr^{-1} dose yielded the most significant increase in total carbon, organic matter content, P_2O_5, and total nitrogen. This same treatment significantly increased GHG emissions for all gases concerned. Similar results were found in the 40 t ha^{-1} 3yr^{-1} and urea for CO_2 and CO_2eq ha^{-1}. TSS application increased soil enzyme activities. According to the microbial diversity results, 80% of the DNA sequences corresponded to 6 main phyla: (from most to least) Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Verrucomicrobia with unclassified material making up an average 10.94% of the sequences. The soil microbial community structure only altered with the 80 t ha^{-1} yr^{-1} dose. The highest dose of TSS applied in this study resulted in the irreversible lodging of the crop and a concomitant decrease in yield.

In the 40 t ha^{-1} 3yr^{-1} treatment, interesting similarities were found with urea alone. In summary, rational application of TSS, such as 40 t ha^{-1} 3yr^{-1} dose, along with urea, trigger a beneficial increase in

Abbreviations: C, carbon; Ca, calcium; $CaCO_3$, calcium carbonate; CCA, canonical correspondence analysis; CH_4, methane; CO_2, carbon dioxide; d, day; DNA, deoxyribonucleic acid; DW, dry weight; ECD, electron capture detector; FID, flame ionization detector; FW, fresh weight; GC, gas chromatography; GHG, greenhouse gas; H′, Shannon-Weaver index; ha, hectare; K, potassium; kg, kilogram; M, molar; mg, milligram; Mg, magnesium; $MgCl_2$, magnesium chloride; N, nitrogen; N_2O, nitrous oxide; Na, sodium; NH_3, ammonia; NH_4, ammonium; NO_3, nitrate; OM, organic matter; OTUs, operational taxonomic units; P, phosphorus; PCoA, principal component analysis; PCR, polymerase chain reaction; RDP, ribosomal database project; rRNA, ribosomal ribonucleic acid; TSS, treated sewage sludge; UPGMA, unweighted pair group method with arithmetic mean; w/w, weight/weight; yr, year.

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microbial activity in soil, that ultimately activates soil metabolism and enhances carbon sequestration possibilities, while GHG emissions remain at the same level as with urea alone. The results support the hypothesis that TSS can induce carbon sequestration without increasing GHG emissions. TSS has proven to exert beneficial outcomes under Mediterranean conditions; additionally, its application offers a viable opportunity for converting this by-product into a fertilizer. However, application rates must be adjusted or it should be used together with mineral fertilization.

1. Introduction

Technological developments have led to important waste water sanitization rates, but collateral residues or by-products are still generated. One such by-product is sewage sludge (SS). Supplementary treatments, such as thermophilic anaerobic digestion, ensure that any potential biological hazards are minimized or removed from the SS. Thermophilic anaerobic digestion (TSS) is an adequate way to remove unpleasant smells, sanities SS and, to a lesser extent, stabilize the generated organic matter. Considering that TSS contains high proportions of organic matter and plant nutrients, applying it agriculturally as a soil amendment or fertilizer is a common practice (Wang, 1997). The physical, chemical and biological properties of soils are positively modified after TSS application; plants are provided essential nutrients and waste recycling is promoted (Singh and Agrawal, 2008). However, regional assessments of long-term application and extreme application rates should be conducted (Paramasivam et al., 2008) to explore the limitations of TSS application to soil, as there are important associated environmental risks (Roig et al., 2012).

Applying SS to soil is known to increase GHG fluxes (Paramasivam et al., 2008). As the organic material in the sludge is mineralized, CO_2 emissions from the soil increase. (Sheppard et al., 2005). However, in certain vulnerable ecosystems where TSS has been applied, soil C and N content have been increased between 40% and 60% (Pavan-Fernandes et al., 2005). Thus, appropriate agricultural management has resulted in important opportunities for GHG mitigation: the optimization of fertilization has resulted in a greater potential to reduce GHG fluxes and increased potential carbon sequestration in soil (Loubet et al., 2011).

An important and increasing number of studies are focusing their efforts on the evaluation of heavy metal evolution, accumulation and translocation in soil in response to TSS application (Lake et al., 1984; Smith, 2009). In Mediterranean ecosystems a similar tendency can be seen. However, only a few studies have assessed the environmental performance of TSS application in terms of GHG fluxes. Arguably, the agricultural application of TSS could induce important N imbalances in soil. Nitrification and denitrification processes could theoretically be exacerbated, increasing N_2O emissions and favouring other nutrient losses (Sheppard et al., 2005; Pezzolla et al., 2012). Moreover, there is a general paucity of research work investigating bacterial community dynamics in response to continued applications of TSS to the soil. Undoubtedly, soil biota plays a key role in the functions that control gaseous losses and nutrient cycling. Alterations at community level after TSS application have been recorded by Dennis and Fresquez (1989), Pascual et al. (2008) and Mattana et al. (2014). Their findings stress the importance of evaluating each individual case in detail, as the TSS composition varies importantly, beginning with the stabilizing and sanitizing treatments, but the effect is also influenced by the dose applied (Zaman et al., 2004).

Therefore, the objective of this research was to determine the possible effects of long-term application of TSS on fertility and soil surface GHG emissions at an agricultural field. Soil chemical, environmental and biological characteristics were assessed during the cropping season of the 20th year after continued treatment application to the soil (TSS; mineral N fertilizer and control). Additionally, the bacterial community was surveyed to investigate its structure and the possible correlations between the bacterial communities and soil enzyme activity and GHG emissions from soil.

2. Materials and methods

2.1. Site description

This research was carried out at a long-term experimental site established in 1992 in Arazuri, Navarra, Spain (42° 48′N−1° 43′W).

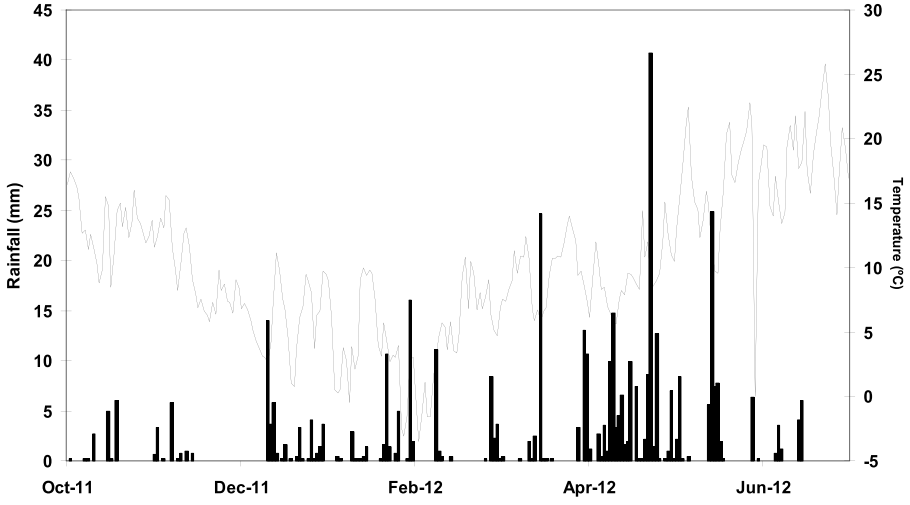

Fig. 1. Daily precipitation (bars) and mean air temperature (line) for 261 days of study in Arazuri.

The area is located in the Arazuri experimental station, adjacent to the wastewater treatment plant of the Pamplona Federation of Municipalities (*Mancomunidad de la Comarca de Pamplona*). The experiment is managed by the *Instituto Navarro de Tecnologias e Infraestructuras Agroalimentarias* (INTIA). According to the agro-climatic classification system of Papadakis (1961), the climate is Humid-temperate-Mediterranean with an annual rainfall of 760 mm and a mean temperature of 12.4 °C. Fig. 1 shows the precipitation levels and air temperatures recorded during the assayed period. The soil is classified as calcaric cambisol (FAO, FAO-UNESCO, 1997) or calcixerollic xerochrept with a silty-clay loam texture. The chemical characteristics of the soils, for each treatment, in the year 2011, can be found in Table 1.

Since the beginning of the experiment, a series of crop rotations has taken place following the sequence "cereal/cereal/non-cereal". Crop rotation is a common practice in this region aimed at managing soil fertility and disease control. Also, nutrient requirements differ between crops, so nutrient exhaustion is avoided. In 2011, the cultivated crop was oats (*Avena sativa* L. var Aintree) sowed at a density of 100 kg ha^{-1}. No weed control was carried out and no supplementary irrigation systems were used. The agricultural management did not differ between treatments.

2.2. Treatments and experimental design

This study involved the application of three doses of TSS, along with an unfertilized control and a mineral fertilizer. The treatment application began in 1992. The TSS was applied to the soil surface and incorporated at a depth of 30 cm around October every year, corresponding with sowing. The chemical and physical characteristics of the TSS are presented in Table 2. The TSS contained 14.1% (w/w) dry matter, 36.75% TOC and 7.4% N Kjendahl. The three doses were: 40 t ha^{-1} yr^{-1}, 80 t ha^{-1} yr^{-1} and 40 t ha^{-1} 3yr^{-1}, this latter treatment received supplemental mineral fertilization every year. The mineral fertilizer used was urea (60 kg N-Urea ha^{-1} split into two applications). The TSS doses were 834 kg N ha^{-1} y^{-1} (80 t), 418 kg N ha^{-1} y^{-1} (40 t), and 139 kg N ha^{-1} y^{-1} (40 t 3yr). The every 3 year treatment received a dose of 120 kg N as urea (split application). In 2011, treatments were applied on October 23rd and oats were sown immediately afterwards. The mineral N was usually split into two applications, corresponding to two plant stages: tillering and stem-elongation. For each treatment an experimental plot of 35 m^2 was identified. The experiment followed a randomized complete block design with four blocks.

2.3. Soil sampling to analyze general soil properties

Before the treatment application, a bulked soil sample comprising four soil subsamples was collected from each experimental plot from the 0–30 cm layer. Since each experimental plot had its own bulk sample, a total of 4 independent samples were analyzed per treatment. The samples were then air-dried at room temperature and stored at 4 °C. The physical and chemical properties of the soil were determined after the samples were sieved through 2 mm mesh. Soil pH was measured in suspension with deionized water (w/w) ratio of 1:2.5. Soil organic matter content was determined using the Walkley–Black organic C method (ISO, 1995). Total nitrogen was measured through ICP-OES after a microwave digestion with H_2SO_4 and H_2O_2 (A.O.A.C., 1984). Phosphorus was determined with the Olsen method. Available K content was determined colorimetrically after extraction using a 0.1 M ammonium acetate solution.

Table 1
Chemical properties of the soil at the Arazuri site (2011-2012) after 20 years of application of the different treatments.

	Total C (% w/w)	O.M (% w/w)	Total N (% w/w)	P_2O_5 (mg/l)	Total P (% w/w)	K_2O (mg/l)	Total K (% w/w)
Control	3.08 c	2.04 c	0.15 c	38 c	0.07 c	169 a	1.05 a
40 t ha^{-1} y^{-1}	3.41 ab	2.23 c	0.16 ab	91 b	0.10 b	149 a	1.06 a
80 t ha^{-1} y^{-1}	3.51 a	2.89 a	0.18 a	117a	0.14 a	154 a	1.08 a
40 t ha^{-1} 3y^{-1}	3.43 ab	2.55 b	0.19 a	98 b	0.11 b	135 a	1.07 a
Mineral	3.16 bc	1.96 c	0.15 c	42 c	0.07 c	169 a	1.09 a

Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{-1} 3y^{-1}. Mineral fertilizer was applied as urea. Different letters within a column indicate Duncan test results between treatments ($P<0.05$; $n=3$).

2.3.1. Soil nitrate and ammonium content

Bulked soil samples were taken from all plots to a depth of 30 cm and analyzed for ammonium and nitrate content on days 1, 3, 7, 11, 13, 24, 58, 107, 112, 130, 154, 164, 169, 176, 197, 212, post-application. The sampling frequency increased after each fertilization event. From the bulked sample, 100 g of soil was extracted with 200 ml KCl (2 M). The extracts were filtered and stored at −20 °C until analysis. The technique employed was the Gries-Illosvay colorimetric method modified by Barnes and Folkard (1951) using a Bran & Luebbe II AutoAnalyzer. An extra soil sample was used to determine gravimetric water content, expressed as the percentage of water-filled pore space (WFPS) with the method described by Menéndez et al. (2009).

2.3.2. Soil enzyme activity

Enzyme activity in soil was analyzed twice during the experiment. First, 15 days after TSS application, and 15 days after the mineral fertilizer had been applied. The soil samples were obtained from three random spots within each plot. The samples were passed through a 2 mm sieve and stored at 4° C to await enzyme activity assays in the subsequent 4 days. Protease, urease, phosphomonoesterase, β-glucosidase were assayed, as well as the hydrolysis of fluorescein diacetate.

Protease activity was tested by determining the quantity of tyrosine released after incubating 0.4 g of soil for 2 h with 1 ml of 200 mM THAM (Tris −hidroxymethyl- aminomethane) buffer (pH 8.0) and 1 ml of 2% Na-caseinate at 50 °C. The remaining substrate was precipitated with 0.92 M trichloroacetic acid and measured colorimetrically using Folin–Ciocalteu reagent at 700 nm (Geisseler and Horwath, 2009). Urease activity was measured using 1 g of soil with 1.75 ml of 100 mM Borate buffer (pH 10.0) and 0.25 ml of 820 mM urea solution at 37 °C for 1 h. Excess urea was extracted with KCl solution and estimated colorimetrically at 670 nm (Kandeler et al., 1999). Phosphomonoesterase (acid phosphatase) activity was assayed using 1 g of soil, 1.6 ml of 20 mM modified universal buffer (pH 6.5), and 0.25 ml of 50 mM *p*-nitrophenyl phosphate. After incubation the enzyme reaction was stopped and centrifuged. Following this, absorbance was measured in the supernatant at 410 nm (Taylor et al., 2002, 2013). β-glucosidase activity was determined using *p*-nitrophenyl-b-D-glucopyranoside (PNG, 0.05 M) as substrate. This assay is based on the release and detection of *p*-nitrophenol (PNP). The amount of PNP was determined using a spectrophotometer at 410 nm (Tabatabai, 1982). The hydrolysis of fluorescein diacetate [3′,6′-diacetylfluorescein (FDA)] was determined using 2 g of soil with 50 ml of 60 mM sodium phosphate buffer (pH 7.6) and 0.50 ml of 4.9 mM FDA lipase substrate solution. After mixing, the samples were incubated, the reaction was stopped and the samples were filtered. The absorbance was measured on a spectrophotometer at a wavelength of 490 nm, based on Shawy and Burns (2005).

The geometric mean of the values of all enzyme activities (Overall Enzyme Activity, OEA) was calculated according to formula [1]:

This indicator has been used previously as an overall indicator of microbial activity on soil quality (García-Ruiz et al., 2008).

Table 2
Physical and chemical properties of the treated sewage sludge during applied at the Arazuri site (2011-2012).

	pH	E.C. (μS/m)	Dry matter (% w/w)	TOC (% w/w)	Nitrogen Kjeldahl (% w/w)	C/N	P_2O_5 (mg/l)	K_2O (mg/l)	CaO (% Ca/DW)	MgO (% Mg/DW)	$N\text{-}NH_4^+$ (% N/DW)
TSS	8.2	1706	14.1	36.75	7.4	4.97	6.12	0.43	5.15	0.89	1.02

Treated sewage sludge (TSS), D.W. Dry weight.

2.3.3. Potential of nitrification

Fifteen days after mineral fertilization, the potential nitrification of soil was measured according to Norton and Stark (2011) in all treatments. 15 g of soil were incubated at 24 °C for 24 h in a solution of 0.2 M KH_2PO_4, 0.2 M K_2HPO_4 and 0.05 M NH_4SO_4. The solution was sampled up to eight times during the incubation. Afterwards, the samples were analyzed for NO_3^- and the rate of NO_3^- production was calculated through a linear regression of the solution concentration over time.

2.4. Soil greenhouse gas emission sampling

On days 1, 3, 7, 11, 13, 18, 24, 42, 48, 77, 100, 102, 105, 107, 112, 120, 133, 144, 154, 159, 167, 175, 188, 206, 219, 242, 248, and 261 after applying the treatment (during the productive period of the crop) N_2O, CO_2 and CH_4 were measured. The frequency of measurements was increased after each fertilization event. Gaseous emissions were determined using the closed chamber technique as in Chadwick et al. (2014) following the recommendations of the Global Research Alliance guidelines (de Klein and Harvey, 2012). Diurnal variations were minimized by a constant measurement in the morning (from 10 to 12 a.m.) (Baggs and Blum, 2004). Air temperature and soil temperature (10 cm depth) were measured just before starting the gaseous emissions sampling. Emission rates and cumulative emissions were calculated, taking into account the concentration increase collected just after closing the chambers and after 45 min (Menéndez et al., 2008). The linearity of the fluxes was checked before the beginning of the experiment and regularly during the experimental period. The samples were analyzed with gas chromatography (GC) (Agilent, 7890A) using an electron capture detector (ECD) for N_2O and a flame ionization detector (FID) for CH_4. To determine CO_2, the gas chromatograph was equipped with a methanizer to reduce CO_2 to CH_4. A capillary column (IA KRCIAES 6017: 240 °C, 30 m × 320 μm) was used. The column temperature ramped from 40 °C to 80 °C and the ECD temperature was 350 °C; a 5% mixture of Ar, with CH_4 was used as the carrier with N_2 as make up (15 ml min^{-1}). A headspace autosampler (Teledyne Tekmar HT3) was connected to the gas chromatograph. Standards were stored and analyzed at the same time as field samples. The cumulative gas production during the experiment was estimated by averaging the fluxes of two successive determinations, multiplying that average flux by the length of the period between the measurements, and adding that amount to the previous cumulative total.

2.4.1. Nitrous oxide production

N_2O production was determined 15 days after TSS application, and again 15 days after the first mineral application. Three soil cores were taken per experimental plot (2.5 cm diameter × 30 cm depth) and incubated in tightly-closed one-liter glass bottles. The bottles were then incubated at ambient soil temperature, in a hole dug adjacent to the experimental plots. Samples from the air headspace were taken at the beginning of the incubation and after 24 h, and were subsequently analyzed using gas chromatography, checking that the accumulation of N_2O was linear over this time.

2.4.2. Denitrification potential

Denitrification potential was measured as denitrifying enzyme activity (Phase 1), as described by Smith and Tiedje (1979), following the method described by Tiedje et al. (1989). 25 g of fresh soil and 25 ml of a solution containing glucose 1 M, KNO_3 1 mM and 1 g l^{-1} chloramphenicol were put into 125 ml flasks. The flasks were sealed with a rubber stopper and repeatedly flushed with N_2 over a 10 min period to create anaerobic conditions. The flasks were divided into two groups (with or without addition of acetylene 5%) to determine denitrification potential up to N_2O or $N_2O + N_2$ by difference (Estavillo et al., 2002).

The flasks were incubated in an orbital shaker at 20 °C and 1 ml of the headspace air was sampled for N_2O determination at 1 and 3 h incubation time, with the increase in N_2O concentration being checked for linearity. The N_2O content was analyzed using gas chromatography.

2.5. Soil bacterial community survey

In order to survey the bacterial communities, soil samples were collected for DNA extraction from the 0–30 cm layer at three random spots on each experimental plot: (i) 15 days after TSS application in 2011; and (ii) after harvest in 2012. A total of 3 independent samples were analyzed per treatment and sampling. Bulked samples were stored at 4 °C and passed through a 2 mm sieve prior to DNA extraction within 24 h of sampling. The DNA was extracted from each individual soil sample using the PowerSoilTM DNA Isolation kit (MoBio, Laboratories Inc., CA), following the manufacturer's instructions. The DNA extraction begins with the chemical lysis of microbial cells through gentle bead-beating; released DNA is purified by being filtered through a silica spin filter and recovered with elution buffer. The DNA yields and quality were checked after electrophoresis in 0.8% (w/v) agarose gel stained with GelRedTM (Biotium) under UV light.

Partial prokaryotic 16S rRNA gene sequences were obtained from the analysis of each individual sample using the coded-primer approach to multiplex pyrosequencing (Binladen et al., 2007). PCR amplification of the hypervariable V3-V5 regions of the 16S rRNA gene was performed for each individual soil DNA extraction using universal primers U341F and U926R (Baker et al., 2003) with an 8 bp bar-coded sequence (Calleja-Cervantes et al., 2015). The PCR mixtures (25 μl) contained 25 pmol of each primer, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, 1× of the corresponding Taq buffer, 5 μl of Taq Master PCR Enhancer, 1 U of Taq Master (5 Prime, USA) and 10 ng of the DNA template. The PCR program involved an initial denaturation step at 94 °C for 4 min, 25 cycles of denaturation at 94 °C for 15 s, primer annealing at 55 °C for 45 s and extension at 72 °C for 1 min, followed by a final step of heating at 72 °C for 10 min. For each sample, amplicons were generated in various replicate PCRs. Amplicons of the same treatment were pooled to reduce per-PCR variability and purified using Ultracentrifugal Filter Ultracel–100 K membranes from Amicon (Cork, Ireland) according to the manufacturer's instructions. After quantification with QuantiFluor dsDNA System (Promega), the samples were combined in equimolar amounts and subjected to pyrosequencing

with the Genome Sequencer Titanium GS-FLX system (454 Life Sciences, Branford, CT, USA) at LifeSequencing S.L. (Valencia, Spain). The sequence files have been submitted to MG-RAST (http://metagenomics.anl.gov/linkin.cgi?project=12480) and are available with the accession numbers ranging from 4617168.3 to 4617197.3.

To taxonomically assign sequence reads, the raw sequences were processed with the Mothur 454 SOP (Schloss et al., 2009), using average quality scores. Those sequences that met any of the following criteria were left out of the analysis: 1) if the bar-code contained more than two errors; 2) if the forward primer sequences contained more than three errors; 3) if the sequences had one or more Ns; 4) if the quality index, according to the .qual file generated during the pyrosequencing process, was less than 20; 5) if the sequences were identified as chimeras using chimera.uchime as the detection method and silva.bacteria as reference; and 6) if one sequence appeared once only, in a single sample (virtual OTU) using the split.abund command with cutoff = 1. Eligible sequences were clustered into operational taxonomic units (OTUs), based on a distance of 3%, by complete linkage clustering. The phyla were assigned using an 80% confidence threshold (Wang et al., 2007). Sequences that could not be classified to a phylum at this level of confidence were excluded from subsequent phylum composition analyses. The resulting clusters were utilized to calculate the Shannon, Shannon Evenness and Inverse Simpson indices, Chao 1 estimator and rarefaction curves at the level of 3% dissimilarity being approximate to species level.

2.6. Plant and soil $\delta^{15}N$ isotope analysis

As a complement for the N analysis, at tillering and stem elongation, in each experimental plot, three different spots with a surface area of 0.5 m^2 were chosen for stem sampling. At harvest 0.5 m^2 per plot were sampled again. The sampled material was bulked to obtain a single composite sample for each experimental plot. The grain was detached from the straw to determine grain yield, and this was adjusted to a 12% moisture content. After harvest, the stems, straws and grain were dried and ground to await further analysis. The $\delta^{15}N$ isotope was determined for ground samples of stem, straw, grain and soil (1 g dry weight) that were sieved through a 0.2 mm mesh. The samples were analyzed via a mass spectrometer (Delta Plus, Thermoquest, Finnigan) coupled to an NC 2500 elemental analyzer (CE Instruments, Milan).

2.7. Statistical analysis

This study involves the mean values of at least four independent samples per treatment. Each independent sample comprised a bulked sampled containing a variable number of sub-samples depending on the analysis, as stated above. In the case of the statistical TSS analyses, one-way ANOVA tests were used. To compare differences between treatments, the Duncan test was carried out with a significance level $P > 0.05$ or $P > 0.01$ using the SPSS software, version 21.0 (IBM Corp., 2012). Correlations were analyzed among variables where appropriate, and the Pearson value was also calculated.

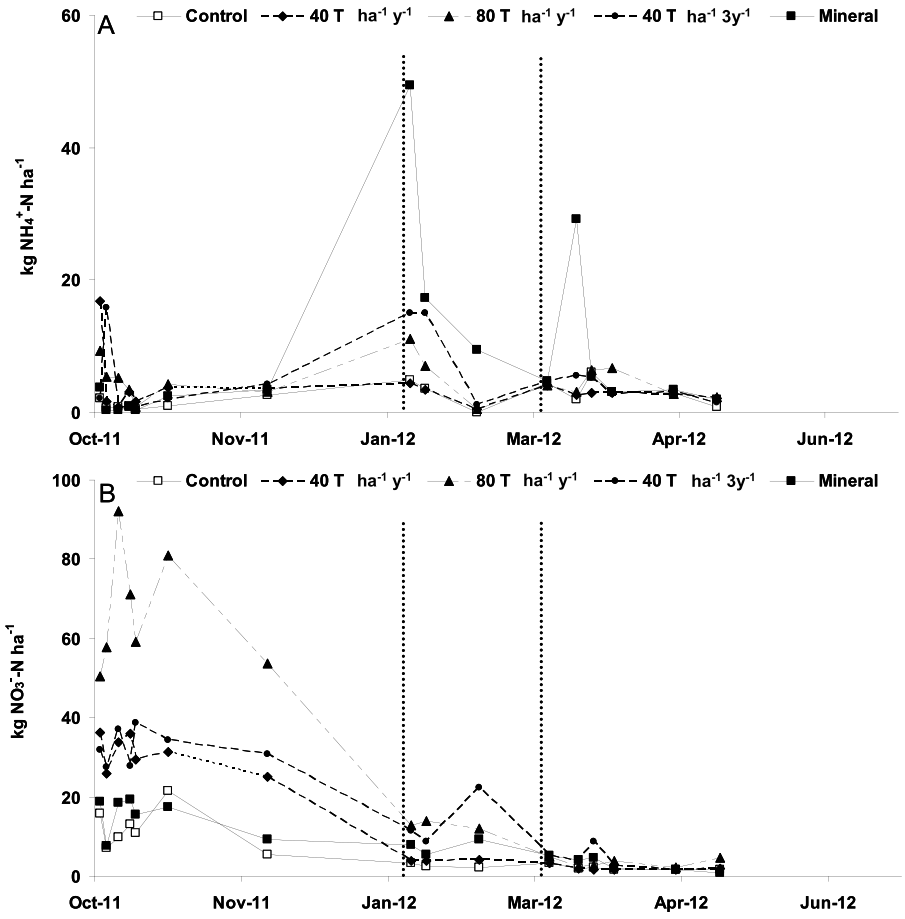

Fig. 2. Soil ammonium content (A) and soil nitrate content (B) in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} $3y^{-1}$, control and mineral (urea). Vertical lines show fertilizer application.

A comparison of bacterial communities was made using the cluster file format conversion and the corresponding matrix was obtained in table form. The distance matrix was completed with the Bray-Curtis index in the Ginkgo software (De Cáceres et al., 2007). This software was used to produce an illustration of the clustering using the unweighted pair group method with arithmetic mean (UPGMA), as well as PCoA and CCA. Finally, a hierarchical clustering was obtained that took into account the abundance of each OTU. Moreover, the five different libraries generated from each soil treatment were pair-wise compared with the STAMP software (Parks et al., 2014) using the parameters recommended by the developers (the statistical test was the two-sided type Fisher's exact test, with a confidence interval of 95% evaluated using the differential Newcombe-Wilson proportions and the FDR multiple test correction of Storey) with samples 40 t ha^{-1} 3yr^{-1} number 3 and Control_3 being eliminated from the analysis as they appeared delocalized in the agglomerative hierarchical clustering.

3. Results

As TSS has a unique composition and its application to soil has different effects; the evaluation of the chemical, environmental and biological performance of a continued application of TSS is important. The results of this study involve the performance aspects corresponding only to the 20th year of treatment application.

3.1. Effects on soil chemical properties

Soil chemical characteristics for each TSS treatment after 20 years of application are presented in Table 1; the nutrient content in soil was increased due to TSS application. Total C and OM rose 14% and 41%, respectively, for the 80 t ha^{-1} yr^{-1} treatment. Nevertheless, the observed increase in total C was not significant in comparison to the other two treatments with TSS. The TSS application once per every 3-yr plus supplementary chemical fertilizer resulted in a significant increase in total C relative to the control, following that of the 80 t ha^{-1} dose. TSS application resulted in higher levels of total N and P_2O_5 compared with control. The most significant changes were recorded in the 80 t ha^{-1} yr^{-1} treatment where total N was 20% more and the amount of P was double that of the control. The extractable K (K_2O) content remained unaffected by the different treatments.

3.1.1. Status of soil ammonium and nitrate N

The total mineral N content (kg-N ha^{-1}) in the soil, and the percentage of different N forms, such as NH_4^+ and NO_3^-, varied between treatments (Fig. 2). Soil NO_3^--N level was highest (ranging from 50.3 to 92.1 kg NO_3^--N ha^{-1}) during the initial 2–3 week period after application of the highest TSS rate (80 t ha^{-1}/yr). There were no significant differences in the soil NO_3^--N levels between the two treatments where TSS was applied either at 40 t ha^{-1}/yr or 40 t ha^{-1}/3-yr rates. In these two treatments, soil NO_3^--N level ranged from 25.9–38.6 kg NO_3^--N ha^{-1} during the initial 2–3 week period after TSS application. Soil NO_3^--N levels were not significantly different between the control and mineral N treatment with values ranging from 7.6 to 21.4 kg NO_3^--N ha^{-1} during the initial 2–3 week period. At the end of the experiment the nitrate content in all the treatments had decreased significantly compared to the initial values. The control and 40 t ha^{-1} treatments, both had similar quantities, ranging from 1.56 to 1.79 kg NO_3^--N ha^{-1}. The mineral treatment showed the lowest NO_3^--N level with 0.85 kg NO_3^--N ha^{-1}; while the 80 t TSS ha^{-1} treatment indicated the highest with 4.54 kg NO_3^--N ha^{-1}. For control and mineral treatments, the highest nitrate content was found around November and decreased thereafter. In contrast, the nitrate content in the TSS treatments remained over 20 kg NO_3^--N ha^{-1} for a period of almost two months, with the 80 t ha^{-1} treatment having the highest observed level, 92.1 kg NO_3^--N ha^{-1}, double that seen in the other two TSS treatments.

The initial soil ammonium content compared to the final values varied less. At the beginning, TSS and mineral application led to increase ammonium in the soil, but this effect rapidly diminished. At the beginning the greatest content was seen in the 40 t and 80 t treatments, with 16.74 kg NH_4^+-N ha^{-1} and 9.25 kg NH_4^+-N ha^{-1}, respectively; at the same time, ammonium in the other treatments was significantly lower, ranging from 2.07 kg NH_4^+-N ha^{-1} (control) to 3.76 kg NH_4^+-N ha^{-1} (urea). An important variation in nitrate and ammonium quantities was observed immediately after the application of the mineral treatment.

3.1.2. Soil enzyme activity assay

Enzyme activity significantly increased in soils that received TSS (Table 3). Urease and protease activity was higher in February than October. β-glucosidase and Phosphatase decreased their activity in February. This indicates seasonal variation, although the pattern of increase remained consistent between the two dates, i.e., it was observed that 80 t ha^{-1} significantly increased enzymes by the following percentages: urease 30% (Oct) and 42% (Feb), protease only in Feb (96%), β-glucosidase 43% (Oct) and 8% (Feb), phosphatase around 28% on both dates, and FDA hydrolysis 96% compared to the control. The 80 t ha^{-1} treatment caused the greatest increase in overall enzyme activity, followed by 40 t ha^{-1} 3y^{-1} and then 40 t ha^{-1}.

3.2. Effects on soil GHG emissions: N_2O, CO_2 and CH_4

Daily N_2O emissions from the control ranged from 0.2 to 8.6 g N_2O-N ha^{-1} d^{-1}, while the 80 t ha^{-1} treatment increased the fluxes up to 39.2 g N_2O-N ha^{-1} d^{-1} (Fig. 3). The emissions for the mineral treatment were close to those of the control during the

Table 3
Enzyme activities in soil 15 days after TSS application (OCT 2011) and 15 days after mineral application (FEB 2012) at the Arazuri site.

Treatment/Enzyme	Urease (mg N-NH^{4+} kg dry soil^{-1} h^{-1})		Protease (mg Tyr kg dry soil^{-1} h^{-1})		β-glucosidase (mg ρ-nitrophenol kg dry soil^{-1} h^{-1})		Phosphatase (mg ρ-nitrophenol kg dry soil^{-1} h^{-1})		FDA hydrolysis (mg fluorescein salt kg dry soil^{-1} h^{-1})	Overall Enzyme Activity	
	2011	2012	2011	2012	2011	2012	2011	2012	2012	2011	2012
Control	23.3 c	25.2 b	18.3 a	34.1 c	301.2 c	136.0 bc	158.4 c	136.3 b	46.8 bc	91.4 b	87.1 bc
40 t ha^{-1} y^{-1}	27.2 b	23.6 b	21.6 a	42.3 bc	392.3 ab	113.7 bc	190.4 a	162.8 b	83.3 bc	112.1 a	91.3 ab
80 t ha^{-1} y^{-1}	31.2 a	35.9 a	20.6 a	67.1 a	431.3 a	147.8 a	199.7 a	177.4 a	90.9 a	120.6 a	121.0 a
40 t ha^{-1} 3y^{-1}	27.8 b	32.8 ab	21.6 a	52.0 bc	414.4 a	137.3 bc	183.9 ab	148.7 b	89.6 ab	113.9 a	102.0 a
Mineral	25.9 bc	30.8 ab	16.2 a	26.5 c	339.8 bc	100.4 c	166.2 bc	135.6 b	30.4 b	95.8 b	79.1 c

Control, TSS doses: 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} 3y^{-1}. Mineral fertilizar was applied as urea. Different letters within a column indicate Duncan test results between treatments ($P < 0.05$; $n = 3$).

entire period, with a maximum rate of 15.5 g N_2O-N ha^{-1} d^{-1}. Treatment application increased cumulative losses (Table 4) of N_2O compared to the control. The mineral fertilizer doubled the losses from the control, while the different TSS treatments caused them to increase by 3 to 6 times, with 80 t ha^{-1} showing the highest cumulative loss (3.5 kg N_2O-N ha^{-1}). The 40 t ha^{-1} and 40 t ha^{-1} 3 yr^{-1} doses resulted in slightly lower cumulative losses. The calculated emissions factors for all the treatments ranged from 0.25% to 0.46%.

Daily CO_2 fluxes from the control treatment ranged between 1.7 and 62.7 kg CO_2-C ha^{-1} d^{-1}, in the same range as the fluxes from urea. The maximum rate of 128.2 kg CO_2-C ha^{-1} d^{-1} was observed in the mineral and 40 t ha^{-1} yr^{-1} treatments around March. TSS application increased daily fluxes compared to the control, and a concomitant effect was observed as TSS application increased cumulative losses (Table 4).

Daily CH_4 fluxes did not differ between treatments. Most of the time the soil acted as a CH_4 sink, with fluxes ranging between −9.3 and 22.5 g CH_4-C ha^{-1} d^{-1} (Fig. 3). Nevertheless, when the soil acted as a source for CH_4 (in various periods), fluxes increased in all treatments. As consequence of this, at the end of the assayed period only the control resulted in a net CH_4 uptake (Table 4). The other treatments presented a net CH_4 increase with no statistically significant differences between them. In terms of Global Warming Potential (GWP) expressed as CO_2 equivalents, the treatments with TSS presented a higher GWP compared to the mineral and control treatments.

3.2.1. Production and emission of N_2O after treatment application

Table 5 shows the production and emission of N_2O fifteen days after organic amendment application at sowing; and then 15 days after the first mineral application at tillering. In October, production rates ranged between 2.01 and 3.59 g N_2O-N ha^{-1} d^{-1} while emissions ranged between 2.07 and 3.16 g N_2O-N ha^{-1} d^{-1}. At that time only the 80 t ha^{-1} yr^{-1} treatment showed statistically different results from the control treatment. In January, production rates increased, ranging between 5.22 and 29.96 g N_2O-N ha^{-1} d^{-1}, while the emissions to the atmosphere ranged between 0.39 and 10.35 g N_2O-N ha^{-1} d^{-1}. At that time, only the 40 t ha^{-1} 3yr^{-1} treatment differed statistically from the other applications in terms of N_2O production and N_2O emissions.

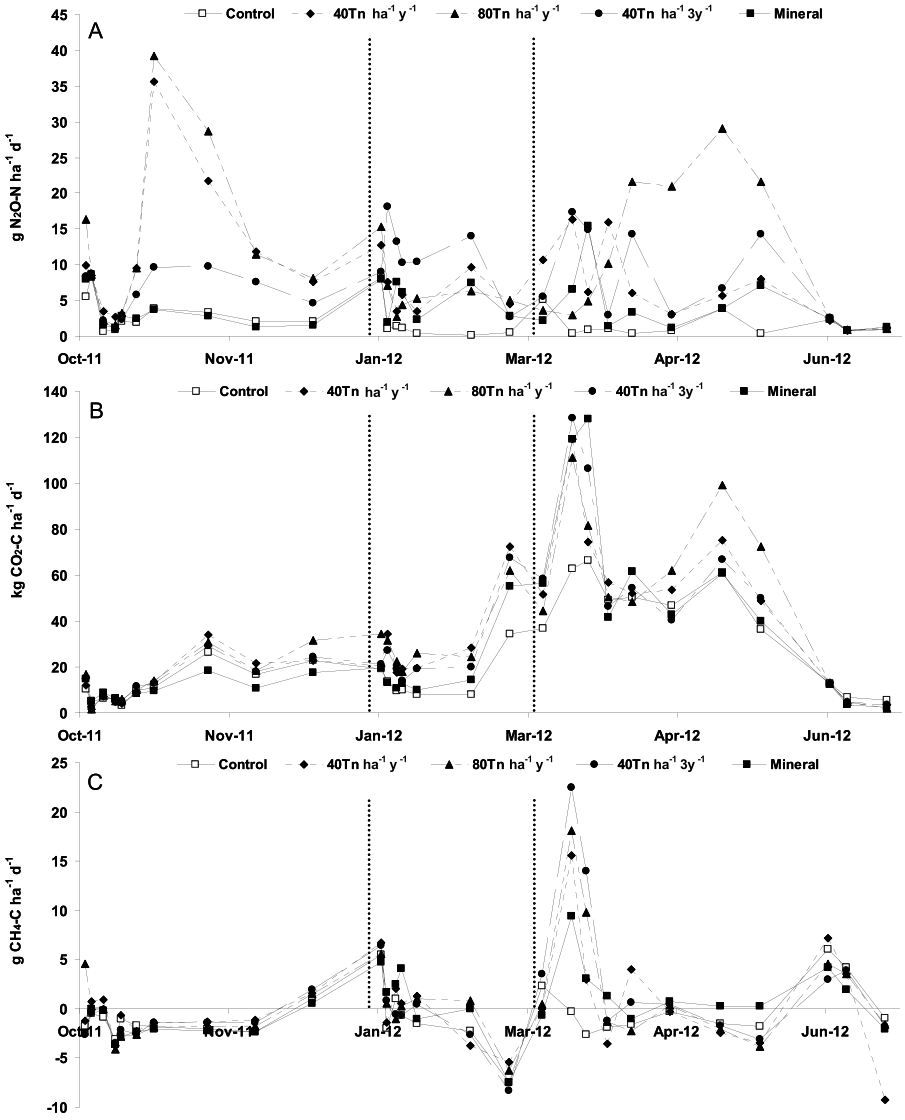

Fig. 3. N_2O (A), CO_2 (B), CH_4 (C) emission rates in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} 3y^{-1}, control and mineral (urea). Vertical lines show fertilizer application.

Table 4
Cumulative nitrous oxide, carbon dioxide and methane emissions, N_2O emission factors (EF) and global warming potential expressed as CO_2 equivalents (GWP) during 261 days at the Arazuri site (2011-2012).

Treatment	kg N_2O-N ha^{-1}	EF(%)	kg CO_2-C ha^{-1}	g CH_4-C ha^{-1}	T CO_2eq ha^{-1}
Control	0.58 d	–	7172 c	−147 b	26.8 c
40 t ha^{-1} y^{-1}	2.41 b	0.46	9629 ab	50 a	39.7 a
80 t ha^{-1} y^{-1}	3.49 a	0.36	10512 a	96 a	41.8 a
40 t ha^{-1} 3y^{-1}	1.92 b	0.25	9315 ab	162 a	36.0 ab
Mineral	0.91 c	0.27	8212 bc	16 ab	31.0 bc

Mineral fertilizer was applied as urea. Different letters within a column indicate Duncan test results between treatments ($P < 0.1$; $n = 4$).

Table 5
Nitrous oxide production in soil and emission to atmosphere (g N_2O-N ha^{-1} d^{-1}) 15 days after TSS application (OCT 2011) and 15 days after urea application (FEB 2012).

Treatment	OCT 2011		FEB 2012	
	Production	Emission	Production	Emission
Control	2.01 b	2.07 b	5.22 b	0.39 b
40 t ha^{-1} y^{-1}	2.94 ab	2.87 ab	5.75 b	3.49 b
80 t ha^{-1} y^{-1}	3.59 a	3.16 a	10.19 b	5.31 ab
40 t ha^{-1} 3y^{-1}	2.94 ab	2.31 ab	29.96 a	10.35 a
Mineral	2.98 ab	2.81 ab	8.35 b	2.30 b

Different letters within a column indicate Duncan test results between treatments ($P < 0.1$; $n = 4$). OCT 2011 corresponds to 15 days after TSS application. FEB 2012 corresponds to 15 days after mineral (urea) application.

3.2.2. Nitrification and denitrification potential

The nitrification potential is shown in Table 6. The control and mineral treatment showed values of 228 μg N kg^{-1} dry soil d^{-1}. The addition of 40 t ha^{-1}, both annually and every 3 years, increased the nitrification potential to 260 μg N kg^{-1} dry soil d^{-1}. The extreme application of 80 t ha^{-1} yr^{-1} significantly amplified the nitrification potential up to 350 μg N kg^{-1} dry soil d^{-1}. There were no significant differences between treatments for denitrification potential up to N_2O. Values ranged from 245 μg N kg^{-1} dry soil d^{-1} to 319 μg N kg^{-1} dry soil d^{-1}. The potential to reduce N_2O to N_2 was significantly higher in all treatments, especially in treatments with TSS, where the rates ranged between 1901 μg N kg^{-1} dry soil d^{-1} and 2576 μg N kg^{-1} dry soil d^{-1}. Even so, the maximum dose (80 t ha^{-1} yr^{-1}) was the only one that was statistically higher compared to the control and urea treatments.

3.3. Diversity and taxonomic composition of bacterial communities

A total of 282,442 reads were obtained for the 5 treatments and their 3 replicates on both sampling dates, with an average of 9415 reads per replicate. Prokaryotic diversity coverage was higher than 87% for all the replicates, except in 2011 40 t ha^{-1} 3yr^{-1} where the coverage was only 81% (2111 reads) (Supplementary Table S1). On the basis of 3% dissimilarity, the number of OTUs varied from 807 to 1762 with a total of 9744 different OTUs from all the replicates. The Chao1 index, calculated with the MOTHUR software, indicated a richness variation from 1220 up to 2845 OTUs per replicate. The Shannon diversity index yielded values ranging from 5.94 to 6.56, i.e., the samples were highly diverse. The compositional difference between the bacterial communities was analyzed through hierarchical agglomerative clustering, which considers the presence or absence of OTUs (Jaccard Analysis); this showed that the samples are grouped into two main, statistically different clusters (Fig. 4). One of the clusters contained all the replicates from the 80 t ha^{-1} yr^{-1} treatment (from both Oct 2011 and July 2012). The second cluster involved the mineral, control and the 40 t ha-1 samples. This means that the main differences are seen in the 80 t ha^{-1} yr^{-1} treatment. However, it should be noted that in the 80 t cluster, both replicates from the control and 40 t ha^{-1} 3yr^{-1} appear. Similar results were obtained when the hierarchical agglomerative clustering was performed, using MOTHUR software (data not shown), taking into account each OTU abundance (Thetayc Analysis; see also Yue and Clayton (2012)). The same results were observed when PCoA was performed with the STAMP software (Parks and Beiko, 2010) and Ginkgo software (De Cáceres et al., 2007) (shown in Supplementary Fig. S1), using total sequences, and normalized number (to the lowest amount found) of each replicate.

The phyla distribution of the microbial communities is shown in Fig. 5 Phyla, as well as Supplementary Table S2. 80% of the sequences found corresponded to 6 main phyla: (from greatest to least) Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi and Verrucomicrobia, with unclassified bacteria making up an average of 10.94% of the sequences. The Protebacteria phylum percentage increased in the 80 t treatment, from an observed abundance of 22.58% in the control to 27.33% in October, and an abundance of 28.84% at harvest from 23.14%. In contrast, the opposite trend was noted for the phylum Acidobacteria. While the control registered the abundance of Acidobacteria as 19.95% in October, it decreased to 13.67% in the 80 t treatment and was only 13.93% at harvest compared with 20.81% in the control. No other significant differences were observed between treatments. However, an increase in the phylum Bacteroidetes was common to all treatments on both sampling dates, but these differences were not statistically significant. The mineral treatment resulted in an increase in the phylum Verrucomicrobia compared to the other treatments and the control, but this was not statistically significant.

Table 6
Nitrification potential and denitrification potential up to N_2O or up to $N_2O + N_2$ at the Arazuri site (2011-2012).

Treatment	Nitrification Potential (μg N kg^{-1} dry soil d^{-1})	Denitrification Potential (N_2O) (μg N kg^{-1} dry soil d^{-1})	Denitrification Potential ($N_2O + N_2$) (μg N kg^{-1} dry soil d^{-1})
Control	228 c	319 a	1901 b
40 t ha^{-1} y^{-1}	260 b	245 a	2271 ab
80 t ha^{-1} y^{-1}	350 a	249 a	2576 a
40 t ha^{-1} 3y^{-1}	263 b	307 a	2263 ab
Mineral	227 c	245 a	1956 b

Mineral fertilizer was applied as urea. Different letters within a column indicate Duncan test results between treatments ($P < 0.05$; $n = 4$).

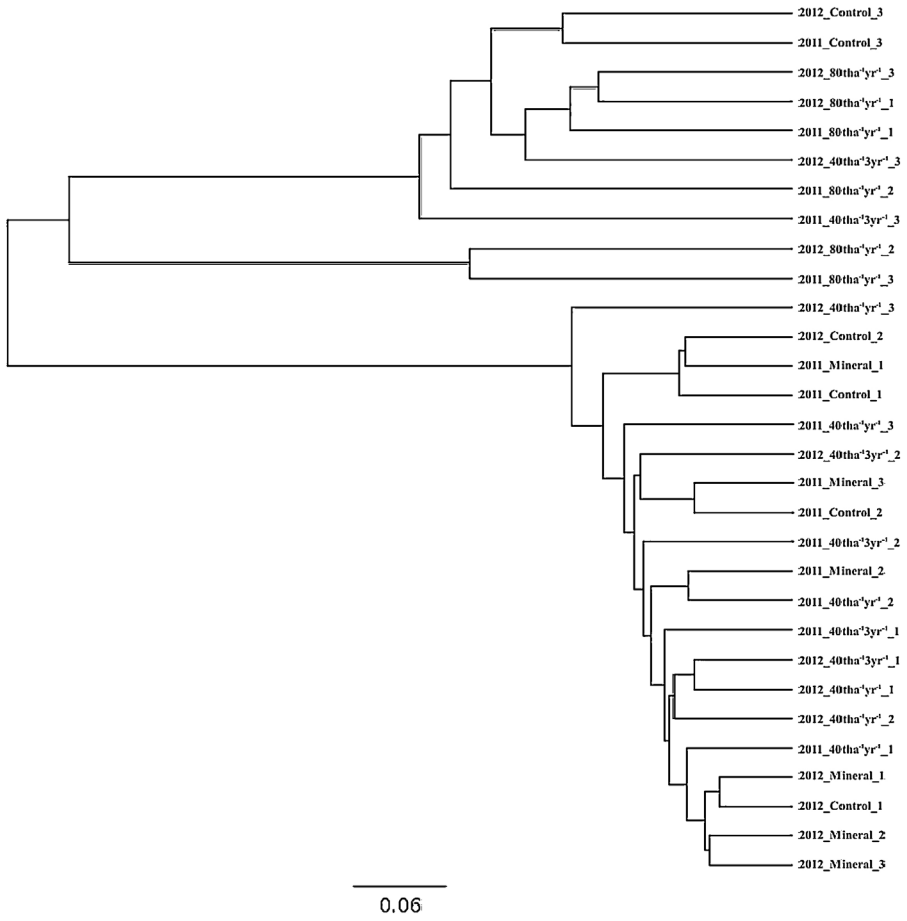

Fig. 4. Cladogram of the different soil bacterial communities based on Jaccard distance (3% dissimilarity). Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} 3y^{-1}, control and mineral (urea). OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1st replicate), R2 (2nd replicate), R3 (3rd replicate).

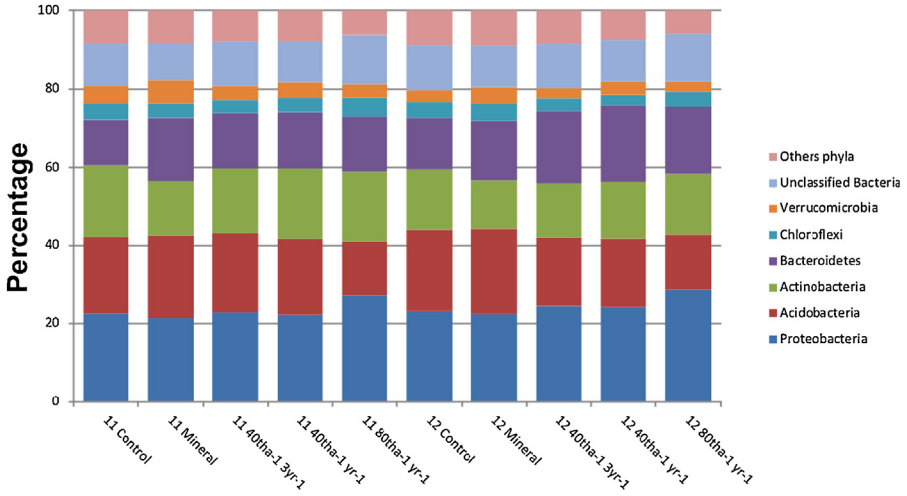

Fig. 5. Percentage distribution of the detected phyla between the different treatments in soil. Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{-1} 3y^{-1}, control and mineral (urea). 11 corresponds to 15 days after TSS application (October). 12 corresponds to harvest (July).

At a lower taxonomic level, only 25.91% of the sequences could be allocated to a genus, i.e., 274 genera were identified (Supplementary Table S3). 74 of the identified genera showed statistically significant differences ($p < 0.05$). The most notable was that the 80 t ha^{-1} yr^{-1} treatment suppressed up to 51 genera including: *Streptomyces*, *Arthrobacter* and *Microbacterium* (phylum Actinobacteria), *Microvirga* and *Bradyrhizobium* (phylum Proteobacteria) and *Bacillus* (phylum Firmicutes) from the bacterial community (Supplementary Table S3). In the other samples, the mentioned genera ranged from 0.14% to 0.59%. The presence of other genera including *Nocardioides* and *Solirubrobacter* (phylum Actinobacteria); *Rhizobium*, *Haliangium* and *Devosia* (phylum

Proteobacteria); and *Terrimonas* and *Niastella* (phylum Bacteroidetes) was greatly reduced in soils treated with 80 t ha^{-1} yr^{-1}. In this case, Acidobacteria Gp3, Gp4, Gp5, Gp6, Gp7 and Gp17 were significantly decreased in the soil. It should be pointed out that the taxonomy of Acidobacteria is only established to class level, thus the number of reads are the same for class and genus.

The observed differences in the phylum Proteobacteria in the 80 t treatment could be due to an increase in genera such as *Arenimonas*, *Dongia*, *Hyphomicrobium*, *Naxibacter*, *Nitrospira* and *Steroidobacter*; and *Serpens* and *Stenotrophomonas* only at the first sampling date (Supplementary Table S3). In the 80 t treatment, a significant increase ($p < 0.05$) was observed for *Flavobacterium*, phylum Bacteroidetes (1.26% on average in all treatments to 2.33%) and *Gaiella*, phylum Actinobacteria (from 3.61% to 4.44%). The canonical correspondence analysis (Fig. 6) showed that differences between the 80 t treatment and the other treatments are mainly due to an increase in the genera *Flavobacterium*, *Gaiella*, *Steroidobacter* and *Microlunatus*, and a depletion of the genera *Streptomyces*, *Arthrobacter*, *Terrimonas*, *Sphingomonas* and *Nocardioides* (Fig. 6).

3.4. Plant and soil effects on $\delta^{15}N$ enrichment (TSS, soil, stem, straw and grain)

Total $\delta^{15}N$ enrichment was significantly influenced by the different treatment applications (Fig. 7). TSS and mineral application affected total $\delta^{15}N$ in soil and grain enrichment. $\delta^{15}N$ enrichment in soil was similar for all the treatments. However, it was lost during the evolution of crop, only persisting in various crop stages of the 80 t ha^{-1} and 40 t ha^{-1} treatments. The 40 t ha^{-1} 3y^{-1} treatment affected straw and grain enrichment differently.

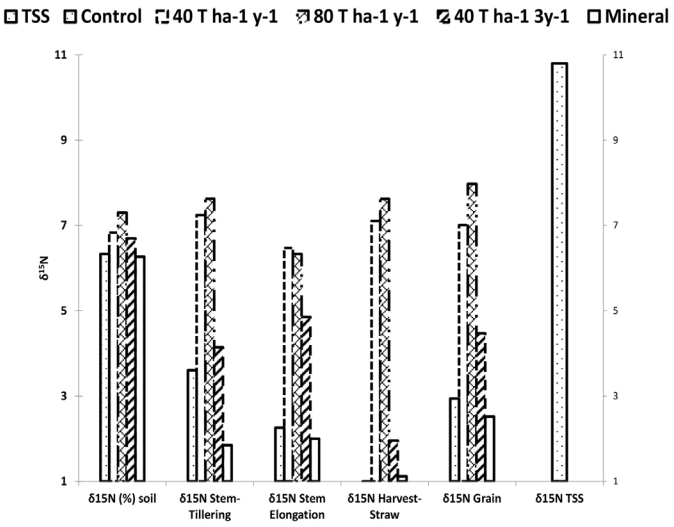

Fig. 7. $\delta^{15}N$ enrichment in soil in the studied treatments: treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{1} 3y^{-1}, control and mineral (urea); stem (at tillering and stem elongation) and straw at harvest, grain and TSS.

The exaggerated applied doses of TSS in this study are not common practice. For instance, in the case of oats, the excessive amount of nitrogen applied to soil leads to the lodging of the crop, causing an irreversible decrease in the yield. It is acknowledged

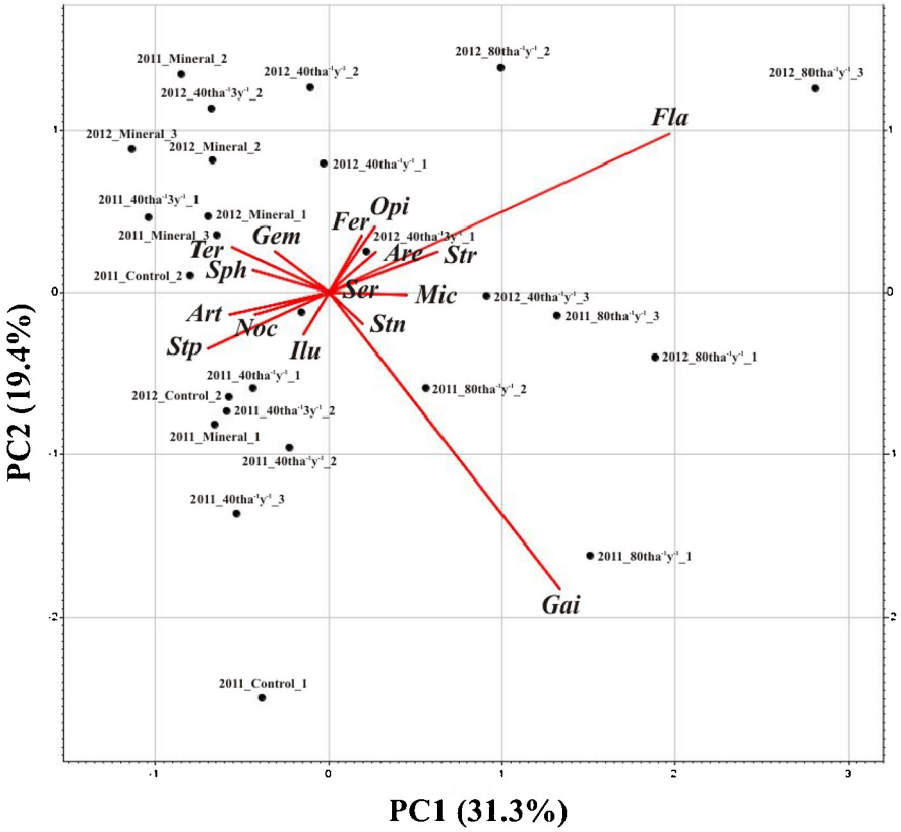

Fig. 6. Plot representing the eigenvectors for the (PCA) of the significant genera. Treated sewage sludge (TSS), 40 t ha^{-1} y^{-1}, 80 t ha^{-1} y^{-1}, 40 t ha^{-1} 3y^{-1}, control and mineral (urea). OCT 2011 corresponds to 15 days after TSS application. JUL 2012 corresponds to harvest. R1 (1 st replicate), R2 (2nd replicate), R3 (3rd replicate). Fla: Flavobacterium; Str: Steroidobacter; Opi: Opitutus; Fer: Ferruginibacter; Are: Arenimonas; Mic: Microlunatus; Ser: Serpens; Stn: Stenotrophomonas; Gai: Gaiella, Ilu: Ilumatobacter; Gem: Gemmatimonas; Ter: Terrimonas; Sph: Sphingomonas; Art: Arthrobacter; Noc: Nocardiodes; Stp: Streptomyces.

that the reported grain yield corresponds to the harvested yield. The unfertilized treatment showed a grain production of 3288 kg ha^{-1} (Table 7). TSS application significantly improved the grain yield compared to the control in a range between 4600 and 4800 kg ha^{-1} when 40 t ha^{-1} was applied. Nevertheless, the dose of 80 t ha^{-1} y^{-1} decreased the yield to 3701 kg ha^{-1} which was no different from the control. The highest yield production was observed for the urea treatment, with a value of 6057 kg ha^{-1}. This latter performance is within the average yield of 5451 kg ha^{-1} expected under similar climatic conditions in Spain (Genvce, 2010).

4. Discussion

The studied doses TSS were designed to evaluate the influence of an extremely high application on various soil parameters. This exaggerated intensity, by increasing the quantity of applied material, results in an important reduction in residues in a one-off effort.

An appropriate agricultural application of TSS as a soil amendment could prevent the unsafe disposal of this material as it induces desirable agronomic effects in soil (Singh and Agrawal, 2008). In this study, nutrient availability in soil was increased due to TSS application and the increase is closely related to the dose and frequency of application. Similar nutrient improvements have already been reported by Parat et al. (2005) in an evaluation of a similar dose. The changes in the 40 t ha^{-1} 3 yr^{-1} treatment represent a novel result. It is hypothesized that the supplemented mineral fertilization, in years that TSS was not applied, triggered increased metabolism in soil, coupled to an activation of root metabolism that induced microbial activity (Garcia-Gil et al., 2004).

The metabolic activity of soils, measured by active enzymes in the soil is known to be a potential indicator of the effects of soil management on soil fertility. Some studies have demonstrated that TSS consistently increases microbial activity (Antolin et al., 2005; Fernandez et al., 2009). In the same way we also observed increased enzyme activity, especially when a 80 t per hectare dose was applied. However, some other studies argue that the presence of heavy metals in TSS reduces and inhibits enzyme expression. Paz-Ferreiro et al. (2012) reported that an increase in the amount of TSS, decreased OEA. Moreover, other studies conclude that fertilization management does not affect enzymes Marschner et al. (2003). None of these assertions have been confirmed by this study. It is important to note that regardless of the annual amount of TSS applied, the enzyme activities did not particularly increase between the plots that received TSS. The difference is based on frequency and mineral application. Chu et al. (2007) reported that balanced fertilization increases the metabolic activity of soils.

In the case of urease activity, the increase must be due to the presence of high amounts of enzymatic substrates in the sewage sludge (Garcia et al., 1993). Urease requires the presence of urea and Ni, which is usually present in sewage sludge (Antonious, 2009), and is therefore not a limiting factor. As expected, the higher inputs of phosphate with the organic amendments induced phosphatase activity, possibly due to the action of the roots as reported by Johansson et al. (1999). However, further studies have to be conducted to confirm the relationship between plant root excretion of this enzyme and microbial action. Protease and FDA hydrolysis establish a noticeable dominance over the other assayed enzymes in the TSS treatments. This may have been because C and N were increased by TSS application, given the provision of organic matter. Also, a repeated exogenous application of soil enzymes could enrich the soil matrix with enzymes.

Even so, the contradictory results reported in the literature on TSS application may have other causes, such as treatment processes, incorporation methods, and crop interaction with soil and differentiated climatic conditions.

4.1. Gaseous emissions

Bøckman and Olfs (1998) stated that the addition of N in a mineral form or from an organic source such as sewage sludge (in which ammonium is released by mineralization) to agricultural soils is a major driver of N_2O emissions. N_2O emissions were increased by treatment application in our study. As expected the highest application dose of 80 t ha^{-1} yr^{-1} induced the highest losses. In spite of the application rate, maximum losses did not exceed the maximum fluxes of 48 g N_2O-N ha^{-1} d^{-1} described by Huérfano et al. (2015) from a winter crop fertilized with mineral fertilizer under humid Mediterranean conditions similar to those of the present study. In a temperate humid marine climate, N_2O fluxes from an oat crop fertilized with mineral N did not exceed 20 g N_2O-N ha^{-1} d^{-1} (Beheydt et al., 2008). The increase induced by the application of sewage sludge was much lower than that described by Scott et al. (2000) after sewage sludge application in grassland. The significant input of exogenous C to the soil should have favored the denitrification process (Bowman and Focht, 1974; Weier et al., 1993). Nevertheless, the denitrification potential to N_2O was not increased by the addition of sewage sludge, although it did increase the denitrification potential up to N_2 (Table 6). This would explain why the observed losses were lower than those described in the literature, as previously discussed. In fact, N_2O production was lower than N_2O emissions (Table 5) suggesting that N_2O was reduced to N_2, which diffused to the atmosphere. However, the N_2O production rates in our experiment are of the same magnitude as those described by Zaman et al. (2004).

Carbon dioxide (CO_2) emissions were regulated by soil water content which is known to modulate O_2 availability in soil. It has been widely described that CO_2 emissions increase when the WFPS is between 55%-60% and decrease when the WFPS is higher (Davidson et al., 1998; Huérfano et al., 2015). Nevertheless, we observed a positive correlation between CO_2 fluxes and soil moisture ($r^2 = 0.841$; $p < 0.000$) when the WFPS was lower than 70%, and a negative correlation ($r^2 = -0.809$; $p < 0.005$) when the

Table 7
Yield production of oats at the Arazuri site (2011–2012).

Treatment	Yield (kg ha^{-1})	GHGI (kg CO_2eq ha^{-1}/kg yield ha^{-1})	Grain N yield scaled Emissions (g N_2O-N ha^{-1}/kg N ha^{-1})
Control	3288 c	7.1 b	8.6 c
40 t ha^{-1} y^{-1}	4856 b	6.8 b	24.3 b
80 t ha^{-1} y^{-1}	3701 c	9.6 a	46.1 a
40 t ha^{-1} $3y^{-1}$	4604 b	6.7 b	20.3 b
Mineral	6057 a	4.4 c	7.3 c

Mineral fertilizer was applied as urea. Different letters within a column indicate Duncan test results between treatments ($P < 0.05$; $n = 4$).

WFPS was higher than 70%. Although it could be expected that global warming has enhanced CO_2 emissions (Kirschbaum, 2000); Xu and Qi (2001) have described a negative correlation between soil temperature and soil respiration. In our study we observed a negative correlation ($r^2 = -0.501$; $p < 0.20$) when the soil moisture was under 70%.

The incorporation of TSS to soil significantly increased the cumulative losses of CO_2. The unfertilized control showed similar values to those described by Huérfano et al. (2015) from an unfertilized wheat crop. However, these values are higher than the 26.1 kg CO_2-C ha^{-1} d^{-1} described by Bortolotto et al. (2015) from an oat crop. In the UK, Scott et al. (2000) observed daily CO_2 fluxes of 84 kg CO_2-C ha^{-1} d^{-1} after sewage sludge application. However, under Mediterranean conditions Quemada and Menacho (2001) describe losses of up to 384 kg CO_2-C ha^{-1} d^{-1} one year after the application of 80 t ha^{-1} yr^{-1} of TSS. The continuous application of TSS over 20 years resulted in daily fluxes that did not exceed 128 kg CO_2-C ha^{-1} d^{-1} (Fig. 3). This reduction in CO_2 flux could be explained by the fact that a single addition of C induced a greater priming effect than repeated and continuous inputs, and consequently a higher CO_2 release (Qiao et al., 2014).

Generally, soils act as CH_4 sinks, with the exception of soils where there is a very high water table, and which are sources of CH_4 emission. In the present study, both mineral and organic fertilization increased CH_4 emissions, resulting in a net source of CH_4. The high NH_4^+ content in the soil as a result of the fertilization could inhibit CH_4 oxidation, probably due to competitive inhibition of methane monooxyegnase by ammonium (Conrad, 1996). The anaerobic decomposition of soil organic carbon, input via the TSS, provided the C substrates for methanogens, controlling methanogenic activity (Segers, 1998). Evidence from this study suggests that maximum CH_4 fluxes took place in spring corresponding with the maximum CO_2 fluxes (Fig. 3), when soil water content and temperature favored organic matter decomposition, providing C substrates for methanogenesis. Maximum daily fluxes of CH_4 were two times higher than the fluxes reported by Ambus et al. (2001) after sewage sludge application. Nonetheless, the cumulative CH_4 losses after 11 months (76 g CH_4-C ha^{-1}) presented by these authors were of the same magnitude as the cumulative losses in this study (Table 4).

TSS application clearly increases $\delta^{15}N$ compared to mineral fertilization. Hogberg (Hogberg and Johannisson, 1993; Hogberg et al., 2014) argued that N processes, like those involved in the decomposition of organic matter, discriminate against ^{14}N, thus enriching ^{15}N. Also, ammonia volatilization and other processes related to the treatment of organic waste are closely related to this enrichment. The mechanisms and processes that drive the links with further plant/grain enrichment are not yet described as physiological routes, but from the reported results there seems to be a clear enrichment during the treatment of SS, and this enrichment is definitely transferred into the soil and the plant/grain.

The addition of mineral fertilizer significantly increased grain yield (Table 7). It is well known that N fertilization is the best way to maximize the production of crops. Sewage sludge is a significant source of organic N that has to be mineralized to be used by crops (Antolin et al., 2005). However, the high organic N inputs with the assayed organic matter resulted in excess mineral N after mineralization, causing plant lodging and decreased grain yield. Fernández et al. (2009) also observed a reduction in grain yield after sewage sludge application as the consequence of an excessive dose. For this reason, the reduction in grain yield and the high N_2O emissions induced by sewage sludge resulted in a significant increase in the N yield-scaled emissions (Table 7). However, the mineral fertilizer did not affect this aspect in the control treatment. In accordance with this, several authors (Venterea et al., 2011; Huérfano et al., 2015) have described the same or even lower N yield-scaled emissions than control treatment when mineral fertilizer is applied.

In terms of Global Warming Potential (GWP) expressed as CO_2 equivalents, the different doses of sewage sludge induced an increment in the CO_2eq losses. If the GWP from each treatment is related to its grain yield and expressed as Green House Gas Intensity (GHGI) (Mosier et al., 2006), the 80 t ha^{-1} yr^{-1} dose significantly increased the GHGI while the mineral fertilizer reduced it. This reduction with respect to the control treatment was also described by Huérfano et al. (2015) after applying mineral fertilizer in humid Mediterranean conditions similar to those in this work. However, the exaggerated applied doses of TSS in this study are not a common practice and the fertilization has to be adjusted and balanced for further studies and field recommendations.

This study shows that the microbial diversity of soil (measured with the Shannon and Simpson inverse indices) and richness (measured with Chao1 index) were not significantly affected by the different amounts of TSS applied to the soil. The high coverage of the analysis, more than 87%, assures an accurate representation of the soil diversity. However, recent observations demonstrated that soil biodiversity and its community composition determine ecosystem functionality (Wagg et al., 2014). For this reason, we investigated the microbial community structure through in-depth-sequencing of the V3-V5 hypervariable regions of the 16S rRNA gene. The results indicate that after fertilization, and also after harvest, there was a statistically significant difference between the TSS at the highest dose compared to the rest of treatments. This means that applying TSS at 80 t ha^{-1} per year over 20 years, results in a differently-structured bacterial community if we consider the presence/absence of OTUs, as well as the specific abundance of each OTU (see Figs. 4 and 6, and Suppl. Fig. S1). This result is in agreement with some reports where the application of manure from farming caused pronounced changes in bacterial community composition (Ge et al., 2010; Ding et al., 2014). However, in that work the difference may be due to the presence of antibiotic sulfadiazine; this is not the case in our study since there are no xenobiotics or other contaminants in the TSS applied. The observed difference in the structure of the microbial community is due to the treatment dose and its persistence in time, since similar communities were detected prior to sowing and after harvest. Moreover, it can be concluded that there is no seasonal effect, since the sampling was carried out both in October and July. The changes observed in the community range from phylum to genus level. The highest dose of TSS resulted in a lower abundance of the phylum Acidobacteria, and an increase of the phylum Proteobacteria; within this latter phylum there is an increase in genera such as *Arenimonas*, *Hyphomicrobium*, *Naxibacter*, *Serpens*, *Stenotrophomonas* and *Steroidobacter*. Interestingly, some of these genera have been identified in microbiome changes correlating with the growth stages of crops (Li et al., 2014), drought resistance (Zolla et al., 2013) and other root-soil interactions that favor disease suppression in arable crops (Yin et al., 2013). Likewise, the genera *Streptomyces*, *Arthrobacter*, *Microbacterium*, *Rhizobium*, *Haliangium*, *Microvirga*, *Bradyrhizobium* and *Bacillus* were either not detected or seen only in very small numbers (Fig. 6) after the application of 80 t per year; some of these genera have been described as bacteria which typically contribute to high soil quality (Ding et al., 2014) and the promotion of plant growth. These results confirm that this dose is excessive for cereal fertilization and could have a negative effect on the microbial community. Additionally, the lack of difference between the other treatments (TSS application versus control or mineral) could indicate benefit to the crops without altering the structure of the bacterial communities.

5. Conclusions

This work stresses the importance of developing a broader approach to the environmental evaluation of agricultural application of recycled materials to soil. The role of waste recycling cannot be fully understood if soil fertility is underestimated within the framework of climate change.

The observed increases in nutrients, enzyme activities and GHG emissions evidence increased metabolic activity after the application of TSS to soil. These positive effects are not consistently beneficial if high doses of TSS are applied to soil and yield is considered. Further research must be done in order to optimize and adjust both doses and frequency.

The combined application of TSS and mineral fertilizer represents a good mitigation opportunity, and further research on possible consequences to soil properties and the environment should be thoroughly examined. Additionally, the influence of crop rotation and root exudates should be further revised to determine possible links between nutrient cycling and bacterial community composition.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.agee.2016.09.021.

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